

**THE EFFECTS OF HEAT STRESS AND FEED RESTRICTION ON THE  
MICROBIOME  
OF LACTATING DAIRY COWS**

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by  
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**THE EFFECTS OF HEAT STRESS AND FEED RESTRICTION ON THE  
MICROBIOME OF LACTATING DAIRY COWS**

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## DEDICATION

I dedicate this thesis to all my family and friends who have supported me throughout this journey. You know who you are.

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# THE EFFECTS OF HEAT STRESS AND FEED RESTRICTION ON THE MICROBIOME OF LACTATING DAIRY COWS

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## ACADEMIC ABSTRACT

Elevated environmental temperatures and relative humidity can cause heat stress in dairy cattle which results in decreased feed intake, reduced milk production, and increased disease incidence. Our objectives were to evaluate the effects of heat stress and feed restriction on the rumen fluid, fecal, bedding, inguinal skin, teat skin, and milk microbiomes of lactating dairy cattle. Feces contribute bacteria to bedding and by evaluating rumen fluid, feces, bedding, inguinal skin, teat skin, and milk samples, we aimed to determine if elevated environmental temperatures induced changes in one microbial niche impacted the other sample types evaluated. We hypothesized that heat stress would result in increased shedding of Gram-negative bacterial phyla (*Bacteroidetes* and *Proteobacteria*) and mastitis-associated bacterial operational taxonomic units (**OTU**) (*Enterobacter*, *Enterococcus*, *Escherichia-Shigella*, *Klebsiella*, *Staphylococcus*, and *Streptococcus*). Our first study evaluated the effects of heat stress on one group of cows (n = 6) in a time course design through pretreatment, heat stress, and recovery periods. Our second study evaluated the effects of elevated and clamped rectal temperature (40°C±0.5; n = 6) or pair-feeding (n = 6) in a sequential design through pretreatment, challenge, and recovery periods. A third group of control cows (n = 6) was maintained under thermoneutral conditions. During these studies, the relative abundances of

dominant bacterial phyla, families, and genera in rumen fluid, feces, bedding, inguinal skin, teat skin, and milk often displayed different responses to heat stress and feed restriction in dairy cattle. Fecal samples were collected from heat-stressed cows in both studies but the relative abundances of *Bacteroidetes* and *Firmicutes* differed between the studies. Rumen fluid and fecal samples contained few of the OTU corresponding to mastitis associated bacteria. *Enterococcus* relative abundance appeared temperature responsive as shown by an increase in abundance in bedding, and on inguinal and teat skin during the heat stress challenge. Because inguinal skin was less exposed to the bedding than teat skin, the rise in the abundance of *Enterococcus* during heat stress highlights the skin's additional contribution to changes within the teat skin microbiome. These studies highlight that the skin microbiome responded to heat stress and may alter the bedding microbiome. Collectively, the results from these studies report specific bacterial OTU that appear heat stress-responsive and support the concept that mastitis-associated bacteria have different responses to heat stress. Additionally, the results from these studies highlight the variability within the dairy cow microbiome over time.

# **CHAPTER I**

## **LITERATURE REVIEW**

### **1.1. INTRODUCTION**

Around the world, mastitis is one of the most prevalent diseases in lactating dairy cattle. Mastitis has negative economic impacts (Cha et al., 2011) and negative impacts on animal health and wellbeing (De Vliegher et al., 2012; Ruegg, 2017). There are many factors that determine incidence and impact of mastitis, including: nutrition, genetics, hygiene, and housing (De Vliegher et al., 2012; Ruegg, 2017). One such factor is season, specifically the proposed link between heat stress in the summer and increased mastitis incidence (Tao et al., 2018). In light of climate change, effects of heat stress on animal productivity and health will likely increase (Key et al., 2014). Therefore, there is a critical need for better understanding of the effects on heat stress on bovine mastitis and that is the overall focus of my thesis.

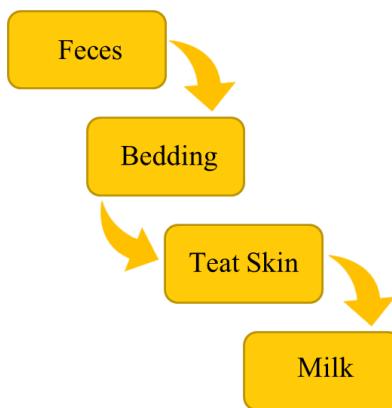
Recent reviews have presented evidence supporting a causal link between heat stress and mastitis (Das et al., 2016; Tao et al., 2018). Indeed, bulk tank SCC (**BTSCC**), an indicator of subclinical mastitis, has been reported to rise during periods of elevated environmental temperatures (Olde Riekerink et al.; 2007, Tao et al., 2018). Another study (Hogan et al., 1989b) has found that bacterial load in bedding and on skin increased during summer. These reports have led to broad acceptance of the idea that summer heat stress predisposes dairy cows to mastitis. However, conclusive supporting evidence remains lacking as does an understanding of the underlying biology. For example, it remains unknown if the reported increased incidence of disease during periods of heat stress is due to increased exposure of the cow to mastitis-causing bacteria, a suppressed

immune system, or other, unknown, factors. Unknown factors could include the impact of uncultivated bacteria or dysbiosis of the normal microbiome.

In the studies described in this thesis, bacterial populations were evaluated using 16S rRNA gene sequencing to determine if microbiome alterations occur during times of heat stress or feed restriction. A change in the bacterial populations coming in contact with the mammary gland could potentially explain the above-mentioned rise in BTSCC during the summer months. Such changes could arise from increased heat and humidity altering several different bacterial reservoirs, thus a comprehensive understanding of environmental impacts on the dairy cow microbiome requires an integrated approach. Therefore, the microbiome of rumen fluid, feces, bedding, inguinal skin, teat skin, and milk samples were characterized during exposure to heat stress or feed restriction. We aimed to determine if changes in one microbial niche were reflected in other niches and if heat stress or feed restriction would alter the bacterial populations of the teat end and/or milk.

At the University of Missouri, the Brody Environmental Units were utilized to control temperature and humidity (**THI**) such that a cows' rectal temperature was elevated and held at  $40+/- 0.5^{\circ}\text{C}$  during defined periods of a heat stress challenge. This enabled us to study the effects of elevated temperature and humidity on the microbiome of lactating dairy cows and their environment. Since heat-stressed cows are known to have reduced feed intake, in our second trial, we included a group of pair-fed cows, maintained under normothermia, to separate effects of heat stress from those of feed restriction alone on the microbiomes under study.

The objectives of these studies were to identify changes in individual bacteria or the overall microbiomes in response to heat stress or feed restriction and, thereby, to identify bacteria that could play a role in the increased BTSCC that commonly occurs in the summer months. We hypothesized that the relative abundance of Gram-negative organisms in feces would increase during experimental heat stress, leading to changes in the bedding microbiome and, ultimately, being reflected in the microbiomes of teat skin and milk (Figure 1.1). The aim of this review is to provide a foundation of the impacts of environmental stressors on the various microbial niches impacting dairy cows and their potential implications for mastitis incidence and control.



**Figure 1.1.** Hypothesized relationship between multiple microbiomes. We hypothesized that the relative abundance of Gram-negative organisms in feces would increase during experimental heat stress, leading to changes in the bedding microbiome and, ultimately, being reflected in the microbiomes of teat skin and milk.

## 1.2. MASTITIS

Mastitis is defined as inflammation of the mammary gland. The average cost of a case of clinical mastitis is between \$95 and \$444 depending on causative bacteria, direct costs (treatment and labor), and indirect costs (future milk loss and culling; Cha et al., 2011; Rollin et al., 2015). Mastitis-causing bacteria are generally placed into two

categories: environmental or contagious; but some bacteria can be spread through both means (Hogan et al., 2017). Environmental mastitis pathogens are found in the cow's environment and are thought to infect the cow between milkings and during the dry period (Hogan et al., 2017). Contagious mastitis pathogens are spread through cow-to-cow transmission, primarily during milking (Hogan et al., 2017).

Mastitis incidence can be evaluated at the herd level or for individual cows. At the herd level, BTSCC can be used to monitor subclinical mastitis incidence. The SCC and SCS can also be measured to monitor an individual cow's incidence of subclinical mastitis. Clinical mastitis in individual cows can be evaluated by observing outward signs such as swelling and redness of the udder and discoloration or altered consistency of the milk.

Bacteria commonly cause intramammary infections (**IMI**) that result in mastitis. The teat end is often regarded as the mammary gland's first line of defense and bacterial entrance through the teat end can result in an IMI (Sordillo, 2018). The streak canal is another component of the mammary gland's physical defense against mastitis. The keratin lining within the streak canal has bacteriostatic properties to prevent bacteria from traveling further into the gland (Sordillo 2018). Non-physical defense mechanisms used by the mammary gland uses to prevent infection include activation of the innate and/or active immune system which can result in inflammation and rapid influx of neutrophils from blood, leading to elevations in the SCC.

Even with physical and non-physical defense mechanisms that protect the mammary gland from mastitis, heat stress poses a challenge and the causes behind the summer rise in BTSCC are not completely understood.

### **1.2.1 FACTORS ASSOCIATED WITH MASTITIS**

Mastitis is a complex disease involving many factors associated with the cow and environment, which are briefly reviewed, below.

#### ***Stage of lactation***

The typical lactation cycle of a dairy cow begins with colostrogenesis and proceeds through lactogenesis, resulting in a dramatic increase in milk production thereafter. The decrease in colostrum production and intense increase in milk production is caused by increases in blood levels of prolactin (Akers, 1985). Peak milk production is reached six to eight weeks post-partum and is followed by a continuous decline of approximately five to seven percent per month until the time of dry-off (Church, 1988; Capuco et al., 2013). The periparturient period, which is the period immediately before and after calving, has been identified as an important risk factor for mastitis and other diseases (Sordillo et al., 2018) and stage of lactation has been reported as a risk factor for reoccurring clinical mastitis (Jamali et al., 2018).

Stage of lactation is an important consideration for mastitis and microbiome studies because treatment effects on early lactation cows can be confounded with the physiological demands required to meet peak milk production. Cows are also more susceptible during the dry period, because of the drastic physiological changes that occur throughout the involution process. Therefore, the following studies presented in this thesis used mid-lactation cows to evaluate the effects of heat stress on the microbiomes.

#### ***Season***

Seasonal impacts on many aspects of milk quality are well established (Hogan et al., 1989; Gao et al., 2017). However, season is a complicated factor that encompasses many potentially confounded elements including effects on feed, housing, photoperiod, and stage of lactation. For the present research, we focused on effects of elevated temperatures.

Milk SCS has been reported to be higher in summer than winter across all regions of the United States (Guinn et al., 2019), and BTSCC has been described to peak in August and September (Olde Riekerink et al., 2007). Additionally, the rate of clinical mastitis (Hogan et al., 1989a) and cumulative incidence of clinical mastitis (Gao et al., 2017) has been reported to be higher during summer months.

Conflicting results regarding IMI during the summer and winter months has been reported. Season has also been reported to impact IMI incidence (Olde Riekerink et al., 2007) while Green et al. (2006) did not find evidence linking a higher IMI rate in the summer to explain the higher SCC. Previously, the cumulative incidence (Gao et al., 2017) and rate of clinical mastitis from low SCC herds (Hogan et al., 1989a) have been reported to be highest during summer months.

Previous studies have reported that, during summer, cows are more likely to be infected by environmental pathogens such as *Escherichia coli* and *Streptococcus uberis* (Zhang et al., 2016; Gao et al., 2017). Similarly, *Escherichia coli* and *Klebsiella* species have been isolated from milk more frequently during summer while *Streptococcus dysgalactiae*, *Streptococcus agalactiae* and other environmental Streptococci have been isolated more commonly in winter (Gao et al., 2017). Furthermore, clinical mastitis caused by *Streptococcus uberis* has been reported to be higher during summer (Osterås et

al., 2006). Contrary to previous reports, infections caused by *Staphylococcus aureus* and *Escherichia coli* were higher in winter and in support of others, infections caused by *Streptococcus dysgalactiae* were higher in winter (Osterås et al., 2006). These conflicting results are likely due to different housing (indoors versus outdoors), location, and bedding.

Additionally, Elmoslemany et al. (2010) reported that coliform counts in bulk tank milk were higher in summer than winter and proposed that bacteria may grow faster during periods of elevated environmental temperatures. Furthermore, isolation rate of *Staphylococcus aureus* from milk has been reported to increase as the THI increases (Zeinhom et al., 2016). These studies highlight that distribution of mastitis pathogens differs across season and further support the concept of potentially altered exposure of the mammary gland to bacteria during periods of elevated environmental temperature.

### ***Exposure to bacteria***

As noted above, the teat end is the cow's first line of defense against IMI. Dairy cows spend over 50% of the day lying down (Gomez et al., 2010; Watters et al., 2013) during which time the teat ends may be in contact with the bedding and other sources of bacteria such as fecal matter. In 1994, Harmon and others proposed the concept that elevated temperatures that cause heat stress do not cause elevated SCC, instead the increase in SCC could be the result of exposure of the teat ends to an increased pathogen load, leading to a higher number of new IMI and clinical mastitis. Contrary to that thought, lying time typically declines in heat stressed cattle (Polsky et al., 2017; Norlund et al., 2019), which would reduce exposure. However, decreased teat end exposure to

bacteria could be negated if superseded by bacterial proliferation in bedding and on teat ends due to more optimal growing conditions during elevated temperature.

The preceding section describes various factors that may influence incidence of mastitis in cows. It is clear that changes in the various bacterial populations that reside in or on dairy cattle and in their environment could impact the exposure of the cow to potentially pathogenic bacteria. Changes in these bacterial communities may explain the common rise in SCC during summer months. Hence, study of the microbiome offers an excellent opportunity to gain novel insights into relevant individual bacteria and entire communities that may impact bovine mastitis.

### **1.3. THE MICROBIOME**

Recent advances in next generation sequencing (**NGS**) techniques have allowed for extensive identification of the microbes that occupy various ecosystems. The impact of the microbiome on human health (Turnbaugh et al., 2007) and livestock health has been explored (Mao et al., 2015; Guevarra et al., 2018). However, the effects of environmental stress on bacterial populations have not been studied extensively. In particular, few studies have evaluated the impact of acute environmental temperature changes on the dairy cow microbiome. The purpose of this section of this review is to discuss the current knowledge of the microbiomes of bovine rumen, feces, skin, milk, and bedding. Additionally, the impact of stress and how bacterial populations interact will be explored.

### **1.3.1 METHODS OF DETECTION**

Using NGS technologies, the microbiome can be evaluated to further understand how a host and its resident microbes work together to maintain their shared and diverse ecosystem. The terms ‘microbiome’ and ‘microbiota’ are similar with slight differences (Table 1.1). Based on the multiple definitions and for the purpose of this review, the ‘microbiome’ will refer to the collection of microorganisms and their genomes, while the ‘microbiota’ will refer to the living microorganisms. For example, the term ‘microbiome’ will be used when addressing results obtained with 16S rRNA sequencing because this technique evaluates bacterial genes and does not distinguish between live and dead bacteria. Conversely, the term ‘microbiota’ would be used when evaluating results obtained with bacterial culture because the living bacteria in the host are being evaluated. Additional definitions of terminology used to describe microbiome measurements are provided in Table 1.1.

<i>Term</i>	<i>Definition</i>	<i>Reference</i>
<i>Microbiome</i>	“The genomes of these microbial symbionts”	Turnbaugh et al., 2007. The human microbiome project
	“The catalog of these microbes and their genes”	Ursell et al., 2012 Defining the human microbiome
<i>Microbiota</i>	“The microorganisms that live inside and on humans”	Turnbaugh et al., 2007. The human microbiome project
	“The microbial taxa associated with humans”	Ursell et al., 2012. Defining the human microbiome
<i>Alpha diversity</i>	“The diversity determined in each sample, using species richness and evenness to calculate the diversity in each environment” Examples: Chao1, Shannon indices	Doyle et al., 2017. Impacts of seasonal housing and teat preparation on raw milk microbiota: a high-throughput sequencing study
<i>Beta diversity</i>	“The diversity between different samples; it provides a measure of dissimilarity between samples” Examples: Principal Coordinate Analysis, Principal Component Analysis	Doyle et al., 2017. Impacts of seasonal housing and teat preparation on raw milk microbiota: a high-throughput sequencing study
<i>Operational taxonomic unit (OTU)</i>	“Make up the microbiota”	Ursell et al., 2012. Defining the human microbiome
<i>Richness</i>	“A calculated metric that accounts for the number of OTU”	Ruegg et al., 2019. A beginner’s guide to the milk microbiome
<i>Diversity</i>	“A metric that accounts for the evenness of OTU”	Ruegg et al., 2019. A beginner’s guide to the milk microbiome

**Table 1.1.** Definitions and references for common microbiome terminology.

Prior to NGS, qPCR was commonly used to quantify gene expression within a sample. Because qPCR requires unique primers for known bacterial species, it cannot be used to measure unknown bacterial species within a population. Both qPCR and NGS technologies can be used to target the 16S gene, but NGS can be used to discover more bacterial species at one time. The universal primers for NGS bind to a highly conserved hypervariable region of the 16S gene and the sequencing of this highly variable region helps us to identify bacteria without having to design primers for individual bacterial species (Ruegg and Metzger, 2018). Due to the sensitivity of NGS, samples must be collected aseptically, and choice of DNA extraction method can alter sequencing results (Bjerre et al., 2019). The results generated with NGS also depend on the reference

sequences within a database; if the sequence fails to find a match within the chosen database, the taxonomy can fail to be classified down to the genus or species level.

Samples for microbial analysis must be collected aseptically to minimize contamination and, after collection, DNA is extracted and the sequence of interest is amplified with PCR (Metzger et al., 2018b). The 16S rRNA gene is of common interest among microbiome studies because it contains nine hypervariable regions, is phylogenetically conserved, and therefore does not transfer laterally through different species (Turnbaugh et al., 2007; Kim et al., 2017). After amplification of a chosen hypervariable region, the chosen hypervariable region of the 16S rRNA gene can be sequenced, and operational taxonomic units (**OTU**) can be assigned based on sequence similarity.

Using NGS, common bacterial phyla including *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, have been reported in the microbiome of humans (Turnbaugh et al., 2007), rodents (Ericsson et al., 2015; Tanca et al., 2018), poultry (Zhu et al., 2019), swine (Guevarra et al., 2018), and cattle (Alipour et al., 2018). Across these studies, the gastrointestinal tract has been predominantly studied for improving human health or animal production and health. An interest in characterizing additional sites in humans (Human Microbiome Project, 2012) and animals (Jami et al., 2014; Mao et al., 2015) to improve health has resulted.

### **1.3.2 THE MICROBIOME OF HUMANS AND LAB ANIMALS**

It has been estimated that the adult human carries one trillion microbes on their skin and 100 trillion microbes in their digestive tract (Luckey, 1972). Similarly, using

NGS, it has been estimated that the healthy human gastrointestinal tract contains between 10 and 100 trillion microorganisms, made up of over 10,000 different bacterial species (Turnbaugh et al., 2007). Knowledge regarding fluctuations within the microbiome could lead to dietary strategies to improve host health, provide biomarkers of illness, and contribute to the development of new treatments for disease.

The concept of microbiome dysbiosis has been proposed to aid in understanding disease susceptibility (Andrews et al., 2019). Dysbiosis can occur when the microbiome is altered and may disrupt normal physiological functions (Thursby and Juge 2017). Previous research in humans has focused on the association between the microbiome and obesity (Gibiino et al., 2018) and Crohn's disease (Sokol et al., 2020). A recent study reported that gastrointestinal tracts of obese humans tend to have more bacteria belonging to the phylum *Firmicutes* and less that belong to the phylum *Bacteroidetes* (Gibiino et al., 2018). It has also been proposed that weight loss is associated with an increase in bacteria belonging to the phylum of Gram-negative bacteria, *Bacteroidetes* (Gibiino et al., 2018). Crohn's disease is another human health issue that has been studied using NGS technologies. After identifying differences between fecal microbiomes from healthy and unhealthy patients, fecal microbiota transplants have been used to aid in remission of Crohn's disease (Sokol et al., 2020). However, more research is needed to better understand how long benefits from fecal microbiota transplants last and to identify other sources for microbiota transplants.

In rats, caloric restriction has been reported to promote health and longevity and the microbiome may contribute to these effects (Tanca et al., 2018). In rats that were feed-restricted (reduced caloric intake, macronutrients unchanged), the *Firmicutes* to

*Bacteroidetes* ratio was decreased and the authors (Tanca et al., 2018) also reported that one week of diet reversion was enough to alter the functional gut microbiome. Tanca et al. (2018) also reported that the microbial enzymes involved in short chain fatty acid synthesis were altered as exhibited by an increase in the propionogenesis pathway with limited changes in butyrogenesis and acetogenesis pathways, indicating metabolic changes due to feed restriction.

Microbiome studies commonly focus on humans to discover ways to treat disease and in lab animals as a proxy for human studies. However, alterations to the microbiomes of livestock and poultry could be beneficial and further research is necessary.

### **1.3.3 THE MICROBIOME OF CATTLE**

The microbiome of cattle has not been studied as extensively as that of humans or mice. Most studies evaluating the cattle microbiome focus on the gastrointestinal tract to find bacteria that are associated with increased feed efficiency and production (Kim et al., 2017; Wallace et al., 2019). Overall, most microbiome studies in cattle report *Actinobacteria*, *Bacteroidetes*, and *Firmicutes* as the dominant phyla in rumen (Lima et al., 2015), feces (Shanks et al., 2011), and milk (Bonsaglia et al., 2017). Other microbial niches, like the skin, have not been studied as extensively.

The microbiota of any niche is inherently variable (Paz et al., 2016; Wagner et al., 2018) due to host factors such as the feeding regime (Henderson et al., 2015; Meale et al., 2016), age (Mariat et al., 2009; Jami et al., 2013; Lima et al., 2017), sex, breed (Paz et al., 2016), and reproductive status (Minuti et al., 2015). Environmental factors such as housing, climate, and location (Shanks et al., 2011) are also known to influence the

microbiome but it is unknown how large of an impact each of these factors has on an animal's microbiome. Previous studies have described the inter-relatedness of dairy cattle microbiomes including rumen fluid, fecal, uterine, bedding, feed, water, dust, and milk to better understand the environmental impact on udder health (Nguyen et al., 2019a; Nguyen et al., 2019b; Wu et al., 2019). The authors reported that some microbiomes, like feces and bedding, were associated with the milk microbiome which highlights the interconnectedness of an animal's microbiomes.

Apart from individual and environmental-induced changes in the microbial communities, various stressors have been reported to alter microbial populations in cattle (Chen et al., 2018; Mir et al., 2019). Changes in behavior, physiology, and hormones can be used as biomarkers of stress, while it remains unknown if there are biomarkers in the microbiota. Stressors such as heat, cold, housing, nutrition, environment, social, weaning, dehorning, and castration have all been reported to significantly alter the microbiome (Meale et al., 2016; Chen et al., 2018; Mir et al., 2019). Whether changes to the microbiota have long term impacts on the health of the animal is currently unknown, and a better understanding of these effects could be informative. Additionally, description of the bidirectionality of flow between microbiome niches could allow us to better understand the interaction between niches and the impact on host health due to altered exposure.

### **1.3.3.1 MICROBIAL NICHES**

#### ***The gastrointestinal microbiome***

The gastrointestinal tract is commonly studied to look for ways to improve feed efficiency. The microbiome has been reported to differ across rumen, duodenum, ileum,

and feces (Frey et al, 2010). Of the gastrointestinal tract samples collected, microbial counts were reported highest in the rumen, followed by ileum and duodenum.

Diet is one of the most important factors to consider when evaluating the gastrointestinal microbiome (Wells et al., 2014) and Shanks et al. (2011) reported that dietary composition (forage, processed grain, or unprocessed grain) was a more important consideration than geographic location. Even though management practices and diets differ, the dominant phyla that are present (*Firmicutes* and *Bacteroidetes*) are similar between different locations (Shanks et al., 2011). Presumably, the microbiota of the multiple segments of the gastrointestinal tract are interdependent but how much so and how changes in one segment impact the microbiome of other segments remains unknown. Better understanding of these relationships holds promise for improving animal health.

### ***The rumen microbiota***

The bovine rumen has been commonly explored in search of bacteria that can be used to improve feed efficiency (Jami et al., 2014; Mao et al., 2015). The rumen microbiome develops as calves age from one-day-old to two-years-old as evidenced by decreases in the relative abundance of aerobic and facultative anaerobic taxa and an increase in the anaerobic taxa (Jami et al., 2013). These changes in bacterial abundance coincide with changes in rumen development and are confounded with effects of diet. The age groups in the previous study included newborn calves fed colostrum, two-month-old calves fed milk and a starter diet, and six-month-old calves and two-year-old calves fed the same diet consisting of roughage and concentrate. Richness, diversity, and intra-

subject similarity of ruminal microbiota also increase with age, which suggests a shift towards a more stable microbiome.

In further support that diet is an important factors to consider when comparing microbial populations across studies, Henderson et al. (2015) collected ruminant and camelid foregut samples from around the world and found differences in microbial compositions were predominantly due to dietary differences and not host differences. They also reported *Prevotella*, *Butyrivibrio*, and *Ruminococcus* to be core bacteria found in the gastrointestinal microbiome. When comparing forage diet eaters to those consuming more concentrates, Henderson et al. (2015) reported *Bacteroidales* and *Ruminococcaceae* to be more dominant in rumen samples from animals consuming more forages while *Prevotella* and *Succinivibrionaceae* were more dominant in those consuming large amounts of concentrates.

In addition to diet composition, amount of feed intake has been shown to alter microbial populations (McEwan et al., 2005; Tanca et al., 2018) and is known to decrease during periods of heat stress. Rumen samples collected from rams fed the same diet were found to differ based on samples collected during long or short days (McEwan et al., 2005). Feed intake was higher during long days and overall bacterial composition was altered, with differences in *Bacteroidetes* abundances suggesting that the amount of feed consumed, day length, or hormones also play a role in the microbiome.

The rumen microbiota includes a diverse population of bacteria, fungi, and protozoa and changes to this community are associated with changes in metabolic rates. As species diversity decreases in rumen samples, the fermentation rate increases (Scharen et al., 2018). A strong positive correlation between the ratio of *Firmicutes* to

*Bacteroidetes* and milk fat yield has been reported, and the authors speculated that, upon further studies, this information could be insightful for rumen inoculations to improve production parameters (Jami et al., 2014).

Beta-diversity in the rumen bacterial communities has been reported to differ between Holstein and Jersey cows fed the same diet although the microbiome still contains the same dominant phyla (*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Fibrobacter*) and families (*Prevotellaceae*, *Lachnospiraceae*, and *Ruminococcaceae*). The previously mentioned study reported that 95% of OTU were shared across breeds while 99% of OTU were shared between sampling methods (rumen cannula and esophageal tubing).

In addition to age, diet, and breed, high environmental temperature has been reported to alter the rumen microbiome. However, few studies have explored the impact of the cow's environment on rumen bacteria. The effects of high environmental temperature and humidity on the rumen microbiome of heifers has been evaluated (Tajima et al., 2007). In each of three studies, heifers were housed in climate-controlled rooms and temperature was 20°C, 28°C, and 33°C, respectively, and humidity was held at 60% to 80% across the studies. There was a tendency for decreased rumen pH as temperature increased in all three sets of experiments. While the richness and Shannon diversity measures were not affected by heat and humidity, there was a decrease in short chain fatty acid concentration as temperature and humidity increased, perhaps reflecting changes in rumen metabolism. This finding is significant, as short chain fatty acids (such as acetate, propionate, and butyrate) are the main energy source for ruminants, and alterations could impact the animal's energy balance.

A core microbiome has been proposed (Jami and Mizrahi, 2012; Jami et al., 2014), and even though there is high variation between animals, the similarity between bacterial taxa across cows was greater than 50%, suggesting that ruminal bacteria share common taxa to meet the functional needs of lactating dairy cows. Even though the rumen microbiome changes as the animal ages, a core microbiome made up of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, has been reported in mature cattle (Mao et al., 2015) and across breeds (Paz et al., 2016). A better understanding of additional factors that alter the rumen microbiota is needed to better understand its response to heat stress.

### ***The fecal microbiota***

Like the rumen microbiome, the fecal microbiome is affected by many factors, including age and diet of the animal and season. Alipour et al. (2018) sampled feces from Ayrshire and Holstein calves within 10 minutes of birth, 24 hours after birth, and one week after birth, and reported that the fecal microbial profile of newborn calves was more similar to the dam's oral microbiome than it was to the cow's fecal or vaginal microbiota. Because the dams and calves were separated immediately after calving, the authors hypothesized that the newborn calves likely acquired bacteria in utero. After one-week, feces collected from calves included more intestinal taxa and were similar in composition to the feces of adult cows. The authors postulated that the calves may be seeded before birth with a diverse microbiome that changes rapidly during early life.

Aside from age, differences in the fecal microbiome between sick and healthy calves have been reported (Gomez et al., 2017); however, management differences between farms may be more responsible for the changes noted. Fecal samples for

microbiome analysis were collected from diarrheic and healthy dairy calves from two different farms with different management practices. The authors also reported that the fecal microbiome of healthy and diarrheic calves was farm-specific, and calves from the same farm, regardless of health status, shared similar microbial communities. The same dominant phyla were found between both farms, with *Firmicutes*, *Actinobacteria*, and *Proteobacteria* making up over 88% of the sequences. Further work needs to be done to determine the effects of different management practices on the fecal microbiome of cattle to determine how large of a role management has on the fecal microbiome.

Studies with differing methods of sequencing report *Bacteroidetes* and *Firmicutes* to be the most dominant phyla in adult dairy cattle feces. Abundance of more rare phyla is more variable (Ozutsumi et al., 2005; Kim and Wells, 2016; Muñoz-Vargas et al., 2018). The genus with the most members in the *Bacteroidetes* phylum across a meta-analysis was *Prevotella*, which belongs to the *Paraprevotella* family, including bacteria known for producing succinate and acetate (Ozutsumi et al., 2005). Within the *Proteobacteria* phylum, *Succinivibrio* was reported as the largest genus and was highly abundant in the feces of cows consuming a corn-based diet. Of the OTU found in rumen and fecal samples, 82/1252 (6.55%) OTU were shared, which further supports the claim by Frey et al. (2010) that different regions of the gastrointestinal tract harbor different bacterial species. In periparturient dairy cows, the same dominant phyla (*Bacteroidetes* and *Firmicutes*) have been reported and while *Spirochaetes* were more abundant than *Proteobacteria* (Muñoz-Vargas et al., 2018), the study had confounding factors that included diet and housing changes.

Microbiome studies commonly focus on bacterial phyla changes to understand the microbiome as a whole and some studies have evaluated specific bacteria that are of cattle or public health significance. For example, *Salmonella* species can cause Salmonellosis in humans and cattle. *Salmonella* shedding in feces from dairy cattle has been associated with the summer season (Likavec et al., 2016), and the authors concluded that for every five-unit increase in the THI, the incidence rate ratio increased by 29%. There was a positive association between the THI and *Salmonella* shedding while fecal microbial diversity did not change. The authors concluded that the thermal environment is a risk factor for periparturient shedding of *Salmonella* in feces.

*Clostridium* species can end up contaminating raw milk and cheese production and are found in feces of cattle (Calamari et al., 2018). *Clostridium* species can survive in extreme environmental conditions and the shedding of *Clostridium tyrobutyricum* into the feces of lactating dairy cows has been shown to increase during the summer season (Calamari et al., 2018). The authors reported an increase in the excreted to ingested spore ratio during summer and postulated that the spores germinate, which allows bacteria to proliferate, resulting in new sporulation in the digestive tract of cows during the summer. The studies by Muñoz-Vargas et al. (2018) and Calamari et al. (2018) suggest that warmer environmental temperatures may play a role in pathogen proliferation which, in turn, may alter the exposure of the cow to pathogenic bacteria. Furthermore, feces have been reported as a source of contamination in milk for processing and human consumption (Doyle et al., 2017); and alterations to the bacterial communities in feces may influence the bacterial populations in milk, possibly resulting in increased risk of disease in cattle.

The feces can harbor mastitis pathogens and the fecal shedding of some mastitis pathogens has been documented. For example, non-aureus *Staphylococcus* can cause mastitis and these bacteria have been reported in the majority (13/25, 52%) of feces collected from healthy cows (Wuytack et al., 2019). Bacteria associated with environmental mastitis, such as, coliforms and *Streptococcus* species, are also found in feces. *Klebsiella* species can cause clinical mastitis and have been reported in approximately 67% of feces and 100% of rumen samples (Zadoks et al., 2011), and *Klebsiella pneumoniae* has been reported in over 80% of feces (Munoz et al., 2006). *Streptococcus uberis* has been reported in approximately 23% of fecal samples (Zadoks et al., 2005). As the THI increases, fecal coliform count has been reported to increase (Zeinhom et al., 2016).

Although fecal shedding of mastitis pathogens has been described, little data on the factors that impact shedding, such as season or stress, has been reported. Environmental stress may alter the composition of feces from cattle and result in increased exposure to pathogenic bacteria. If the cow is exposed to more pathogenic bacteria, the animal may have an increased risk of disease. It is logical that fecal shedding should influence the microbiota of bedding, which, in turn, represents another potential reservoir of pathogens.

### ***The bedding microbiota***

It is well-established that bacterial populations of bedding increase after use and that increased bacterial count in bedding increases the exposure of the teat end to potential pathogens (Rendos et al., 1975). This is true across various bedding materials, including sawdust, wood shavings, and straw. However, various bedding types typically

contain different counts of common mastitis causing pathogens. For example, total coliform counts were reported to be highest in sawdust bedding, while Streptococci counts were highest in straw and Staphylococci were lowest in shavings (Rendos et al., 1975). Those counts corresponded with counts from teat samples, highlighting the link between bacterial counts in bedding and on teat ends. In another study using traditional culturing methods, Hogan et al. (1989) compared organic (sawdust or straw) and inorganic (sand or crushed limestone) bedding sources across seasons and reported greater teat end exposure to Gram-negative pathogens in bedding during summer and fall. Gram-negative organisms such as coliforms include the genera *Escherichia*, *Klebsiella*, and *Enterobacter* (Fairchild et al., 1982) and commonly cause environmental mastitis in cattle. Hogan et al. (1989) postulated that the higher coliform counts in organic bedding during summer and fall could be due to higher ambient temperatures. In a more recent study, a positive association between relative humidity and percentage of moisture in bedding has been reported (Robles et al., 2019) which, in turn, was associated with bulk milk bacterial counts. This highlights the importance of providing clean and dry bedding to dairy cows. Additional recent studies (Patel et al., 2019; Rowe et al., 2019) have reported a higher prevalence of IMI in the summer versus winter. In contrast to Hogan et al. (1989), Patel et al. (2019) reported no association between clinical mastitis and Gram-negative counts in bedding, but they concluded that it may be due to greater variation within their dataset possibly caused by differences in bedding age and many farms with different bedding types and management strategies. Nevertheless, further work needs to be done to better understand the relationship between bacterial communities in bedding and the impact on udder health.

In addition to differing bacterial counts in various bedding, the relationship between bacteria in bedding and on teat ends has been evaluated (Zdanowicz et al., 2004; Rowbotham and Ruegg, 2016). Using traditional culture methods, *Streptococcus* counts were reported to be ten times higher on the teat ends of cows housed on sand compared to cows housed on sawdust bedding, which had twice the number of coliforms and six times the amount of *Klebsiella* on the teat ends compared to sand bedded cows (Zdanowicz et al., 2004). The cows that were housed on sand had poorer udder hygiene than those housed on sawdust, but this was not consistently correlated with teat end bacterial counts. Rowbotham and Ruegg (2016) compared deep bedded new and recycled sand and deep and shallow bedded manure solids and found Strep-like organisms were present in all bedding types, but *Streptococcus* and Strep-like organism counts were highest in shallow bedded manure solids. They postulated that Strep-like organisms may therefore not be influenced by bedding type. New sand contained much lower Gram-negative counts compared to all other bedding types and cows housed on new sand had the lowest Gram-negative teat end counts. Overall, results from the previous studies suggest that various bedding types harbor different abundances of environmental pathogens that may predispose the cow to disease (Zdanowicz et al., 2004; Rowbotham and Ruegg, 2016).

Few studies have evaluated the microbiome of bedding and the relationship between the bedding and mammary gland microbiomes. On two farms that used automated milking systems, the bedding microbiome was associated with the milk microbiome (Wu et al., 2019); this further supports previous findings regarding the influence of bacteria in bedding on the mammary gland. The authors commented that bedding is a source of skin contaminants, and therefore, bedding management remains of

utmost importance to minimize exposure of the cow's udder to mastitis pathogens. A recent study reported that the milk microbiome is associated with the bedding microbiome (Wu et al., 2019), so alterations to the microbial communities in bedding may impact the teat end and milk microbiome.

The bedding microbiota is influenced by more than just the bedding material; it includes contaminants from skin, fecal matter, milk, dust, flies, and water. Therefore, elevated environmental temperatures may alter the bacterial populations found in bedding which could, in turn, alter the bacterial populations found on teat ends.

### ***The skin microbiota***

Like the ruminal and fecal microbiota, the dominant bacterial phyla found on the teat apex and in the teat canal were *Firmicutes*, followed by varying abundances of *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (Derakhshani et al., 2018). Teat skin and feces have been reported as important sources of contamination in milk (Doyle et al., 2017) suggesting that the cow's environment can play a large role in exposing the teat and thus the mammary gland to bacteria. In studies in which bacteria were identified using traditional culture, bacterial populations on teat ends were impacted by bacteria found in different types of bedding (Rendos et al., 1975; Zdanowicz et al., 2004; Rowbotham and Ruegg, 2016); and increased exposure of the teat end may alter the mammary gland microbiome—increasing the risk of mastitis.

Studies have evaluated the teat skin microbiome from healthy, subclinical, and clinical mastitis glands and have reported higher diversity on teat ends from non-infected cows (Braem et al., 2012). Even on teat ends of clinically healthy cows, bacterial species

are diverse; and diversity is lower on teat ends from cows with subclinical or clinical mastitis (Braem et al., 2012). When evaluating the teat end microbiome of clinically healthy cows, a low bacterial load has been reported (Braem et al., 2013).

The effects of stressors on cattle, such as elevated temperature, have not been studied extensively on the skin microbiome, and the response of the skin to changes in the cow's environment remains unknown. More specifically, the impact of changes on the teat end microbiome on the mammary gland and milk microbiome warrants further evaluation. Further research is needed to determine if the teat end microbiome could alter the milk microbiome and predispose the cow to potential pathogens.

### ***The milk microbiota***

It has been thought that the mammary gland is a sterile organ. However, recent studies have identified microbial DNA in milk and colostrum (Young et al., 2015; Lima et al., 2017). The dominant phyla in colostrum (Lima et al., 2017) and milk (Braem et al., 2012) are *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*. The prevalence of strictly anaerobic microorganisms (like *Fusobacterium*, *Prevotella*, *Bacteroides*, and *Clostridiales*) in colostrum suggests that these bacteria were not contaminants because they would not survive outside of the mammary gland (Lima et al., 2017). Bacteria within the mammary gland could enter the gland via the teat orifice and come from multiple sources such as the environment, bedding, streak canal, and teat cistern. Additional sources of bacteria in milk may include contamination of teat ends by feces (Doyle et al., 2017). Bacterial DNA found in the mammary gland could also come from contaminants or be introduced during sample processing and sequencing (Dahlberg et al., 2019). Previously, using PCR, it has been reported that based on sampling technique

(cistern versus conventional), culture results may differ (Hiitio et al., 2016). The previously mentioned studies support the concept of a mammary gland microbiome.

The bovine udder is a complex organ with multiple factors that exist to prevent disease. The hypothesis of an entero-mammary pathway for bacteria to enter the mammary gland via the gastrointestinal tract has been proposed (Young et al., 2015; Tong et al., 2019) but remains debated. Young et al. (2015) evaluated blood, milk, and feces of lactating dairy cows and found some genera (*Ruminococcus* and *Bifidobacterium*) to be common to all three sample types. The overall community structure of milk was like that of feces, and the authors suggested that bacteria are entering the mammary gland from an entero-mammary pathway, or via contamination of the teat end, allowing bacteria to surpass the keratin lining and proceed up and into the gland. Tong et al. (2019) used 16S sequencing to evaluate milk samples collected from cows with subclinical *Streptococcus agalactiae* mastitis, and speculated that the bacteria from the rumen are transferred to the mammary gland or fecal contamination in the cow's environment results in bacteria in milk.

Recently, the impacts of sampling method and bedding type on the milk microbiome of cows with low SCC and no history of clinical mastitis were evaluated (Metzger et al., 2018a). Different bacterial DNA has been found between the different sampling collection methods: composite milk, quarter-level milk, and milk collected via needle aspiration of the gland cistern (Metzger et al., 2018a). Additionally, multiple bedding materials were sampled—manure solids, recycled sand, new sand, and sawdust—and the authors reported that bacterial community composition in milk collected from the gland cistern differed by bedding type. These results highlight

differences in the milk microbiome due to sampling technique and bedding type and illustrate the concept that the cow's environment can contribute bacteria to the mammary microbiome and alter SCC.

Recently, studies have attempted to find associations between the milk microbiome and mastitis incidence (Falentin et al., 2016; Metzger et al., 2018a; Pang et al., 2018). Bacterial communities in milk have been reported to differ between healthy and subclinical or clinical cows (Oikonomou et al., 2012; Kuehn et al., 2013; Andrews et al., 2019). These changes in the microbiome could predispose the cow to other mammary infections or could be a response to pathogens within the udder (Kuehn et al., 2013). In dairy cows with a history of clinical mastitis, a lower diversity in the teat canal has been reported in unhealthy quarters compared to healthy quarters (Falentin et al., 2016). The authors also reported no difference in diversity in the teat canal samples from cows with a possible history of subclinical mastitis. In contrast, higher diversity has been reported in teat canal samples from healthy quarters versus quarters with subclinical mastitis (Pang et al., 2018). Milk samples collected from different farms with low or high incidence rates of subclinical mastitis contain some genera that are commonly found in the gastrointestinal tract (*Prevotella*, *Ruminococcus*, *Bacteroides*, *Rikenella*, and *Alistipes*; Pang et al., 2018). These authors reported that the presence of *Escherichia-Shigella*, *Klebsiella*, *Streptococcus*, and *Corynebacterium* in the milk microbiome are biomarkers for subclinical mastitis. In a recent study, infected quarters were more variable than healthy quarters and were commonly dominated by a single OTU (Andrews et al., 2019). Additionally, the dominant OTU was not always the same organism that was cultured and that could be due to its inability to be cultured using traditional culturing methods.

Microbes of teat end skin had similar microbial composition whether from healthy or infected quarters. Andrews et al. (2019) reported that microbial composition clustered in a principal coordinate analysis more by time than infection status. The authors did not find evidence of an association between the teat end and subclinical mastitis incidence. In contrast to Falentin et al. (2016) and Pang et al. (2018), Andrews et al. (2019) reported a lack of difference between infected and healthy quarters in alpha diversity (richness and evenness). There were unexpected management changes during the study, which may account for the lack of differences reported. Reports of long-lasting impacts on the microbiome of cows that have previously experienced clinical (Falentin et al., 2016) and subclinical mastitis (Metzger et al., 2018a; Pang et al., 2018) highlights the need for a better understanding of the microbiota of the mammary gland and the role it plays in disease susceptibility.

Intramammary antimicrobials are commonly used to treat clinical mastitis and the effects of these antimicrobials on the milk microbiome have been explored. Antimicrobials are also commonly used at dry-off and have been reported to not drastically alter the milk microbiome at the onset of the next lactation as evidenced by the lack of shift on a principal component analysis (**PCA**) (Bonsaglia et al., 2017). The previously mentioned study housed cows in a freestall barn during the prepartum period and then moved cows into a tie-stall barn for the first 60d postpartum (Bonsaglia et al., 2017). The authors concluded that either the antimicrobial induced shift was negligible, or a shift was reversed by the time the samples were collected seven days post calving in the following lactation (Bonsaglia et al., 2017). However, samples were collected within the first week after calving and additional factors in early lactation such as changing from

colostrogenesis to lactogenesis, drastic increases in milk production, a state of negative energy balance, or housing changes (free-stall prepartum to tie-stall postpartum) likely confound the lack of observed changes to the milk microbiome.

Antibiotics have been used for a long time to treat mastitis and with concerns surrounding their use growing, probiotics are being evaluated for the treatment of this disease. Lactic acid bacteria have been identified as Generally Recognized As Safe status for probiotic use and while some strains are potential candidates for colonization of the mammary gland, their effects have not been studied on the mammary microbiome (Bouchard et al., 2015). Determining new ways to treat and prevent mastitis are necessary but their effects on the healthy microbiota also need further determination.

Internal teat sealants are often used in combination with antimicrobials as dry cow therapy and a recent study detected bacterial communities in milk in the following lactation (Derakhshani et al., 2018). These effects could be due to introduction of contaminants during infusion of the teat sealant and antimicrobial or for the cows that only received the teat sealant, it could signify that the internal teat sealant did not completely prevent bacteria from entering the gland during the dry period.

Stressors such as environmental stress may alter the microbiota within the mammary gland and could increase the cow's risk of disease. Season was reported to affect changes in richness and diversity in the milk microbiome, while lactation stage was not (Metzger et al., 2018a). Milk samples can be difficult to sequence due to their low bacterial biomass and a higher sequencing success rate has been reported in milk samples from healthy quarters, with greater bacterial richness in the summer versus the winter (Metzger et al., 2018a) suggesting that the number and relative abundance of OTU were

greater in summer than winter. The Shannon index tended to be more even in the spring than winter, and community composition differed across seasons. Another study has reported that the highest Shannon index (evenness) occurred in June while the lowest value was in December (Li et al., 2018). That study also reported the greatest richness in June and the least in December. In agreement with Falentin et al. (2016) and Pang et al. (2018), Metzger et al. (2018) also reported lower diversity in unhealthy mammary quarters. Like previously mentioned studies, Metzger et al. (2018a) postulated that bedding could be a source for the bacterial DNA that was found in milk.

The role that environmental stress plays in altering the milk microbiome and how it impacts risk of disease remains unknown. Furthermore, the interaction between multiple microbial niches and the impact of environmental stress on multiple niches has not been thoroughly evaluated.

### **1.3.3.2 THE MICROBIOTA ACROSS MULTIPLE NICHES**

Across all the previously mentioned microbial niches, the same dominant bacterial phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*) are present but in vastly different relative abundances. These differences in abundance between gastrointestinal segments, environment, skin, and mammary secretions are likely due to differences in nutrient availability and environmental conditions such as temperature.

Within a microbiome niche, like feces, differences based on diet, location, breed, and sex have been reported. Within rumen fluid, the microbiome has been reported to differ based on sampling time and location (Li et al., 2009). Across the gastrointestinal

segments, rumen and fecal samples collected from the rectum have been evaluated to determine the impacts of weaning on the microbiome (Meale et al., 2016). Weaning is often regarded as one of the most stressful times in a calf's life and weaning itself has been shown to alter the composition of rumen and fecal microbiome more than weaning strategy (Meale et al., 2016). Rumen and fecal samples were collected from dairy calves prior to (d36) and after (d54) weaning and weaning method (abrupt versus gradual) was evaluated. Method had less of an impact on the rumen microbiome than weaning itself. The authors reported higher alpha diversity in rumen samples prior to weaning and in contrast, lower alpha diversity in feces, while the dominant phyla (*Bacteroidetes*, *Firmicutes*, and *Proteobacteria*) remained dominant prior to and after weaning. However, the relative abundances of these phyla varied with stage of development. Meale et al. (2016) concluded that the shift in gastrointestinal microbiome did not account for the declines in weight gain and feed intake that occurred after abrupt weaning. Additional samples throughout the weaning transition could have been beneficial to better differentiate between microbiome changes due to the stress of weaning and the weaning strategies used. While weaning stress alters the gut microbiome, it remains unknown if these effects can be controlled to minimize the stress on the animal as they mature. Even within the mammary gland microbiome, bacterial populations have been reported to differ between the teat apex, teat canal, colostrum, and milk niches. Nevertheless, the same dominant phyla are present across sample types, albeit in different relative abundances (Derakhshani et al., 2018).

Recent studies in dairy cattle have evaluated the relationships between microbiomes of rumen, feces, milk, water, feed, dust, and bedding on farms with

automated milking systems (Wu et al., 2019) and between microbiomes of uterine, feces, milk, dust, and bedding during summer and winter (Nguyen et al., 2019a; Nguyen et al., 2019b). These studies have investigated the inter-relatedness of microbiomes and reported that the milk microbiome is associated with bedding and that the most abundant taxa varies across sample types. The previously mentioned association highlights the importance of the cow's environment and the potential alteration in the milk microbiome due to altered exposure of the mammary gland to bedding.

Alterations to the microbiomes of dairy calves and cows during periods of stress have been reported as mentioned previously. The impact of other stressors, like environmental temperature, have been briefly explored in dairy cattle but more studies evaluating the impact of stressors on the microbiome are needed to define the acute impact of stressors on the microbiome, to understand the potentially long-lasting impacts of these stressors on the microbiome, and the role these changes may play in disease susceptibility.

#### **1.4. HEAT STRESS**

##### ***Introduction***

Heat stress has been well studied in dairy cattle due to the large annual losses that impact producers worldwide and range from \$897 to \$1.5 million in the United States (St-Pierre et al., 2003). This estimated cost rose to over \$1.2 billion in 2010 due to milk loss from heat stress alone and these costs are expected to rise further as environmental temperatures continue to increase (Key et al., 2014). A THI value of as little as 68 can cause heat stress and alter dairy cattle performance through changes in milk yield, milk

components, and reproduction performance (Guinn et al., 2019). For example, a combination of 26.5°C and 0% relative humidity or 22°C and 45% relative humidity both equate to a THI of 68. At a THI of 68, mild heat stress occurs and as temperature and relative humidity increase, the severity to the animal increases.

### ***Physiological response***

Physiological responses to heat stress include increased heart rate acutely followed by a decrease during prolonged heat stress, decreased feed intake, and increased water intake, and respiration rate (Bouraoui et al., 2002; Beatty et al., 2006). The decrease in feed intake that results from heat stress is associated with a decline in milk yield, however, the reduced nutrient intake only accounts for approximately 50% of the decrease in milk yield (Rhoads et al., 2009; Wheelock et al., 2010). Rhoads et al. (2009) studied the effects of heat stress on cows housed in temperature-controlled chambers and used a group of pair fed cows to control for the direct (higher temperatures) and indirect (reduced feed intake) effects of heat stress. The previous study did not report a decrease in milk fat percentage like the authors expected and they postulated that additional seasonal factors play a role in reduced milk fat percentages.

Heat-stressed dairy cattle have decreased dry matter intake (Beatty et al., 2006), but the effects of reduced feed intake without heat stress has not been extensively explored. A previous study evaluated the effects of feed restriction in dairy cattle during a period of mild heat stress (15-25°C; Beatty et al., 2008). The authors reported that feed restricting cows did not increase or decrease the body temperature of cattle (Beatty et al., 2008). While the environmental temperature was high enough to cause a decline in feed intake, the cows did not express additional outward signs (panting and sweating) of heat

stress and were feed restricted for a short period of time—three out of ten days. The authors concluded that conductive heat loss from the rumen decreased at the same time as heat production from the rumen and therefore, the effects were negated. The effects of heat stress on dairy cow production and physiology have been documented but the impact on the microbiome has not been studied extensively.

### ***Microbiome response***

As mentioned previously the effects of elevated environmental temperature on the microbiome of dairy cattle have not been explored as thoroughly as the effects of heat stress on production. Recently, Chen et al. (2018) studied the effects of heat stress on the microbiome of lactating dairy cows that were classified as low- or high-heat sensitive based on clinical signs (i.e. panting scores). Fecal samples were collected at one time point from each of the four groups: heat stressed (more heat sensitive or less heat sensitive) or not heat stressed (more heat sensitive or less heat sensitive). The authors reported that heat stress reduced the diversity of the fecal microbiome and increased the rectal temperatures, and the main clusters of the heat map separated into either heat-stressed or non-heat stressed, suggesting each contained different microbial community structure. Cows that were exposed to slight or no heat stress had greater richness than those exposed to high temperatures. The relative abundances of *Firmicutes* were higher in non-heat stressed groups while the relative abundances of *Bacteroidetes* were lower in non-heat stressed groups. The study by Chen et al. (2018) highlights the impact of heat stress on the fecal microbiome on one day of sampling and future time course studies are needed to determine the onset and duration of these impacts.

Previously, it has been established that the rumen pH continually increases during starvation (Meiske et al., 1958). Decreased rumen pH can result after feeding and persist for greater than five hours (Mao et al., 2013). These changes in pH can alter microbial populations and VFA production in the rumen as shown by increased *Actinobacteria* and *Firmicutes* and decreased *Bacteroidetes* and *Proteobacteria* when subacute ruminal acidosis is induced by diet alterations (Mao et al., 2013). Feed restricted bulls had an increase in acetate to propionate ratio which corresponded to changes in *Methanobrevibacter* species (McCabe et al., 2015). These studies highlight that although few studies have been done to evaluate the impact of elevated temperature on the rumen microbiome, based on this information, we expect there to be a change in the rumen bacterial abundance during heat stress.

Similar to cattle, in goats, the effects of heat stress on the rumen bacterial communities have been evaluated. Zhong et al. (2019) reported that heat stress had no effect on alpha diversity, but the most dominant phyla transitioned from *Firmicutes* to *Bacteroidetes* as THI increased. Total VFAs and butyrate decreased while acetate and the acetate to propionate ratio increased as THI increased, and the authors postulated that this change may be due to decreased feed intake.

Outside of ruminants, there is more information regarding the effects of heat stress on the gastrointestinal microbiome of poultry and swine. Wang et al. (2018) studied the effects of increased temperature on the ileum microbiome of broilers and found that microbiomes of heat-stressed broilers clustered separately from that of control animals. Chao richness was reported to be higher in ileum samples from heat stressed broilers while Shannon diversity was not different between the treatments. In the heat-

stressed birds, *Firmicutes* relative abundance was higher while *Proteobacteria* and *Bacteroidetes* abundance was lower. In cecum samples collected from heat stressed broilers (Shi et al., 2019), feed intake and average daily gain decreased while the feed conversion ratio increased. Similarly, to Wang et al. (2018), *Firmicutes* relative abundance increased and *Bacteroidetes* abundance decreased in the heat-stressed broilers. In contrast to the previously mentioned study, Shi et al. (2019) reported an increase in *Proteobacteria*, *Tenericutes*, *Anaeroplasma*, and *Lactobacillus*. The same dominant phyla (*Firmicutes*, *Bacteroidetes*, and *Proteobacteria*) have also been reported in laying hens, whether heat stressed or not (Zhu et al., 2019). In contrast to the studies using broilers, heat-stressed laying hens had a decreased relative abundance of *Firmicutes* and an increased abundance of *Bacteroidetes*. Therefore, changes in the abundances of *Firmicutes* and *Bacteroidetes* may be associated with the heat stress induced decrease in feed intake.

Like dairy cows, fecal samples from heat stressed ducks clustered on a Principal Coordinate Analysis (**PCoA**) based on whether the animals were heat stressed or not (He et al., 2019). Alpha diversity (Chao and Shannon indices) was not different between the treatments while some taxon specific changes were noted. He et al. (2019) postulated that the lower abundance of *Bacteroidales* in heat-stressed ducks may be related to disrupted gut homeostasis and enhanced inflammatory response. Total short chain fatty acids were decreased with heat stress and enhanced inflammatory responses produce short chain fatty acids which may also be related to the disrupted gut homeostasis and energy imbalance. This finding in ducks has also been reported in cattle and is important as short chain fatty acids are the main energy source for ruminants.

### ***Summary of heat stress***

As previously discussed, heat-stressed dairy cattle have decreased milk production and increased risk of disease. Elevated environmental temperatures also impact milk quality as exhibited by the association between THI and BTSCC. Aside from physiological effects, like decreased DMI, altered exposure of the cow to bacteria could partially explain the increase in BTSCC during summer months.

### **1.5. CONCLUSION**

Currently, the response of the microbial populations of different body sites (such as the gastrointestinal tract and skin) to environmental changes is unknown. To date, researchers have explored various microbial niches and highlighted alterations to the microbiota due to changes in diet and health, and noted that the dominant phyla, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, remain abundant across some species and sample types. The following studies presented herein offer an evaluation of changes over time in the various microbiome of various sites of lactating dairy cattle and responses to heat stress and pair-feeding.

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**CHAPTER II**

**STRESS IMPACTS THE MICROBIOME OF LACTATING DAIRY COWS: I.**

**HEAT STRESS MODIFIES BACTERIAL COMMUNITIES IN FECES**

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## **HEAT STRESS MODIFIES BACTERIAL COMMUNITIES IN FECES**

### **2.1 ABSTRACT**

Heat stress results in decreased milk production and increased incidence of disease. However, the effects of heat stress on the microbiome have not been extensively studied in dairy cattle. Therefore, the objective of this study was to evaluate the effects of increased environmental temperature and relative humidity on the fecal microbiome of lactating dairy cows. We hypothesized that heat stress would trigger increased shedding of Gram-negative organisms and mastitis pathogens into the feces of stressed cows. Six Holstein cows were housed in tie stalls in an environmental chamber. Cows were given a 5-d pretreatment period to allow them to acclimate to the chambers (d -5 to 0; temperature-humidity index (**THI**) averaged 64.7) and were then subjected to constant heat stress for 16 d (d 0 to 16; average THI  $75.4 \pm 0.07$ ), followed by a 9-d recovery period (d 16 to 24; average THI  $66.1 \pm 0.09$ ). Fecal samples were collected per rectum on d -1, 0, 6, 13, 16, 20, and 24. The V4 hypervariable region of the 16S rRNA gene was sequenced to evaluate the fecal microbiome. Overall, the richness and diversity of bacterial populations in the feces were higher during heat stress compared to pretreatment. Principal coordinate analysis and hierarchical clustering revealed that fecal samples clustered by experiment day. *Bacteroidetes*, a phylum of Gram-negative bacteria, displayed higher relative abundance in feces after prolonged heat stress compared to pretreatment or early heat stress. Interestingly, this increase remained through the recovery period, indicating extended effects of heat stress on the fecal microbiome. Of the selected mastitis pathogens, *Escherichia-Shigella* relative abundance was lower during recovery compared to pretreatment and heat stress. In conclusion, heat

stress altered the fecal microbiome and such changes appear to persist beyond the stress period. Identification of Operational Taxonomic Units (**OTU**) that changed in abundance during the study may provide biomarkers of heat stress in cattle. Further investigation into associated functional changes related to altered microbial populations is warranted.

## 2.2 INTRODUCTION

Heat stress in dairy cattle causes elevated respiratory rate and body temperature, reduced feed intake and reproductive rates (Das et al., 2016; Tao et al., 2018), and an increased risk of metritis and retained placenta (DuBois and Williams, 1980). As a consequence of heat stress, decreased milk production has been estimated to cost the dairy industry over \$1.2 billion in 2010, and this cost is expected to rise as environmental temperatures increase (Key et al., 2014). Decreased milk production during times of heat stress may be partially related to increased prevalence of mastitis, demonstrated by elevated bulk tank SCC and a higher incidence of clinical mastitis during the summer months (Hogan et al., 1989; Green et al., 2006).

Increased rates of clinical mastitis coincide with increased bedding bacterial counts, and higher numbers of Gram-negative organisms in bedding have been reported in the summer months (Hogan et al., 1989). A positive association between bedding bacteria counts and the odds of an IMI has been recently reported (Rowe et al., 2019). However, others failed to detect the same association between clinical mastitis and Gram-negative bacteria counts in bedding (Patel et al., 2019). In that study, traditional culture methods were used on thawed samples and freezing of the samples could have introduced error and influenced results (Patel et al., 2019). Both recent studies reported a higher proportion of mid- and late-lactation cows with an IMI on test days in the summer versus

the winter (Patel et al., 2019; Rowe et al., 2019), and the authors suggested that bedding management practices must be in place to mitigate the risk of IMI.

Bacterial populations in bedding change with differences in dry matter or organic matter of the bedding (Zdanowicz et al., 2004). For example, in organic bedding, both moisture content and numbers of coliforms, such as *Escherichia coli* and *Klebsiella* species, have been reported to increase during the summer (Hogan et al., 1989; Rowbotham and Ruegg, 2016). Environmental contaminants, such as feces and soil, can change organic matter content of bedding. Because feces represent a reservoir of environmental bacteria, changes in the fecal microbiota of dairy cattle may alter the number and type of pathogens to which cows are exposed. For example, an increase in abundance and diversity of bacterial populations has been reported in the feces of heat-stressed cows (Chen et al., 2018). It remains unclear if these changes in the bacterial diversity due to heat stress are consistent across studies or if the changes in the fecal populations with heat stress could explain the increased incidence of mastitis in the summer. Other stressors such as dehorning, castration, and weaning have also been shown to alter the fecal microbiome of dairy calves (Meale et al., 2016; Mir et al., 2019).

The objective of this study was to determine alterations in the fecal microbiome of lactating dairy cows subjected to heat stress. We hypothesized that heat stress would result in increased abundance of Gram-negative organisms in feces. We also hypothesized that there would be increased shedding of mastitis pathogens, including Gram-positive organisms such as *Streptococcus*, *Staphylococcus*, and *Enterococcus*, and Gram-negative organisms such as *Escherichia-Shigella* and *Klebsiella* genera.

## **2.3 MATERIALS AND METHODS**

### ***Cow selection***

This study was approved by the University of Missouri Animal Care and Use Committee (Protocol Number 9283). Holstein cows from the University of Missouri Foremost Dairy Research and Teaching farm were selected based on quarter milk cultures, parity, days pregnant, DIM, daily milk production, and lactational average SCC (Hirtz et al., 2018). Quarter-level milk samples were collected using aseptic technique prior to enrollment to determine if cows had an IMI (Middleton et al., 2017). Milk samples for bacterial culture were frozen for approximately 24 h, thawed at room temperature, and then plated on Columbia Blood Agar (**CBA**; 5% Sheep Blood, Remel) using a sterile cotton-tipped applicator (approximately 10 µL). Plates were incubated at 37°C for 24 h and read at 24 h and 48 h. Milk samples with ≥ one bacterial colony (approximately 100 CFU/ml) were considered to have an IMI (Dohoo et al., 2011). Bacteria were identified using MALDI-TOF mass spectrometry analysis (Wilson et al., 2019). Of the cows selected for enrollment (n = 6), milk samples from four cows had no growth and two cows had a *Staphylococcus chromogenes* IMI. None of the cows had been treated for mastitis during the current lactation.

### ***Study housing design***

Selected cows were transported to the University of Missouri Animal Science Research Center and housed in tie stalls within one chamber of the Brody Environmental Unit for the duration of the study (30 d). For this study, heat stress was defined as rectal temperature 1°C above normothermia (Rodrigues et al., 2019). Elevations in environmental temperature and humidity were used to induce an increased body

temperature. Initially, cows were given 5 d to acclimate to the chamber (rumen temperatures ~39.0°C). Ambient temperature-humidity index (**THI**) averaged 64.7 (20.2°C and 34.6% relative humidity) over the 2 d (d -1 and d 0), prior to initiation of heat stress. During the heat stress period (rumen temperatures ~ 39.8°C), average THI was 75.4 (27.1°C and 58.1% relative humidity) and was maintained from d 0 to d 16. Temperature and humidity were adjusted only to maintain cows at the target fecal temperature of 1°C above normothermia (Beatty et al., 2006). The chamber was programmed to hold body temperature constant during the heat stress phase of the study (Hirtz et al., 2018). At termination of the 16-d heat stress period, cows were monitored during an 8-d recovery period (d 16 to 24), at an average THI of 66.1 (20.2°C and 61.5% relative humidity) to maintain normothermia (rumen temperatures ~ 38.8°C).

Throughout the study, cows were milked and fed twice daily. The feed provided was the same TMR that was fed on the farm prior to the onset of the study. The TMR was formulated to meet or exceed nutrient requirements for milk yield (NRC, 2001), and was fed to achieve at least 10% refusal. Further details on environmental conditions and cow variables are presented in Hirtz et al. (2018).

### ***Sample collection***

Fecal samples were collected from all cows on d -1, 0, 6, 13, 16, 20, and 24. Samples collected on d -1 and 0 were baseline samples from the pretreatment period. Samples collected on d 6, 13, and 16 represent increasing duration of heat stress and samples collected on d 20 and 24 represent 4 or 8 d, respectively, of post-heat stress recovery. Each fecal sample was collected from the rectum without using lubricant, using a new pair of disposable gloves (Latex Exam Gloves; AmerisourceBergen; Oceanside,

CA), and placed in a sterile, 50 mL conical tube (ThermoFisher Scientific; Waltham, MA). Fecal samples were frozen at -20°C immediately after collection until further processing.

#### ***DNA extraction and sequencing***

All fecal samples were thawed, and DNA was extracted from all fecal samples (n = 42) at the University of Missouri Metagenomics Center using QIAamp PowerFecal DNA Kits (Qiagen; Germantown, MD). Per the manufacturer's instructions, approximately 0.25 g of feces were added to a bead tube and cells were lysed using a TissueLyser II (Qiagen). After multiple wash steps and centrifugations through a spin column, purified DNA was isolated. Concentration of DNA in each sample was determined using a Qubit 2.0 fluorometer (ThermoFisher Scientific) with the quant-iT BR dsDNA reagent kit (Invitrogen; Carlsbad, CA). The University of Missouri DNA Core Facility constructed the 16S rRNA libraries and performed sequencing with the Illumina MiSeq platform. The library was constructed by amplifying the V4 region of the 16S rRNA gene with universal primers (U515F/806R) flanked by Illumina standard adapter sequences (Caporaso et al., 2011). Chimeras were detected and removed with Qiime v1.8. Operational Taxonomic Units (**OTU**) were assigned based on ≥97% nucleotide identity (Caporaso et al., 2010) and were annotated against the Silva.v132 database (Quast et al., 2013) using NCBI BLAST (McGinnis and Madden, 2004).

#### ***Statistical analysis***

Alpha diversity was evaluated with the Chao1 and Shannon indices and analyzed in SigmaPlot 13.0 (Systat Software Inc., San Jose, CA, USA), using one-way repeated measures ANOVA with pairwise comparisons via Bonferroni t-tests. Data were cube root

transformed and a heat map was created to evaluate the top 25 most variable OTU and to describe the compositional differences between different days of the experiment (Xia et al., 2009). To analyze community composition, a one-way permutational multivariate analysis of variance of Bray-Curtis weighted distances was conducted using Paleontological statistics software (Hammer et al., 2001). The relative abundance of OTU were fourth root transformed to normalize the data. Temporal changes in individual phyla and genera profiles were evaluated using a repeated measures analysis with a randomized complete block design in PROC GLIMMIX (SAS Institute Inc.; Cary, NC SAS, 2010), with treatment, time and their interaction as fixed effects. Data were rank transformed due to the lack of normality, as determined by Shapiro-Wilk testing, of *Escherichia-Shigella*, *Enterococcus*, and *Streptococcus*. Statistical significance was declared at  $P < 0.05$  for individual phyla and genera profiles, and the results are reported as least squares means  $\pm$  standard error.

## 2.4 RESULTS

### ***General observations***

All the cows completed the study, and no adverse clinical health events were detected during the experiment (Hirtz et al., 2018). A total of 42 fecal samples were collected and all were successfully sequenced with an average read count of 64,699.

### ***Richness and diversity***

Heat stress increased Chao1 richness (number of OTU;  $P < 0.001$ ) of the fecal microbiome by approximately 10% compared to pretreatment d -1 (Figure 2.1A). Likewise, the Shannon index indicated an approximate 5% increase ( $P < 0.03$ ) in diversity of bacterial populations during d6 and 16 of heat stress compared to d-1 of the

pretreatment period (Figure 2.1B). Bacterial diversity returned to pretreatment levels by d20. Bacterial richness also declined after heat stress but was not fully restored to d -1 levels until d 24 ( $P < 0.05$ ). These results indicate that the number of OTU and their abundance across samples differed, due to imposition of hyperthermia followed by return to normothermia in the recovery period.

### ***Principal coordinate analysis***

Principal coordinate analysis (**PCoA**), based on Bray-Curtis weighted distances, displays samples clustered by experiment day (Figure 2.2). Coordinates one and two captured 17.5% and 10.4% of the total variation, respectively. There was a difference in the bacterial populations that comprised the samples, and the largest shift in sample composition occurred during heat stress between d13 and 16. This analysis revealed that the samples clustered together according to experimental time points ( $P < 0.001$ ;  $F = 2.59$ ). The two clusters that contain samples from the pretreatment period (d-1 and 0) overlap almost entirely and this suggests that these samples contain similar bacterial communities. The samples collected in early (d6) and mid-heat stress (d13) formed two clusters that overlapped slightly, while the samples from late heat stress (d16) overlapped only slightly with mid-heat stress (d13) but not with early heat stress (d6). Like the pretreatment samples (d-1 and 0), samples from the recovery period (d20 and 24) formed two overlapping clusters; however, those clusters did not overlap with the pretreatment clusters. This indicates that the microbial composition of feces did not fully return to that of pre-heat stress, even after 8 days of recovery under normothermia. Overall, the PcoA analysis indicates that fecal composition changed over the course of the experiment.

### ***Relative abundance of bacterial phyla***

The fecal samples comprised a total of 19 bacterial phyla, including one unidentified phylum that were present in at least six samples. Across all samples, the dominant phyla were *Firmicutes*, *Bacteroidetes*, and *Tenericutes*, which remained abundant throughout the experiment (average, approximately 51%, 42%, and 3%, respectively). Phyla relative abundance varied between cows and between experimental days. Of the major phyla, the abundance of *Firmicutes* (Figure 2.3A) and *Bacteroidetes* (Figure 2.3B) varied over experimental day, implying effects of exposure to, and recovery from, heat stress. More specifically, the abundance of *Firmicutes* was higher during pretreatment and heat stress than on d24 (day 8 of recovery;  $P < 0.05$ ). The relative abundance of *Firmicutes* was stable from early to mid-heat stress and then decreased on d16 of the heat stress period ( $P < 0.02$ ). Conversely, *Bacteroidetes* were more abundant during recovery compared to pretreatment and to d6 and 13 of heat stress ( $P < 0.05$ ). Like *Firmicutes*, the relative abundance of *Bacteroidetes* remained stable from early to mid-heat stress but, in contrast, the abundance of *Bacteroidetes* increased on d 16 of heat stress ( $P < 0.02$ ). This denotes that the most striking change in the relative abundance of Gram-negative phyla, *Bacteroidetes*, occurred in late heat stress.

#### ***Relative abundance of bacterial families***

Overall, 165 bacterial families were identified, including those that belong to an ambiguous taxon ( $n = 4$ ) or were identified as uncultured organisms ( $n = 29$ ). Of these, the top 20 families are displayed (Figure 2.4), while all others were combined to form the group “Other”. Like phyla abundance, there was variation in family relative abundance between cows, throughout the experiment. However, the less abundant bacterial families appear more variable compared to the more abundant families. The dominant families

across all samples were *Ruminococcaceae*, *Rikenellaceae*, and *Bacteroidaceae*, and these families remained abundant throughout the experiment (approximately 36%, 22%, and 7% respectively). The *Ruminococcaceae* family belongs to the *Firmicutes* phylum, while the *Rikenellaceae* and *Bacteroidaceae* families belong to the *Bacteroidetes* phylum. Like the dominant phyla, the most prevalent families remained dominant throughout the study while some families appear more responsive to heat stress as shown by the numerical changes in relative abundance (Figure 2.4).

### ***Hierarchical clustering***

The 25 most variable OTU were subjected to hierarchical clustering, which revealed a pronounced influence of experiment day on patterns of OTU abundance in fecal samples (Figure 2.5). Samples distributed into two main clusters, with two large subclusters within each major cluster. One main cluster included samples exclusively from days 16, 20, and 24, representing late heat stress and recovery. Notably, the three tertiary subclusters within this large group generally distinguished different sample days. Similarly, the second main cluster included two large subclusters, one containing exclusively samples from pretreatment (d-1 and 0) and the other comprised almost exclusively (12/13 samples) samples from early heat stress (d6 and 13). These results indicate that the relative abundance of the 25 most variable OTU differed across sample days, consistent with a response to imposition and cessation of heat stress.

### ***Top 25 most variable OTU***

The taxonomic information (Table 2.1) for the 25 most variable OTU (from Figure 2.5) show that these OTU belonged mostly to the *Bacteroidetes* and *Firmicutes* phyla (6/25 and 14/25 OTU, respectively). The OTU belonging to the *Bacteroidetes*

phyla were numerically higher during recovery compared to the pretreatment period. Except for two OTU, the OTU in the heat map that belonged to the *Firmicutes* phyla were less abundant during recovery compared to pretreatment. Other phyla represented among the 25 most variable OTU were *Proteobacteria*, *Kiritimatiellaeota*, *Cyanobacteria* and *Tenericutes*. The consistency of changes across, and grouping within, experiment day suggests that these OTU varied with cow body temperature and time. These OTU appear to be hyperthermia responsive.

#### ***Individual mastitis pathogens at the genera level***

To assess potential links between heat stress-related changes in fecal bacterial composition and fecal abundance of mastitis pathogens, we evaluated changes in abundance of *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Escherichia-Shigella*, and *Klebsiella* genera. No *Staphylococcus* or *Klebsiella* genera were identified in the samples. There was no difference in the relative abundance of *Streptococcus* or *Enterococcus* genera throughout the study ( $P > 0.50$ ). However, the relative abundance of *Escherichia-Shigella* tended to be higher over time ( $P = 0.07$ ). Specifically, *Escherichia-Shigella* was higher ( $P < 0.01$ ; Figure 2.6) during pretreatment and heat stress, compared to recovery. Of the mastitis pathogens evaluated, only *Escherichia-Shigella* differed over time.

## **2.5 DISCUSSION**

The results from this study provide evidence that the fecal microbiome of lactating dairy cows is affected by exposure to, and recovery from, heat stress, as reported previously (Chen et al., 2018; Li et al., 2019). Prior experiments to determine changes in the fecal microbiome of heat stressed dairy cows have been performed on a single day, comparing cows that appeared clinically heat stress to those that were not

showing clinical signs (Chen et al., 2018) or carried out over multiple seasons (Li et al., 2019). Contrasting previous studies, the present study design allowed for sampling of cows immediately prior to, during, and after heat stress which allowed for detection of changes in microbial populations at the onset of heat stress and over a prolonged period. By using environmental chambers and inducing heat stress, we were able to control variables that are known to affect the fecal microbiome, such as cow, diet, stage of lactation, and age (Mao et al., 2015; Kim et al., 2016; Paz et al., 2016).

The richness and diversity of bacterial populations in the fecal samples were higher during heat stress and began to return to pretreatment levels by the last sampling day (Figure 2.1). These findings conflict with a recent study by Li et al. (2019) who reported decreased Shannon diversity and a trend for decreased richness in the summer versus spring in the feces of heat-stressed cows. Chen et al. (2018) also reported greater richness in the fecal samples of non-heat stressed dairy cows compared to heat-stressed cows. However, the previous study (Chen et al., 2018) evaluated two groups of high- or low-heat sensitive cows, basing sensitivity on panting score, milk yield, DIM, and parity, under heat stress or thermoneutral conditions. For the present study, cows were sampled and assessed over time, providing the ability to determine changes at the onset, after prolonged, and during recovery from heat stress. The current study evaluated cows that were subjected to constant heat stress over a period of 16 days which can explain some of the differences seen in richness and diversity between studies.

Aside from the studies by Chen et al. (2018) and Li et al. (2019), there is limited information regarding the effects of heat stress on the fecal microbiome of dairy cattle. However, several studies have examined the effects of other stressors. Weaning stress in

general has been reported to alter the rumen and fecal microbiomes of dairy calves more so than weaning strategy (abrupt vs gradual; Meale et al., 2016). Dehorning and castration of dairy calves have also been reported to alter the fecal microbiome in light and heavy-weight calves (Mir et al., 2019). A decrease in diversity of bacterial populations after dehorning and castration was reported, which was more pronounced in light-weight calves. The previously mentioned studies differed from the present study in that they collected samples from dairy calves vs mature animals and at different and fewer time points. The current study found diversity and richness to be greater during heat stress compared to the pretreatment period, but both returned to pre-treatment levels by the last day of sampling, which conflicts with Mir et al. (2019) who reported greater richness and diversity prior to treatment. These effects cannot be fully attributed to dehorning, castration, and weaning due to the confounding issues of animal maturation and diet. Nevertheless, the common feature with our study is that various stressors potentially cause changes in the fecal microbiome. How such changes may relate to animal health and performance remains to be established. Interestingly, even with animals of vastly different ages compared to the present study, the same dominant phyla were present consistently in feces collected from calves and cows across all the previously mentioned studies which suggests that there may be a core microbiome.

The bacteria within the *Bacteroidetes* and *Firmicutes* phyla are responsible for producing short chain fatty acids, like Butyrate, in the rumen which supplies energy to the cow. Several studies have reported that these phyla are the dominant bacterial phyla in the feces of dairy cows and calves (Meale et al., 2016; Li et al., 2018; Mir et al., 2019). Alterations to the relative abundances of these phyla during heat stress might alter energy

metabolism in the cow and their abundances have been reported to differ between heat stressed and non-heat stressed cows (Chen et al., 2018). Chen et al. (2018) reported that the abundance of *Firmicutes* was lower and *Bacteroidetes* was higher in heat stressed cows compared to non-heat stressed cows. However, the present study found the relative abundance of *Firmicutes* and *Bacteroidetes* changed markedly and in opposite directions, but only after prolonged heat stress and continued into recovery (Figure 2.3). This finding indicates that these bacterial populations may be slow to change, both in response to hyperthermia and upon return to normothermia. Hence, longer periods of heat stress and recovery may be needed to determine how long these effects persist. Like this heat stress study, dehorning and castration also resulted in a decreased *Firmicutes* to *Bacteroidetes* ratio (Mir et al., 2019). In contrast, weaning stress resulted in decreased relative abundance of *Bacteroidetes* and increased relative abundance of *Firmicutes* compared to feces collected pre-weaning (Meale et al., 2016). In general, the fecal microbiome changes in response to stressors and changes may occur at different speeds, depending on the type of stress.

Within a phylum, multiple families and genera responded differently to the effects of, and relief from, heat stress. Based on the two main clusters in the heat map (Figure 2.5), there appear to be prolonged effects of heat stress on the fecal microbiome as evidenced by the more similar composition between samples collected on the last day of heat stress and during recovery, which is also noted in Figure 2. These results indicate that heat stress has prolonged effects on the fecal microbiome, specifically shown by alterations in the abundance of some OTU.

The OTU belonging to the *Alistipes*, *Clostridium sensu stricto 1*, and *Romboutsia* genera were reported in this study (Table 2.1) and a recent study by Chen et al. (2018). *Alistipes* and *Romboutsia* were reported to be more abundant in the feces of heat stressed cows (low-heat sensitive, high-heat sensitive, respectively) in the study by Chen et al. (2018) and in the current study, during the heat stress period. Also, *Clostridium sensu stricto 1* was more abundant in the feces of high-heat sensitive heat stressed cows in the study by Chen et al. (2018). However, in the present study, this OTU was less abundant during heat stress. These genera did not show the same responses to heat stress in the two studies, which is likely due to different sampling times and overall study design. However, because their relative abundance is altered by stressors, it is plausible that these genera are important in maintaining gut homeostasis during times of stress. In the current study, abundance of *Blautia*, a member of the *Firmicutes* phylum, decreased post-heat stress, and this decrease has also been reported in feces of calves post-weaning (Meale et al. 2016). A study conducted in poultry also reported lower relative abundance of *Blautia* in the feces of heat stressed laying hens compared to controls (Zhu et al., 2018). *Firmicutes* produce butyrate and decreased in late heat stress while some genera within this phylum increased during heat stress (*Blautia*, *Clostridium sensu stricto 1*, and *Romboutsia*). It is possible that these genera increased to compensate for decreases in other *Firmicutes* genera.

In milk samples, OTU that are typically found in gastrointestinal and skin samples such as those in the phylum *Bacteroidetes*, have been reported (Metzger et al. 2018). In the current study, several of the genera associated with mastitis were not detected in feces, and changes in the abundance of *Escherichia-Shigella* differed over time (Figure

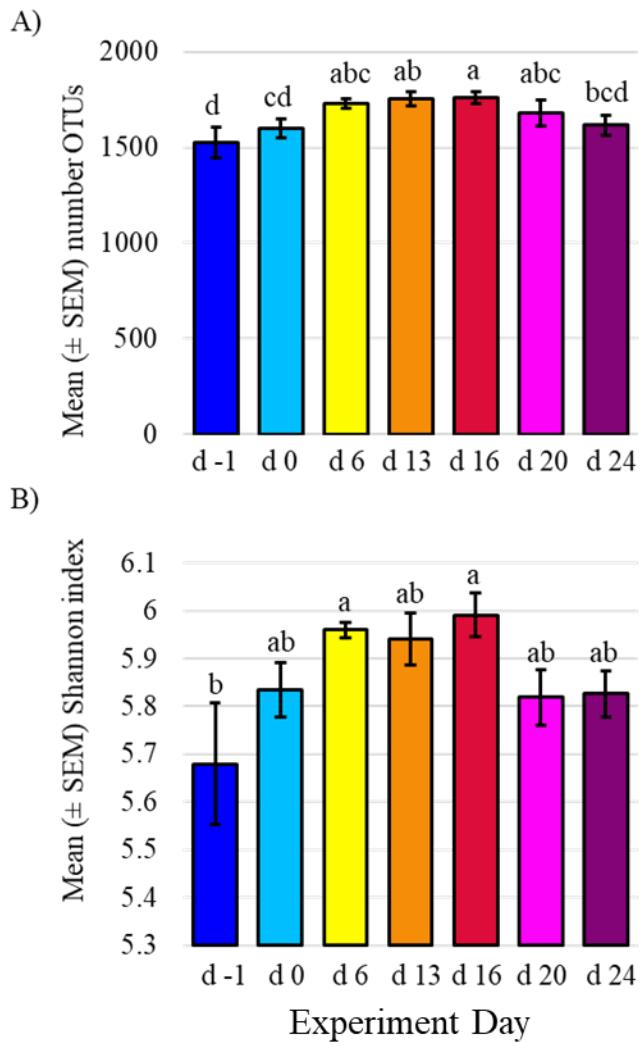
6). During recovery, *Escherichia-Shigella* was lower compared to the pretreatment and heat stress periods. Abundance of *Streptococcus* and *Enterococcus* did not differ over time. Although others have identified *Staphylococcus* (Waytack et al., 2019) and *Klebsiella* (Zadoks et al, 2011) in the feces of dairy cattle using traditional culture, these genera were not found in the samples in this study; potentially due to a very low abundance or lack of these organisms in feces compared to other, more dominant, microbiota. The abundance of *Streptococcus* in the feces of calves has been shown to be low prior to weaning and decrease post-weaning (Meale et al., 2016). Additionally, *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Escherichia-Shigella* genera have been detected in the fecal microbiome of calves (Alipour et al., 2018). Of note, *Staphylococcus* was only present (2.5% abundance) in samples from newborn dairy calves collected within 10 minutes of birth while *Streptococcus* was present in newborn, 24-hour and 7-day samples with abundance decreasing over time (2.7%, 0.2% and 0.1%, respectively). *Escherichia-Shigella* and *Enterococcus* were only present in the 24-hour samples (76.8% and 0.8% abundance, respectively). It is possible that these *Staphylococcus*, *Streptococcus*, and *Enterococcus* genera are more abundant in younger animals and decrease as the animal matures. It is also possible that other factors, such as farm, diet, and environment play a role in the presence of these organisms in the feces of adult dairy cattle. Further studies are needed to explore these possibilities.

During this experiment, the changes in the fecal microbiome were monitored over a period of 30 days (16 days of constant heat stress), and it is currently unknown if the changes observed would be sustained, or magnified, during longer periods of heat stress. The current study has provided new insights into changes within the fecal microbiome

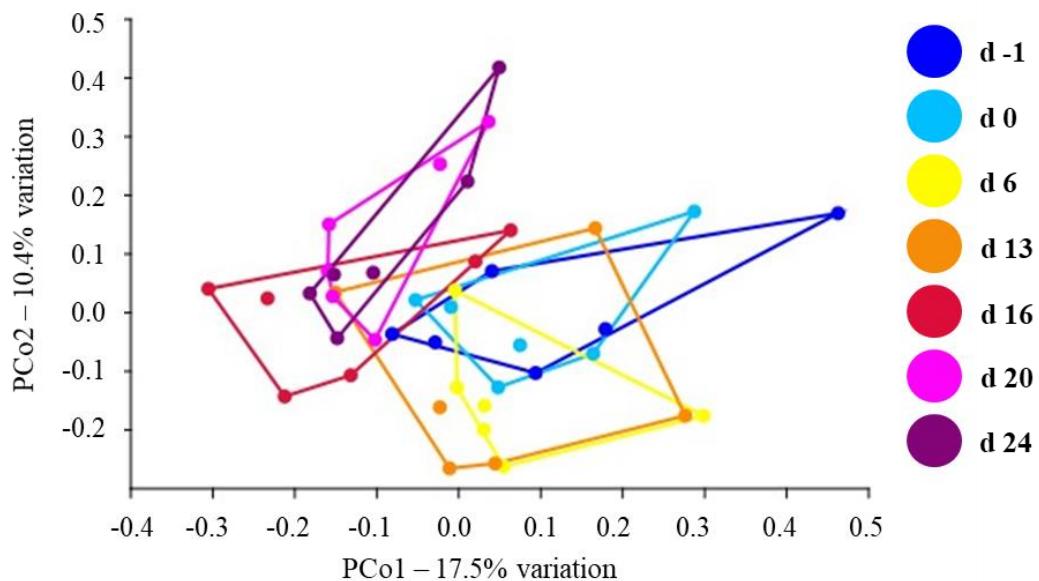
and future work will be done to determine if these changes are persistent in other microbial niches within the cow. It is also unknown if the changes in the composition of the fecal microbiome during heat stress eventually revert, after the cessation of heat stress, to a composition more like pre-treatment. It is clear from this and previous studies that the fecal microbiome changes in response to stressors, but the nature and degree of changes is highly variable and requires further study. Like other time course studies, there were inherent limitations in the experimental design of this study, including the inability to distinguish effects of heat stress from confounding effects of time on the study; those effects could include reduced feed intake, advancing lactation and gestation, accumulated stress of restraint in stanchions within the chamber, and potential acclimation to elevated THI. Nevertheless, the most plausible interpretation of these results is that the fecal microbiome was affected by imposition of, then relief from, hyperthermia.

## **2.6 CONCLUSIONS**

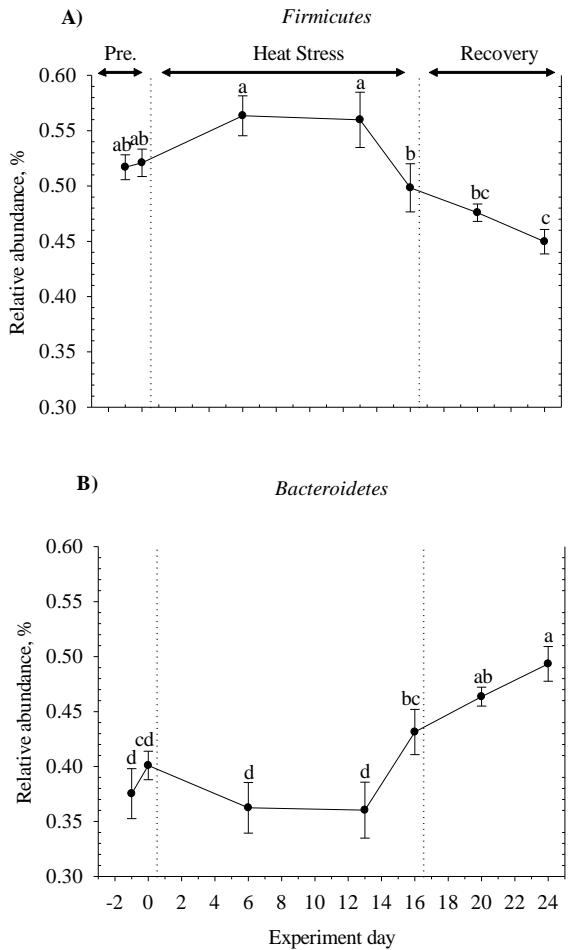
We conclude that 16-d of constant heat stress altered the fecal microbiome of lactating dairy cows affecting the richness and diversity of bacterial populations. Individual OTU responded differently to heat stress, enabling us to identify hyperthermia responsive OTU in feces.



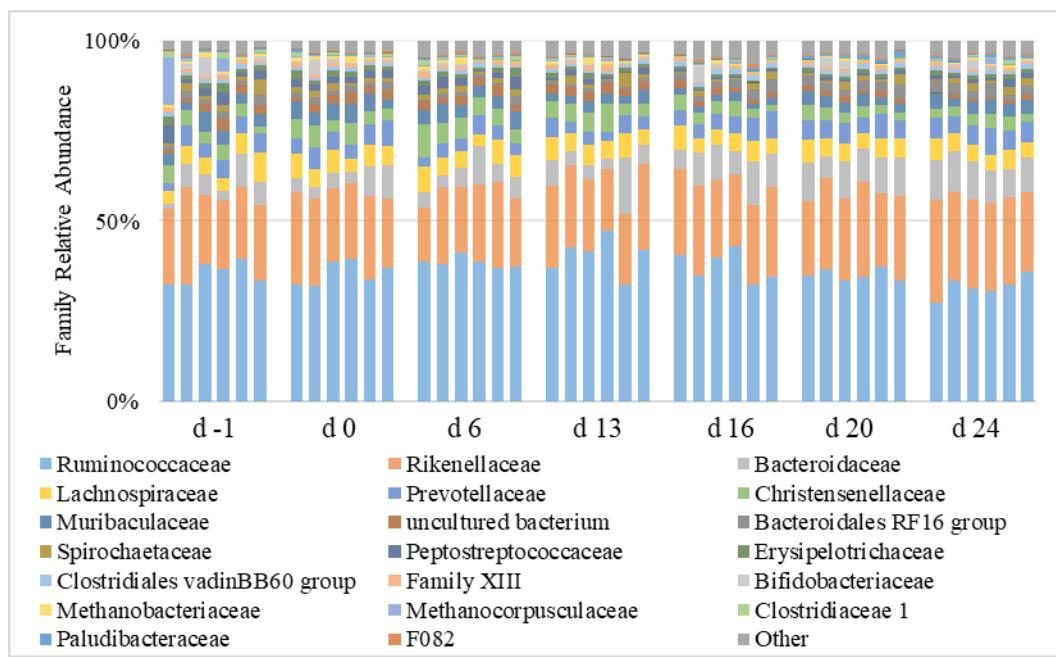
**Figure 2.1.** Changes in A) richness (number of OTU) and B) Shannon diversity of fecal microbiome in cows subjected to 16 days of heat stress followed by an 8-day recovery period. Richness was calculated using the Chao1 index ( $P < 0.001$ ,  $F = 8.92$ ); diversity was calculated within sample day using a Shannon index ( $P = 0.007$ ,  $F = 3.76$ ). Samples were collected during pretreatment (d -1 and 0), heat stress (d 6, 13, and 16), and recovery (d 20 and 24). <sup>a,b,c,d</sup>Within panel, means lacking a common superscript letter differ ( $P < 0.05$ ).



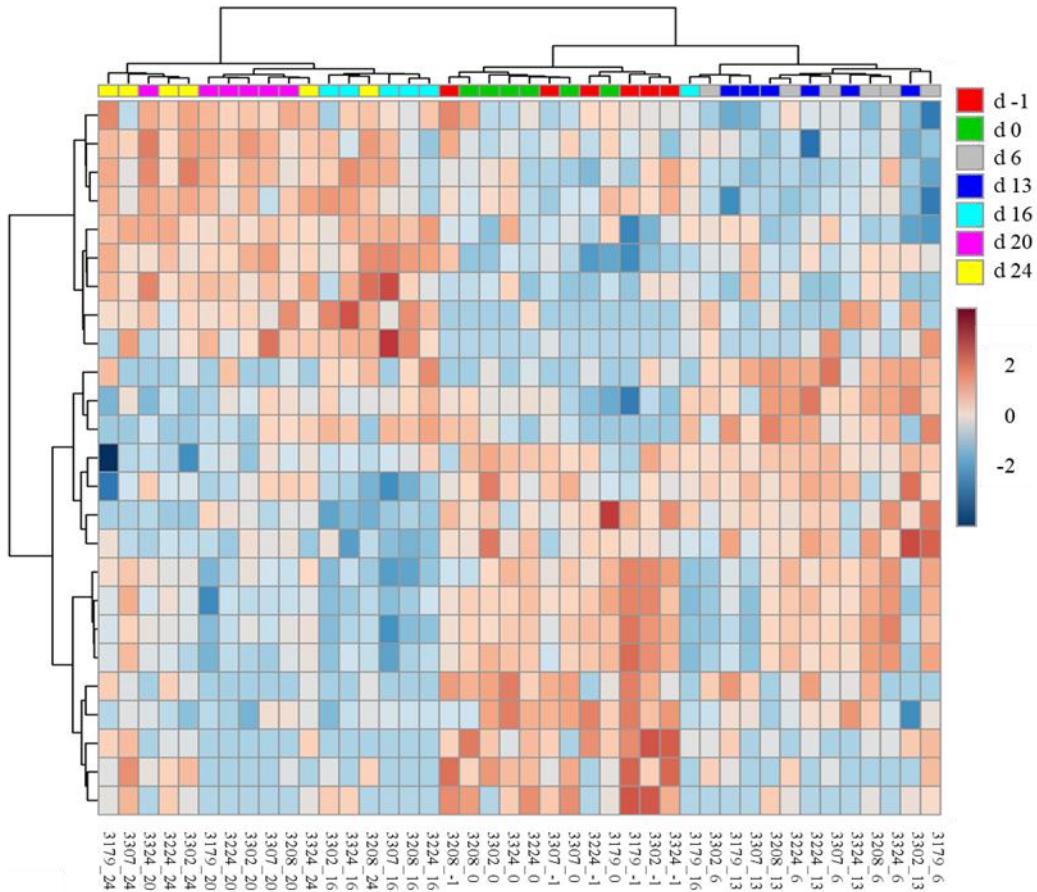
**Figure 2.2.** Changes in the fecal microbiome of cows subjected to heat stress shown as principal coordinate analysis clustering of the bacterial communities that make up the samples based on Bray-Curtis (weighted) distances ( $P < 0.001$ ;  $F = 2.59$ ). Coordinates 1 and 2 account for 17.5% and 10.4% of the variation, respectively. Different colors in the legend to the right represent days of sample collection. Fecal samples were collected during pretreatment (d -1 and 0), heat stress (d 6, 13, and 16), and recovery (d 20 and 24).



**Figure 2.3.** Changes in relative abundance of major phyla identified in feces of lactating cows before, during and after exposure to elevated THI. A) *Firmicutes*. B) *Bacteroidetes*. Samples were collected during pretreatment (Pre; d -1 and 0), heat stress (d 6, 13, and 16), and recovery (d 20 and 24). <sup>a,b,c,d</sup>Within panel, means lacking a common superscript letter differ ( $P < 0.05$ ). Bars represent standard error of the mean.



**Figure 2.4.** Relative abundance of bacterial families in fecal samples collected from cows ( $n = 6$ ) prior to (d -1 and 0), during (d 6, 13, and 16) or after (d 20 and 24) exposure to heat stress. The top 20 most abundant families are displayed; all other families were combined into the “Other” category. Individual cows are listed in the same order, left to right, within each sample day.



**Figure 2.5.** Hierarchical heat map displaying changes in relative abundance of the most variable OTU in feces of lactating cows exposed to heat stress. The 25 OTU that varied most across the experiment are represented by rows; each sampling day for each cow is represented by a column ( $n = 42$ ). The map was self-assembled using hierarchical clustering. The “heat” scale shows relative abundance of a specific OTU (red = higher abundance; blue = lower abundance). Each sample collection day is represented by a different colored box (in key and across the top of the map): pretreatment (d -1 and 0), heat stress (d 6, 13, and 16) and recovery (d 20 and 24). Dendograms show clustering of samples by day (across top) or by OTU (at left).

**Table 2.1.** Top 25 most variable OTU identified in the hierarchical heat map<sup>1</sup>

Phylum	Class	Order	Family	Genus	Pretreatment		Heat Stress		Recovery	
					Mean <sup>2</sup>	SEM <sup>3</sup>	Mean <sup>2</sup>	SEM <sup>3</sup>	Mean <sup>2</sup>	SEM <sup>3</sup>
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidales RF16 group	Ambiguous taxa	1.68E-03	5.72E-04	7.78E-04	1.67E-04	3.10E-03	4.91E-04
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidales RF16 group	uncultured Porphyromonadaceae bacterium	2.16E-03	2.95E-04	1.56E-03	3.16E-04	3.26E-03	3.24E-04
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidales RF16 group	uncultured bacterium	1.16E-02	1.61E-03	1.16E-02	1.68E-03	2.13E-02	2.01E-03
Bacteroidetes	Bacteroidia	Bacteroidales	Paludibacteraceae	uncultured	2.00E-03	4.98E-04	1.47E-03	3.21E-04	6.23E-03	8.87E-04
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	<i>Alistipes</i>	3.71E-02	3.07E-03	4.32E-02	2.99E-03	5.41E-02	1.66E-03
Bacteroidetes	Bacteroidia	Bacteroidales	uncultured	uncultured bacterium	1.44E-03	2.34E-04	2.85E-03	3.09E-04	3.63E-03	3.05E-04
Cyanobacteria	Melainabacteria	Gastranaerophilales	uncultured rumen bacterium 4C0d-2	uncultured rumen bacterium 4C0d-2	2.59E-05	1.15E-05	1.40E-04	2.47E-05	2.07E-05	8.48E-06
Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	4.61E-05	7.84 E-06	1.54E-05	5.17E-06	9.98E-06	3.37E-06
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	<i>Brevibacillus</i>	5.80E-05	1.39E-05	8.71E-06	2.81E-06	9.83E-06	4.14E-06
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	<i>Paenibacillus</i>	6.02E-05	1.43E-05	8.41E-06	3.26E-06	1.98E-05	7.41E-06
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	<i>Paenibacillus</i>	6.63E-05	1.60E-05	1.12E-05	3.27E-06	1.11E-05	4.50E-06
Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	<i>Clostridium sensu stricto 1</i>	6.94E-03	1.38E-03	3.62E-03	8.69E-04	2.62E-03	4.55E-04
Firmicutes	Clostridia	Clostridiales	Family XIII	<i>Mogibacterium</i>	5.94E-04	7.03E-05	6.28E-04	1.05E-04	3.67E-04	2.92E-05
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Acetitomaculum</i>	2.44E-04	3.82E-05	2.32E-04	3.55E-05	1.62E-04	2.50E-05
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Blautia</i>	1.33E-03	2.10E-04	9.45E-04	1.19E-04	6.45E-04	6.41E-05
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	<i>Paeniclostridium</i>	1.07E-02	1.82E-03	5.61E-03	1.22E-03	3.50E-03	6.92E-04
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	<i>Romboutsia</i>	1.10E-02	1.25E-03	6.53E-03	1.20E-03	5.29E-03	4.80E-04

**Table 2.1.** Top 25 most variable OTU identified in the hierarchical heat map (continued)<sup>1</sup>

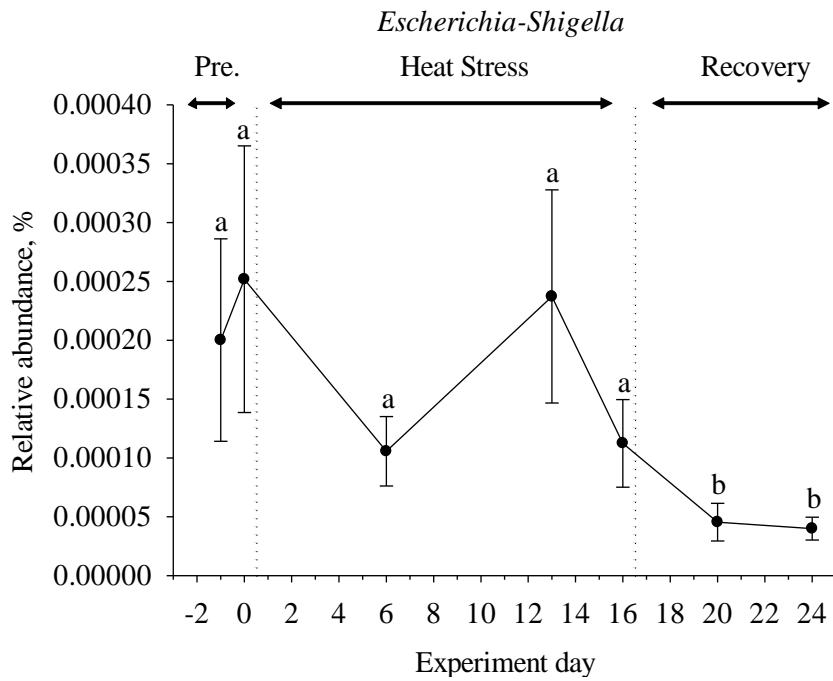
Phylum	Class	Order	Family	Genus	Pretreatment		Heat Stress		Recovery	
					Mean <sup>2</sup>	SEM <sup>3</sup>	Mean <sup>2</sup>	SEM <sup>3</sup>	Mean <sup>2</sup>	SEM <sup>3</sup>
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Ruminiclostridium</i> 5	1.15E-03	8.70E-05	6.95E-04	5.33E-05	6.22E-04	3.97E-05
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Ruminococcaceae NK4A214 group</i>	2.75E-03	2.37E-04	4.38E-03	2.34E-04	2.88E-03	1.94E-04
Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	<i>Turicibacter</i>	9.75E-03	1.51E-03	5.08E-03	1.10E-03	3.49E-03	8.45E-04
Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	uncultured	3.70E-06	3.70E-06	1.20E-04	4.46E-05	7.43E-05	2.03E-05
Kiritimatiellaeota	Kiritimatiellae	WCHB1-41	uncultured Kiritimatiellaeota bacterium	Kiritimatiellaeota bacterium	1.30E-06	1.30E-06	6.07E-05	2.74E-05	5.13E-05	1.56E-05
Proteobacteria	Alphaproteobacteria	Rickettsiales	Mitochondria	metagenome	8.05E-06	3.12E-06	4.73E-05	7.22E-06	1.35E-05	5.60E-06
Proteobacteria	Gammaproteobacteria	Betaproteobacterales	Burkholderiaceae	<i>Parasutterella</i>	2.08E-05	6.74E-06	8.31E-05	4.62E-05	1.47E-04	3.67E-05
Tenericutes	Mollicutes	Mollicutes RF39	uncultured bacterium	uncultured bacterium	2.25E-02	2.39E-03	2.10E-02	1.28E-03	1.10E-02	1.39E-03

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<sup>1</sup>The top 25 most variable OTU that correspond to the heat map rows starting at the first row of the map and continuing down (Figure 5) and their taxonomic information for each. Species are not listed as most resulted in “ambiguous taxa” or “uncultured bacterium”.

<sup>2</sup>Mean indicates the average percent relative abundance of each OTU per period of the experiment.

<sup>3</sup>SEM corresponds to the standard error of the mean percent relative abundance.



**Figure 2.6.** Changes in relative abundance of *Escherichia-Shigella* in fecal samples from lactating cows before (Pre; d -1 and 0), during (heat stress; d 6, 13, and 16), and after (recovery; d 20 and 24) exposure to heat stress. <sup>a,b</sup>Within figure, means lacking a common superscript letter differ ( $P < 0.05$ ).

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**CHAPTER III**  
**INFLUENCE OF PCR CYCLE NUMBER ON 16S rRNA GENE AMPLICON**  
**SEQUENCING OF LOW BIOMASS SAMPLES**

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## **INFLUENCE OF PCR CYCLE NUMBER ON 16S rRNA GENE AMPLICON SEQUENCING OF LOW BIOMASS SAMPLES**

### **3.1 ABSTRACT**

The objective of this study was to evaluate the effects of increased PCR cycle number on sequencing results from samples with low microbial biomass, including bovine milk, and murine pelage and blood. We hypothesized that subjecting DNA from such samples to higher PCR cycle numbers would increase 16S rRNA sequencing coverage. DNA was extracted from matched samples of each type and multiple PCR cycle numbers were evaluated to generate a total of 96 libraries from 24 milk samples, 46 libraries from 23 pelage samples, and 170 libraries from 85 blood samples. 16S rRNA sequencing was performed on the Illumina MiSeq platform, and the coverage per sample, detected richness, and beta-diversity were evaluated. Across all sample types, higher PCR cycle numbers were associated with increased coverage. Surprisingly however, while higher PCR cycle numbers resulted in greater number of useable datapoints, no differences were detected in metrics of richness or beta-diversity. While reagent controls amplified for 40 cycles yielded similarly increased coverage, control and experimental samples were clearly differentiated based on beta-diversity. The results from this study support the use of higher PCR cycle numbers to evaluate samples with low microbial biomass.

### **3.2 INTRODUCTION**

Next-generation sequencing of targeted amplicon libraries (e.g., 16S rRNA or internal transcribed spacer [ITS] libraries) allows researchers to characterize the taxonomic composition of complex bacterial or fungal communities. As these libraries

rely on PCR to amplify a variable region of the microbial genome, they can be performed with relatively small amounts of total bacterial biomass. When extracting DNA from feces, for example, the vast majority of the sample weight is bacterial biomass and one-quarter of a mouse fecal pellet provides more than enough DNA. There is however an emerging interest in the bacterial communities present in other biological samples, such as blood and milk (Young et al., 2015; Metzger et al., 2018b). Such samples are often difficult to sequence using next-generation sequencing technologies due to low microbial biomass and excessive contamination with host cells. While there are commercially available kits designed to separate eukaryotic and prokaryotic DNA, they are not practical in situations where there is also relatively low bacterial biomass, because of the large quantity of starting material required to achieve enough bacterial biomass.

During library generation using fecal DNA, most laboratories use relatively low PCR cycle numbers, most commonly 25 cycles (Ericsson et al., 2015; Young et al., 2015). Previous studies have evaluated the effects of PCR cycle number on next-generation sequencing results (Wu et al., 2010; Sze and Schloss, 2019). However, these studies commonly used moderate to high microbial biomass samples such as soil and feces, or mock communities. Studies that evaluated samples with low microbial biomass, such as milk or blood, often used much higher PCR cycle numbers such as 35 (Andrews et al., 2019; Dahlberg et al., 2019) and 40 (Young et al., 2015; Metzger et al., 2018a; Metzger et al., 2018b), but did not show direct comparisons of libraries created at different cycle numbers from matched samples. Higher cycle numbers have been shown to lead to decreased data quality when working with high biomass samples (Sze and Schloss 2019), however low biomass samples often return uninterpretable data (Bjerre et

al., 2019) due to low coverage or a complete absence of PCR amplification. For low biomass samples, the potential benefit of increased coverage may outweigh concerns of read quality, which can be filtered out during the bio-informatics stage of analysis.

The objective of this study was to evaluate the effects of PCR cycle number on the sequencing results from three different sample types, which are known to contain low microbial biomass and variable host DNA content. We hypothesized that an increased PCR cycle number would allow for the successful sequencing of low microbial biomass samples. DNA was extracted from matched milk, furred pelage, and blood samples, amplified using different PCR cycle numbers (25, 30, 35, or 40 cycles in the case of milk; 25 and 40 in the case of pelage and blood), and then sequenced on the Illumina MiSeq platform to compare data quality and agreement between libraries created using differing numbers of PCR cycles.

### **3.3 MATERIALS AND METHODS**

#### *Sample collection*

Lactating Holstein dairy cows were selected for inclusion in the study based on somatic cell count (SCC, < 100,000 cells/mL). Quarter level milk samples were collected from cows (n = 10) at the University of Missouri Foremost Dairy Research and Teaching farm just before milking. Milk samples were collected aseptically, according to the National Mastitis Council recommendations (Middleton et al., 2017). Briefly, the cows' teats were pre-dipped with an iodine-based teat dip, wiped with an individual cloth, and foremilk was stripped. Then, teat ends were cleaned with 70% Isopropyl alcohol, 2-3 streams of milk were discarded, and approximately 30 mL of milk was collected into 50

mL sterile conical tubes (Falcon, Corning, New York). The samples were frozen at -20°C until further processing.

Full thickness dorsal pelage samples, measuring approximately 3 × 2 cm, were collected post-mortem from 23 adult outbred CD-1 mice of both sexes. Mice were bred and group-housed under barrier conditions in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) international-accredited facility, in individually ventilated microisolator cages, on a 14:10 light:dark cycle, and received autoclaved standard chow (Purina LabDiet 5058) and autoclaved, acidified water ad libitum. Samples were collected using aseptic technique on a downdraft table, immediately placed in sterile Eppendorf tubes, and frozen at -20°C until further processing.

Blood samples were collected postmortem via cardiac puncture, placed in anti-coagulant (EDTA)-tubes and frozen at -20°C until further processing. While these mice were from a different cohort than that used for the pelage samples, the generation and maintenance of mice was exactly as described above.

#### ***Milk culture***

Milk samples for culture and sequencing were thawed at room temperature (approximately 25°C) and five aliquots of 1.5 mL were prepared using sterile 2 mL tubes (Thermo Scientific, Waltham, MA). One aliquot of each milk sample was cultured with traditional methods to determine if an intramammary infection was present (Middleton et al., 2017). Culturing was performed using a sterile cotton-tipped swab to spread approximately 10 µL of milk onto one-half of a Columbia Blood Agar plate (5% Sheep Blood, Remel, Lenexa, KS). The plates were incubated at 37°C and read at 24 and 48

hours. Only samples that had no bacterial growth at the 48-hour reading were used for downstream sequencing ( $n = 24$ ).

### ***DNA Extraction***

#### ***Milk sample preparation***

The remaining four aliquots of 1.5 mL each, were centrifuged at  $13,000 \times g$  for 20 minutes to separate the fat layer from the supernatant. After centrifugation, a sterile cotton tipped swab was used to remove the layer of fat. Samples were then vortexed to resuspend the pellet. Of the four aliquots of each milk sample ( $n = 96$ ), 800  $\mu$ L of milk was used as the starting material for DNA extraction.

#### ***DNA Extraction and quantification***

DNA was extracted from the milk samples (800  $\mu$ L/sample), full thickness pelage, and blood (800  $\mu$ L/sample) with a PowerFecal DNA Isolation Kit (Qiagen, Germantown, MD) following the manufacturer's instructions, with the exception that samples were initially subjected to mechanical cell lysis with a TissueLyser II (10 min at 30 Hz, Qiagen), rather than using the vortex adaptor described in the protocol. After extraction, DNA was quantified via fluorometry (Qubit 2.0) using 10  $\mu$ L of sample DNA and the quant-iT Broad Range dsDNA assay (Life Technologies, Carlsbad, CA).

#### ***Library preparation and sequencing of the 16S rRNA gene amplicons***

For milk, 96 libraries were generated using four matched DNA samples from 24 quarter level milk samples. There were 24 libraries generated using each of the four PCR cycle numbers tested, including 25, 30, 35, and 40, all of which were sequenced on a single flow cell. For pelage, 46 libraries were generated using two matched DNA samples from 23 mice (23 generated using 25 PCR cycles, and 23 generated using 40 cycles) on a

single flow cell. For blood, 170 libraries were generated, using two matched DNA samples from 85 mice (85 generated using 25 cycles, an 85 generated using 40 cycles); only the latter were sequenced, again on a single flow cell. Library preparation and Illumina sequencing were performed at the University of Missouri DNA Core Facility. The V4 region of the 16S rRNA gene was amplified with the use of previously published universal primers (U515F/806R) (Caporaso et al. 2011) and flanked with Illumina adapter sequences (Walters et al., 2011). Dual-indexed forward and reverse primers were used in all reactions. PCR was performed in 50 µL reactions containing 100 ng metagenomic DNA, primers (0.2 µM each), dNTPs (200 µM each), and Phusion high-fidelity DNA polymerase (1U). Amplification parameters were  $98^{\circ}\text{C}^{(3:00)} + [98^{\circ}\text{C}^{(0:15)} + 50^{\circ}\text{C}^{(0:30)} + 72^{\circ}\text{C}^{(0:30)}] \times 25$  to 40 cycles (as indicated)  $+72^{\circ}\text{C}^{(7:00)}$ . Amplicon pools (5 µL/reaction) were combined, thoroughly mixed, and then purified by addition of Axygen Axyprep MagPCR clean-up beads to an equal volume of 50 µL of amplicons and incubated for 15 minutes at room temperature. Products were then washed multiple times with 80% ethanol and the dried pellet then resuspended in 32.5 µL EB buffer, incubated for two minutes at room temperature, and then placed on the magnetic stand for five minutes. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system, quantified using quant-iT HS dsDNA reagent kits, and diluted according to Illumina's standard protocol for sequencing on the MiSeq instrument. All libraries, except those using blood DNA and amplified for 25 cycles, were sequenced on the Illumina MiSeq platform following the manufacturer's protocol.

### ***Bioinformatics***

The DNA sequences were assembled and annotated at the MU Informatics Research Core Facility. Primers were designed to match the 5' ends of the forward and reverse reads. Cutadapt (version 2.6; <https://github.com/marcelm/cutadapt>) was used to remove the primer from the 5' end of the forward read. If found, the reverse complement of the primer to the reverse read was then removed from the forward read as were all bases downstream. Thus, a forward read could be trimmed at both ends if the insert was shorter than the amplicon length. The same approach was used on the reverse read, but with the primers in the opposite roles. Read pairs were rejected if one read or the other did not match a 5' primer, and an error-rate of 0.1 was allowed. Two passes were made over each read to ensure removal of the second primer. A minimal overlap of 3 with the 3' end of the primer sequence was required for removal.

The Qiime2 dada2 plugin (version 1.10.0) was used to denoise, de-rePLICATE, and count ASVs (amplicon sequence variants), incorporating the following parameters: 1) forward and reverse reads were truncated to 150 bases, 2) forward and reverse reads with number of expected errors higher than 2.0 were discarded, and 3) Chimeras were detected using the "consensus" method and removed. R version 3.5.1 and Biom version 2.1.7 were used in Qiime2. Taxonomies were assigned to final sequences using the Silva.v132 database, using the classify-sklearn procedure.

### ***Statistical methods***

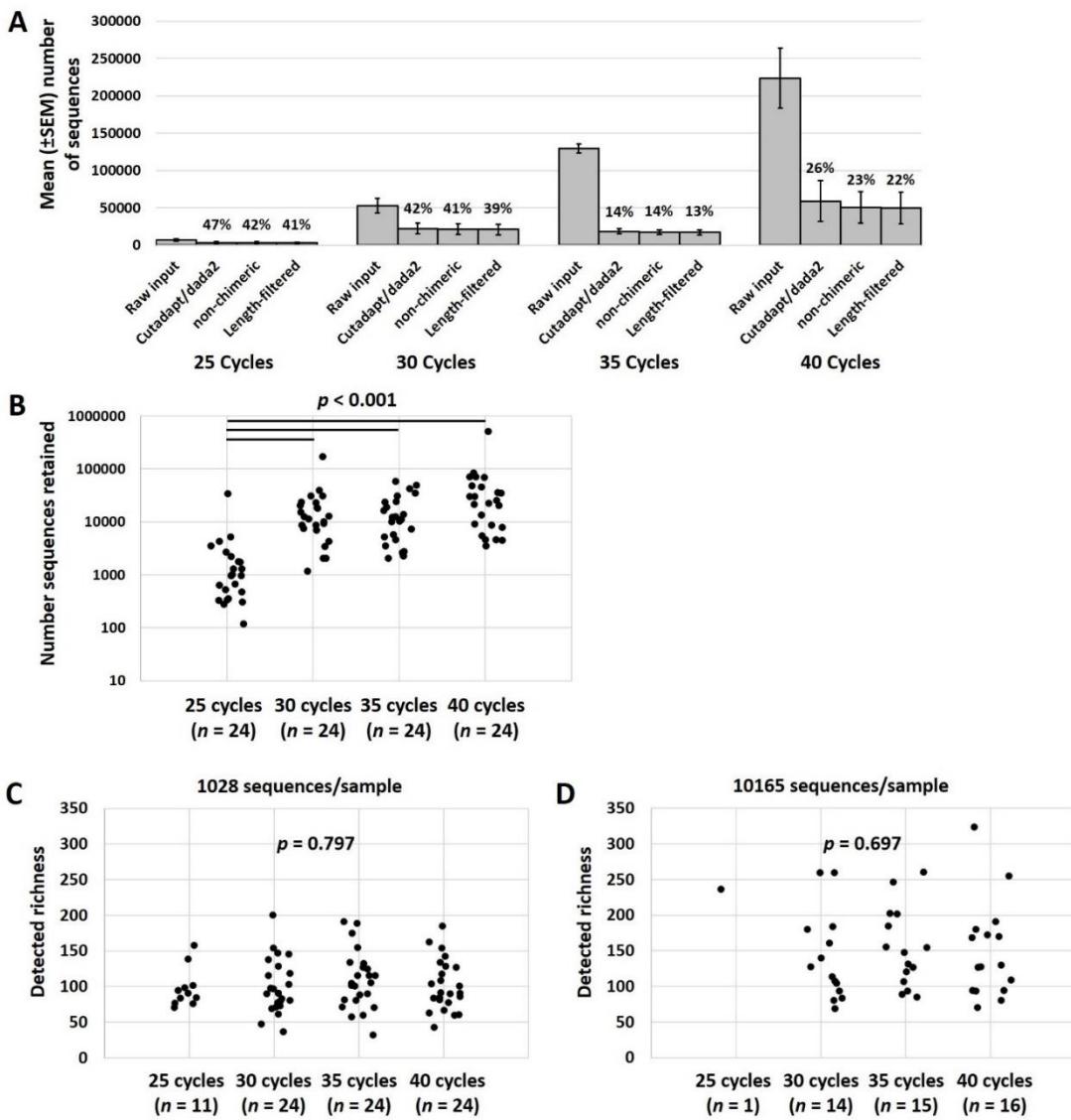
The number of detected sequences and OTUs were rank-transformed due to lack of normality as determined by the Shapiro-Wilks test, and a repeated measures ANOVA was performed, using SigmaPlot 14.0. Beta diversity was determined with the weighted Bray-Curtis and unweighted Jaccard similarities in PAST (Hammer et al., 2001),

analyzed with a permutational analysis of variance (PERMANOVA), and displayed in a Principal Coordinate Analysis (PCoA) based on Bray-Curtis distances. The proportion of changes in coverage (i.e., the number of high-quality sequences retained) per subject with increasing cycle numbers, relative to 25 PCR cycles, was also evaluated. Statistical significance was determined at  $p < 0.05$ .

### 3.4 RESULTS

Of the aliquoted milk samples used for sequencing ( $n = 96$ ), only eight had detectable DNA yields (Median = 5.12 ng/ml; Range: 4.08-6.20 ng/ml) based on fluorometric values obtained using broad-range reagents, which provide a lower limit of detection of 2 ng of DNA. Among all 96 samples, a total of 2,168,824 high-quality sequences were obtained with 25, 30, 35, and 40 cycles of PCR. Despite the paucity of DNA based on fluorometric values, the mean ( $\pm$  standard deviation) read count was  $22592 \pm 456199$  sequences with a minimum of 120 and a maximum of 513,457 sequences retained from libraries generated at 25 and 40 cycles, respectively. Of note, while the proportion of filtered reads increased in libraries amplified for 35 and 40 cycles, the greater total number of raw (i.e., unfiltered) sequences was sufficiently increased due to increased cycle number to offset the increased filtering (Figure 1A). Supplementary Table 1 summarizes the total number, and proportion of raw reads represented by that number, remaining in each group of samples at each stage of filtering.

The overall number of sequences detected in the milk samples increased ( $p < 0.001$ ) with higher PCR cycle numbers (Figure 1B). Samples that were subjected to 40 cycles returned the greatest number of sequences followed by 35, 30, and 25 cycles.



**Figure 3.1.** Histograms showing the number of sequences retained at each level of data filtering from DNA extracted from matched milk samples ( $n = 24$ ), and amplified for 25, 30, 35, or 40 PCR cycles (A). Dot plots show the total number of sequences retained at each cycle number (B), and the detected richness in datasets subsampled to a uniform depth of 1028 sequences (C) or 10165 sequences (D) per sample. Bars indicate significant ( $P < 0.001$ ) differences (Friedman's repeated measures ANOVA on ranks due to lack of normality).

For each set of four replicate samples from 24 quarter level milk samples, the highest mean read count was obtained with 40 PCR cycles followed by 35, 30 and 25 cycles (Table 1). However, 30 and 35 cycles of PCR yielded higher read counts than 40 PCR cycles in 6/24 and 4/24 of samples, respectively.

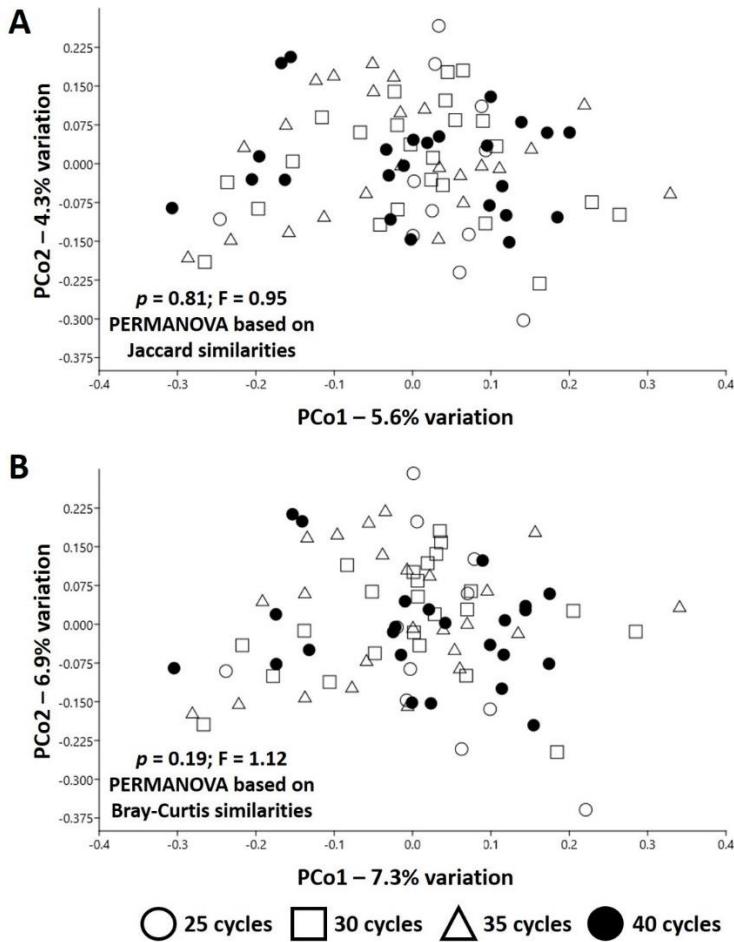
PCR Cycle Number	<sup>a</sup> Highest read count (%)	<sup>b</sup> Read count ( $\pm$ SEM)
25	0/24 (0.0)	2783 (1410)
30	6/24 (25.0)	20838 (6995)
35	4/24 (16.7)	17042 (3242)
40	14/24 (58.3)	49704 (20776)

**Table 3.1.** The highest proportion of read counts obtained with each cycle threshold.  
<sup>a</sup>The proportion of samples with the highest read count for a given cycle number. <sup>b</sup>The average read count for each PCR cycle threshold ( $\pm$  standard error of the mean).

To determine if the increase in coverage was associated with an increase in detected richness, the number of detected OTUs were compared. When data from milk samples were subsampled to a uniform read count, there were no differences in detected richness among samples achieving the subsampling threshold. Thus, while the use of higher PCR cycle numbers increased the number of useable data points (Figures 1C-D), no differences in detected richness were found regardless of the subsampling threshold.

Lastly, beta-diversity was assessed to determine how increased cycle number affected overall community composition. Based on Jaccard similarities, PERMANOVA detected no difference ( $p = 0.114$ ) across PCR cycle number (Figure 2A). Similarly, comparison based on Bray-Curtis similarities (Figure 2B) detected no significant difference between cycle numbers ( $p = 0.311$ ). This suggests that while additional rare

taxa were detected with higher cycle numbers, the rare taxa did not alter beta-diversity substantially. Thus, data from samples processed at 25 cycles still accurately profiles the dominant taxa and general beta-diversity, while libraries generated using increased cycle numbers provided more comprehensive profiles.

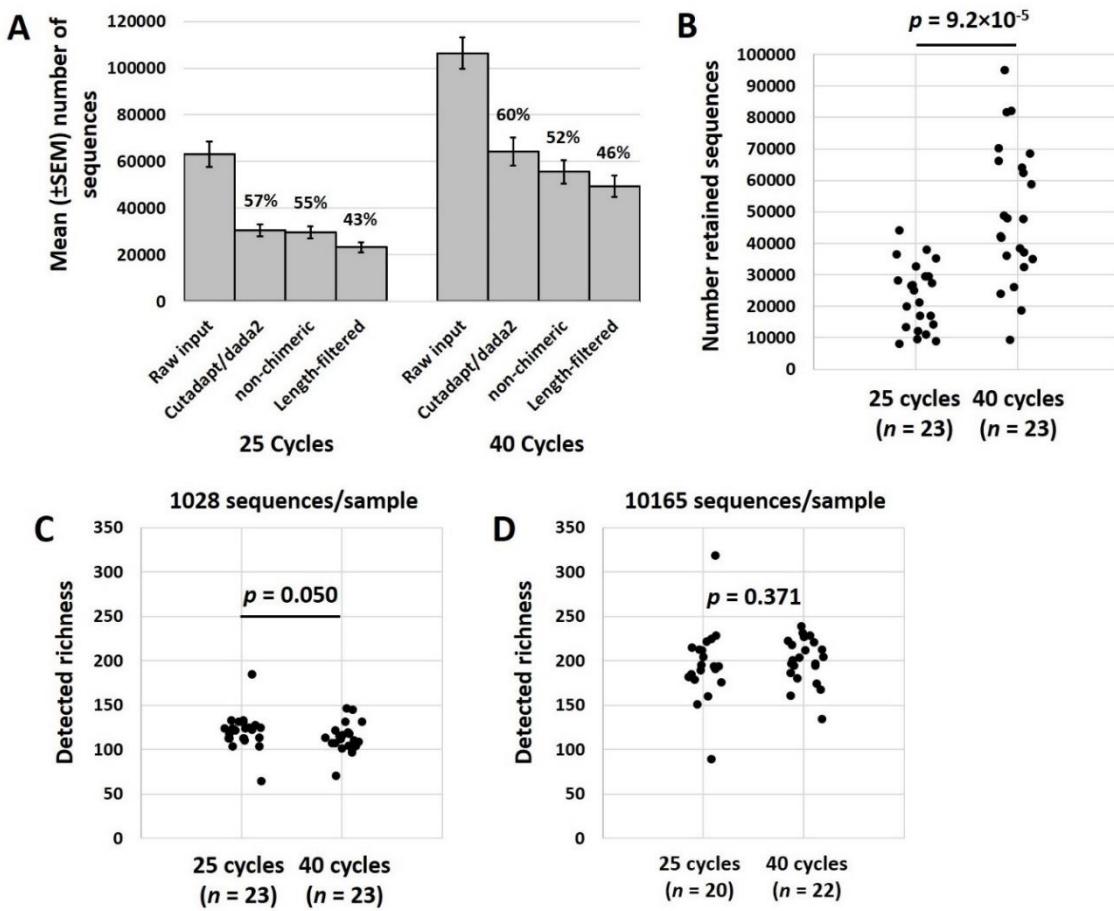


**Figure 3.2.** Comparisons of beta-diversity, based on Jaccard (A) and Bray-Curtis (B) similarities via principal coordinate analysis, of libraries prepared from matched milk samples ( $n = 24$ ) and amplified for 25, 30, 35, or 40 cycles (legend at bottom). Results of one-factor PERMANOVA are provided on each plot.

To determine if increased cycle number could similarly benefit amplification and sequencing of other low biomass sampled, additional samples were processed including portions of furred pelage, and aliquots of blood, collected from healthy mice maintained

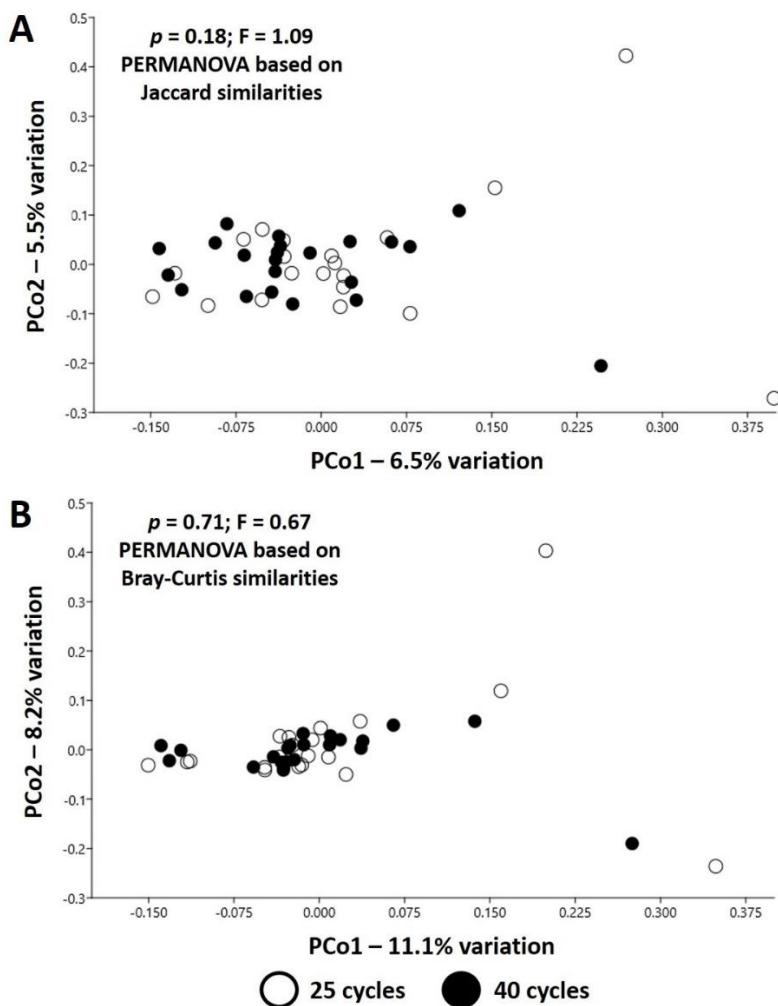
in an SPF facility. To conserve resources, libraries were generated from matched DNA using only 25 or 40 PCR cycles for these sample types.

DNA yields from pelage were below the limit of detection of our broad range fluorometric assay for all samples. Comparison of the total number of sequences and rate of filtering revealed a similar proportion of sequences were retained from the pelage libraries generated at 25 cycles (43%) and 40 cycles (46%) (Figure 3A, Supplementary Table 1). However, as the number of unfiltered reads was so much greater in the pelage libraries amplified for 40 cycles, the final number of retained sequences was also higher in those libraries ( $p = 9.2 \times 10^{-5}$ , paired t-test; Figure 3B). As with the milk sample data, there was no difference in the detected richness of pelage samples in libraries generated with 25 or 40 cycles, regardless of how data were subsampled. All 23 samples met the lower threshold of 1028 sequences per sample (Figure 3C), but at the higher subsampling threshold of 10165 sequences per sample, three libraries amplified for 25 cycles and one library amplified for 40 cycle were not included (Figure 3D).



**Figure 3.3.** Histograms showing the number of sequences retained at each level of data filtering from DNA extracted from matched pelage samples ( $n = 23$ ) and amplified for 25 or 40 PCR cycles (A). Dot plots show the total number of sequences retained at each cycle number (B), and the detected richness in datasets subsampled to a uniform depth of 1028 sequences (C) or 10165 sequences (D) per sample.

As with data generated from milk samples, comparisons of  $\beta$ -diversity between mouse pelage libraries amplified with 25 or 40 cycle numbers failed to detect significant differences, regardless of the similarity metric used and despite the relatively high number of samples ( $n = 23/\text{method}$ ) (Figure 4).

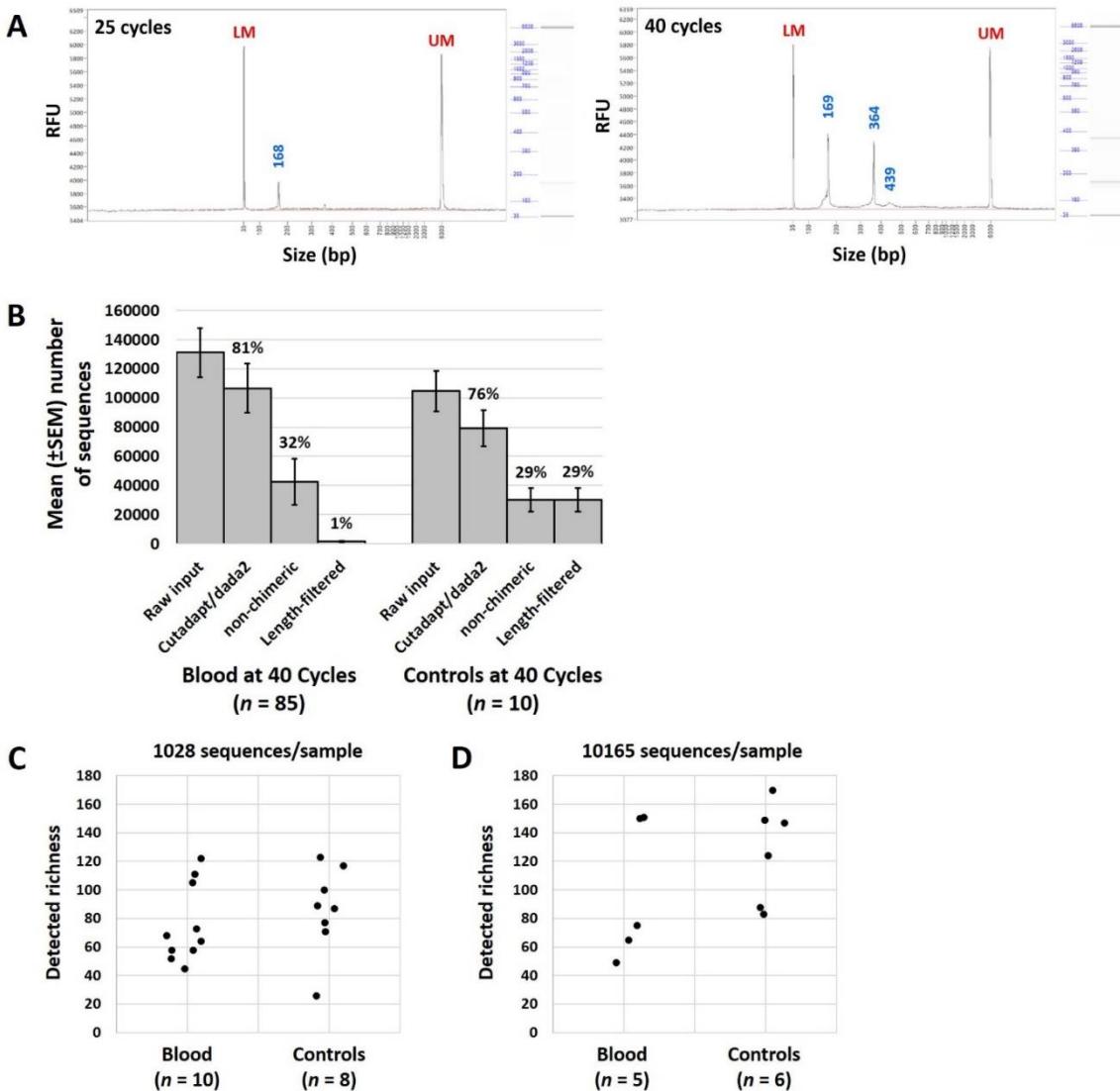


**Figure 3.4.** Comparisons of beta-diversity, based on Jaccard (A) and Bray-Curtis (B) similarities via principal coordinate analysis, of libraries prepared from matched pelage samples ( $n = 23$ ) and amplified for 25 or 40 cycles (legend at bottom). Results of one-factor PERMANOVA are provided on each plot.

Lastly, to evaluate the effect of increased cycle number on samples with extremely low bacterial biomass, murine blood from was used as a source of DNA. While there is not an actual microbial community colonizing peripheral circulation of healthy animals, microbial signatures can be retrieved from blood samples, likely reflecting bacterial DNA within phagocytic leukocytes (e.g., macrophages). Blood samples from 85 healthy mice with a mean ( $\pm$ SD) volume of  $391 \mu\text{L}$  ( $\pm 131 \mu\text{L}$ ) yielded  $2.47 \mu\text{g}$  ( $\pm 1.09$

$\mu$ g) total DNA, presumably of leukocyte origin. Following library generation at either 25 or 40 cycles using matched DNA, libraries were pooled and analyzed on a Fragment Analyzer to evaluate overall amplification. Analysis of all 85 amplicon libraries generated using 25 cycles produced no characteristic peak at 439 bp, representing the expected amplicon size. Due to the lack of detectable amplification, this pool was not sequenced. In contrast, libraries generated using 40 cycles produced a small peak at the expected size (Figure 5A) and were thus sequenced on a single flow cell. Recognizing the increased potential for amplification of contaminating DNA with increased PCR cycle numbers, we also amplified and sequenced blank reagents ( $n = 10$ ), as negative technical controls, on a separate flow cell.

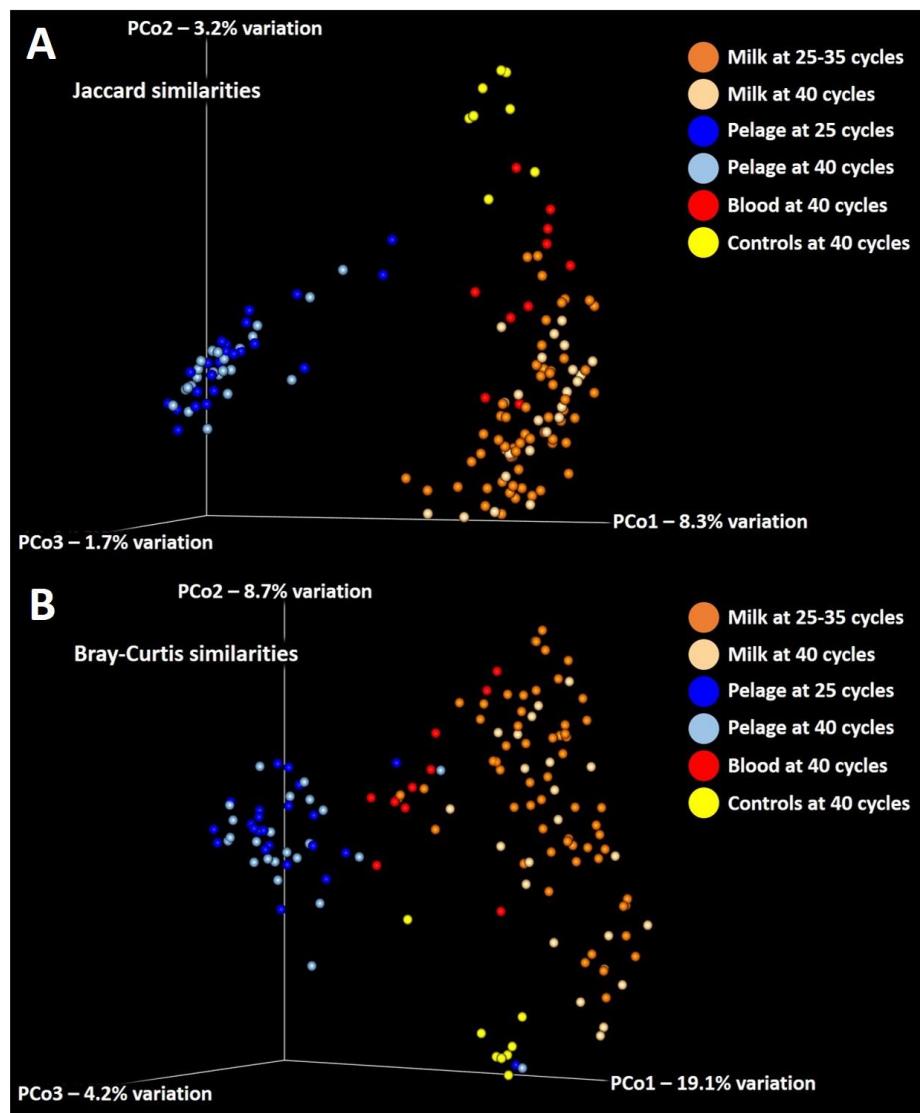
While the blood samples generated a total of over 130,000 raw sequences, filtering resulted in removal of all but 1.1% of those reads (Figure 5B, Supplementary Table 1). Nonetheless, data were generated for all 85 samples when amplified for 40 cycles, with a mean read count of 1377 ( $\pm 432$ ) sequences per samples. Notably however, reagent controls amplified for 40 cycles also yielded surprisingly high sequence counts (Figure 5B), bringing into question the veracity of other sequence data generated using 40 PCR cycles. Moreover, subsampling of data from blood and negative controls suggested similar richness, albeit substantially less than that detected in pelage and some milk samples.



**Figure 3.5.** Quantification of amplified DNA at 439 bp in pooled 16S rRNA libraries generated from murine blood ( $n = 85$ ) amplified for 25 or 40 cycles (A); histograms showing the number of sequences retained at each level of data filtering from DNA extracted from blood samples ( $n = 853$ ) and reagent controls ( $n = 10$ ), and amplified for 40 PCR cycles (B). Dot plots show the detected richness in datasets subsampled to a uniform depth of 1028 sequences (C) or 10165 sequences (D) per sample.

Lastly, considering the negative control data, beta-diversity among all experimental and control samples was compared using data subsampled to a uniform depth of 1028 sequences per sample to maximize the number of samples included in the analysis. Regardless of the similarity metric used, PCoA demonstrated clear separation of

all sample groups, with control samples clustering distinctly from all experimental groups, particularly with regard to the milk and pelage samples (Figure 6A-B). As seen previously, libraries from matched sample types showed no separation based on cycle number. Collectively, we interpret these data as support for the use of increased PCR cycle number with low biomass samples, provided reagent controls are processed alongside experimental samples and taken into consideration in downstream analyses.



**Figure 3.6.** Three-dimensional PCoA showing beta-diversity among all amplified libraries, subsampled to a uniform depth of 1028 sequences per sample, and ordinated according to Jaccard (A) or Bray-Curtis (B) similarities; legend at right.

### **3.5 DISCUSSION**

The current study explored the impact of PCR cycle number (25, 30, 35, and 40 cycles) on 16S rRNA sequencing results of healthy bovine milk samples. In this study, bovine milk samples were used as an example of a sample type known to contain low microbial biomass. Low, medium, and high biomass samples have been defined as samples with 10, 1,000, and 100,000 16S rRNA copies per microliter (Bender et al., 2018). Samples that are of low microbial biomass are generally those that were once considered ‘sterile’ and include body sites such as milk and blood, and environmental sites, such as glacial ice, air, rocks. These low biomass samples often fail sequencing using next-generation sequencing technologies (Eisenhofer et al., 2019) due to low bacterial biomass. There are concerns with using a PCR cycle number greater than 25 when working with high biomass samples due to effects on read quality. However, the results of the current study indicate that, while read quality may or may not be adversely affected by an increased PCR cycle number, the increase in pre-filtering read counts more than compensates for any increased in losses due to filtering.

Previous studies have determined that using PCR cycle numbers greater than 25 can result in increased error rate and chimera formation (Sze and Schloss, 2019). However, the previously mentioned study was conducted using fecal DNA at the standard concentration for library preparation, likely resulting in loss of efficiency in PCR at higher cycles due to saturation of reagents. Chimeras can result from biological similarity, sequencer error, or poor-quality alignment (Edgar et al., 2011) and can cause non-existent organisms to be identified (Wang and Wang, 1996). However, chimera removal standardly occurs after sequencing and before sequence annotation, therefore

minimizing this concern. Another apprehension with using PCR cycles greater than 25 is that PCR error could be higher, which may result in inflated alpha-diversity measures that do not accurately represent the true population (Bokulich et al., 2013). Therefore, beta diversity measures should be calculated and reported in combination with alpha diversity to evaluate changes in overall community composition. Moreover, subsampling at multiple depths will greatly mitigate these concerns.

All of the above notwithstanding, the optimized PCR parameter of 25 cycles is preferred when working with high microbial biomass samples like feces or soil, where  $\mu$ g-level masses of DNA are available, and a precise amount of template DNA can be used during library prep. The current study was performed using extremely low microbial biomass milk samples where error rate and chimeras are of less concern than complete sample failure or sequencing to such a low depth that data are uninterpretable.

The present study and others (Young et al., 2015; Dahlberg et al., 2019) have evaluated milk samples from cows with low SCC (< 100,000 cells/mL). Additionally, in the current study, only samples that had no growth of bacterial colonies with traditional culture methods were sequenced. Normal milk appearance, low SCC, and negative culture results indicate that these samples were from cows without an intramammary infection and free of subclinical or clinical mastitis. Previous studies evaluating the milk microbiome have reported higher (Metzger et al., 2018b) and lower (Young et al., 2015; Andrews et al., 2019; Dahlberg et al., 2019) read counts than the study at hand. However, the previously mentioned studies evaluated healthy and infected quarters (Metzger et al., 2018b; Andrews et al., 2019), potentially contributing to successful amplification.

Results from the present study suggest that a higher PCR cycle number allows for increased sample coverage and thus the possibility of increased group sample sizes, without altering the overall community composition noticeably. Ultimately, however, the goals of a specific project, or convenience in combining multiple sample types on one plate for library preparation, might dictate whether or not an increased PCR cycle number is warranted. Coverage can also theoretically be increased by reducing the number of libraries sequenced on a single flow cell, although this brings a concomitant increase in the cost per sample. It should also be noted that reduced multiplexing on a single flow cell would be expected to result in similar increases in read counts from negative controls.

In the present study, reagents controls yielded high read counts, supporting the introduction of contaminating bacterial DNA at some stage of sample processing or sequencing. While these data certainly raise questions regarding experimental data generated using the same PCR parameters, the fact that detected richness was substantially higher in many experimental samples (even at lower coverage), and that beta-diversity was consistent within experimental groups yet different from the controls, we believe that a significant portion of the experimental data are valid. While beyond the scope of the current study, multiple approaches are available allowing for removal of potential contaminants found in control samples from experimental data, and data generated from low biomass samples should always be analyzed in the context of control data, particularly when extending the PCR parameters.

Based on the results of this study, using PCR cycle numbers greater than 25, such as 35 or 40 cycles, for samples with low microbial biomass will improve 16S rRNA

sequencing by allowing for the detection of more rare taxa while not influencing overall bacterial composition. These results contribute to our current understanding of the influences of PCR parameters on the results of 16S rRNA sequencing and provide support for the use of high cycle numbers for samples that contain low microbial biomass.

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**CHAPTER IV**

**THE IMPACT OF STRESS ON THE MICROBIOME OF LACTATING DAIRY  
COWS: II. MICROBIAL COMMUNITIES OF RUMEN AND FECES ARE  
ALTERED BY HEAT STRESS OR FEED RESTRICTION**

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## MICROBIAL COMMUNITIES OF RUMEN AND FECES ARE ALTERED BY HEAT STRESS OR FEED RESTRICTION

### 4.1 ABSTRACT

The objective of this study was to identify changes in rumen and fecal microbiomes during heat stress and feed restriction. We hypothesized that Gram-negative bacteria and mastitis-associated bacteria in rumen and feces would increase in abundance during heat stress. We also hypothesized that diversity of bacteria in rumen and feces would increase during feed restriction. Cows ( $n = 18$ ) were housed in environmental chambers for 27 days and allocated to one of three treatments: control cows (**CL**), heat-stressed cows (**HS**), or pair fed cows (**PF**). All cows were housed under thermoneutral conditions for 5d prior to initiation of treatments (d-5 to d0; temperature-humidity index (**THI**) ~66). The CL cows remained in thermoneutral conditions and received feed ad libitum for the duration of the experiment (d-5 to d21). The HS cows were subjected to elevated THI (~80) during the challenge period (d0 to d14), followed by an 8d recovery period (THI ~66). The PF cows were kept at thermoneutral THI (~66) but were pair fed to match the HS group throughout the challenge and recovery periods. Rumen fluid samples were collected via rumenocentesis on d-3, 7, 13, and 21. Fecal samples were collected per rectum on d-3, 0, 4, 7, 10, 13, 18, and 21. Sequencing of the V4 region of the 16S rRNA gene was used to evaluate the rumen and fecal microbiomes. Richness and diversity of rumen fluid was lower in the PF cows than the HS and CL cows, whereas fecal microbiome did not differ by treatment. Principal coordinate analysis (**PCoA**) and hierarchical clustering displayed changes in microbiomes of rumen and feces based on time and treatment. In the PF cows, the abundance of *Bacteroidetes* (phylum of Gram-

negative bacteria) was greater in rumen fluid on d7 and in feces on d4. Of several mastitis-associated bacteria evaluated (*Enterobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Staphylococcus*, and *Streptococcus*) only *Escherichia* was more abundant in feces during heat stress and feed restriction. The results demonstrate changes in the microbiome of rumen and feces that are associated with heat stress, feed restriction, and experimental conditions. Further studies are needed to determine how long effects from these stressors persist and the potential impact on the mammary gland microbiome to determine mastitis risk during periods of elevated THI.

## 4.2 INTRODUCTION

The economic impact of heat stress has been well-documented (Key et al., 2014) and is largely attributed to reduced feed intake. However, reduced feed intake only accounts for up to 50% of the decrease in milk production that occurs during heat stress (Rhoads et al., 2009; Wheelock et al., 2010). Therefore, understanding the impact of reduced DMI and heat stress on the microbiota could potentially explain part of the loss in milk production in addition to reduced feed intake.

The microbiota of the rumen (Jami and Mizrahi, 2012) and feces (Shanks et al., 2011) has been evaluated and authors found the same dominant phyla present, albeit at different relative abundances, which provides a more in-depth investigation into the bacterial communities that reside within an animal compared to traditional bacterial culture. Additional studies have provided information that can be used to better understand the role of the microbiome in feed efficiency (Jami et al., 2014) and milk production (Scharen et al., 2018; Xue et al., 2020). Feed restriction without heat stress has been associated with an increase in rumen microbial diversity (McCabe et al., 2015)

but it is unknown if these effects occur in cattle that exhibit reduced feed intake as a consequence of heat stress.

Heat stress has broad negative effects on health and production. For example, retained placenta incidence has been reported to increase during the summer (DuBois and Williams, 1980) and recently, increased rates of clinical mastitis, retained placenta, and puerperal disorders have been associated with increased temperature-humidity index (**THI**; Gernand et al., 2019). In addition, bulk tank SCC (**BTSCC**), an indicator of subclinical mastitis, has been reported to increase during summer months (Hogan et al., 1989; Olde Riekerink et al., 2007). Additionally, Gram-negative bacteria (like *Escherichia coli* and *Klebsiella* species) were more frequently isolated from milk samples, collected in summer versus winter (Makovec and Ruegg, 2003).

While it is known that heat stress alters bacterial populations of rumen (Uyeno et al., 2010; Zhao et al., 2019) and feces (Chen et al., 2018; Li et al., 2020) of dairy cows, it is unknown if fecal shedding of mastitis-associated bacteria increases during heat stress. Alpha diversity in the rumen has been associated with a higher SCC (Zhong et al., 2018) and a higher SCS has been reported in the summer versus winter (Guinn et al., 2019). Feces are a known source of bacteria in bedding and increased bacterial counts in bedding have been associated with increased bacterial counts on teat ends (Zdanowicz et al., 2004). Furthermore, Gram-negative bacterial counts in bedding have been reported to increase in the summer (Rowbotham and Ruegg, 2016). Therefore, an increase in mastitis-causing bacteria in feces could increase exposure of teat ends to pathogens, leading to increased prevalence of mastitis, and may partially account for increased BTSCC during the summer months.

While it has been established that heat stress impacts the rumen and fecal microbiota, results are not consistent across studies and previous studies have not evaluated the relationship between these sample types within cows. Previous studies have not included untreated controls to account for changes over time nor can they distinguish effects of elevated temperature from effects of decreased feed intake because they did not include pair fed cows kept in thermoneutral conditions. Therefore, the objective of this study was to determine effects of heat stress or feed restriction on rumen and fecal microbiota of dairy cows. We hypothesized the abundance of Gram-negative bacteria in rumen fluid and feces would increase during heat stress. We also hypothesized that reduced feed intake would result in increased diversity of bacteria in the rumen and feces of cattle during feed restriction. Lastly, we hypothesized that heat stress but not feed restriction would result in increased fecal shedding of mastitis-causing pathogens.

Therefore, temporal profiles of operational taxonomic units (**OTU**) belonging to Gram-negative phyla (*Bacteroidetes* and *Proteobacteria*) and OTU belonging to known mastitis-causing genera (*Enterobacter*, *Enterococcus*, *Escherichia-Shigella*, *Klebsiella*, *Staphylococcus*, and *Streptococcus*) were evaluated during and after exposure to heat stress.

#### **4.3 MATERIALS AND METHODS**

##### ***Selection and enrollment of animals***

The University of Missouri Animal Care and Use Committee approved this study (Protocol Number 9283). Mid-lactation Holstein cows (n = 18) from the University of Missouri Foremost Dairy Research and Teaching farm were selected based on similar

parity, days pregnant, SCC, daily milk production, and DIM (Rodrigues, 2019). Of the cows enrolled, SCC from the previous month of testing was < 200,000 cells/mL.

### ***Experimental design***

Cows were blocked based on MY, DIM, and parity and cows were randomly allocated, within a block, to one of three treatments: control (**CL**; n = 6), heat stressed (**HS**; n = 6) or pair fed (**PF**; n = 6). The experiment was conducted at the University of Missouri Brody Environmental Units over a period of 27 days (d-5 to 0, pretreatment; d0 to 14, challenge; d14 to 22, recovery). All cows were fitted with radio telemetric rumen bolus SmartStock to monitor rumen temperature (Rodrigues et al., 2019). The CL cows were kept at thermoneutral conditions (THI ~ 66) to maintain a rumen temperature of approximately 39.5°C and were fed ad libitum for the duration of the experiment. The THI was adjusted to induce heat stress by raising the rumen temperature by 1°C above the cow's (Beatty et al., 2006) normothermia and the environmental chamber was monitored to keep the cow's body temperature elevated for the duration of the challenge period (Rodrigues, 2019). The HS cows received feed ad libitum and were maintained at thermoneutral conditions during pretreatment and recovery (THI ~ 66; rumen temperature maintained at ~ 39.5°C), but were subjected to constant, non-cyclical, heat stress during the challenge period (THI ~ 80; rumen temperature ~ 40.6°C). To distinguish the effects of reduced feed intake without heat stress, a group of cows was pair fed to match intake of the HS cows within block. Cows on PF were maintained at thermoneutral conditions throughout the study (THI ~ 66) to maintain a rumen temperature of approximately 39.5°C. Cows were milked and fed a TMR twice daily. The TMR was formulated to meet or exceed the predicted nutrient requirements (NRC,

2001). Further details including mammary gland biopsy methodology and production results can be found in Rodrigues, 2019.

### ***Sampling***

Rumen fluid samples were collected from all cows approximately three hours after morning milking (~9AM) using previously described rumenocentesis methodology (Chako, 2014). Samples from all cows except for four ( $n = 68$ ) were collected during the pretreatment (d-3), challenge (d7 and 13), and recovery (d21) periods. Approximately 5 ml of rumen fluid were collected from each cow into a non-sterile container for pH determination immediately after collection using a handheld meter (HI98107; Hanna Instruments Inc., RI, USA). Another 5 ml were collected from each cow into a sterile 50 ml conical tube (ThermoFisher Scientific; Waltham, MA) and stored at -80°C prior to amplicon sequencing. The pH of rumen fluid samples was normally distributed (Shapiro-Wilk,  $P > 0.88$ ) and data were analyzed with a two-way repeated measures ANOVA in PROC GLIMMIX (SAS Institute Inc., 2010).

Fecal samples ( $n = 144$ ) were collected from all cows per rectum approximately three hours after morning milking. Feces were collected during the pretreatment (d-3 and 0), challenge (d4, 7, 10, and 13), and recovery (d18 and 21) periods. Samples were immediately placed into sterile, 50 mL conical tubes (ThermoFisher Scientific) and frozen at -20°C until further processing.

### ***DNA extraction, 16S rRNA sequencing, and bioinformatics***

Rumen fluid ( $n = 68$ ) and fecal ( $n = 144$ ) samples were thawed at room temperature and DNA was extracted using QIAamp PowerFecal DNA kits (Qiagen; Germantown, MD). Approximately 800  $\mu$ l of rumen fluid and 0.25 g of feces from each

sample were added to separate bead tubes and a TissueLyser (Qiagen) was used to lyse the cells. Per the manufacturer's instructions, multiple wash steps and centrifugations through a spin column were used to isolate purified DNA. The quant-iT BR dsDNA reagent kit (Invitrogen; Carlsbad, CA) was used to determine the DNA concentration of the samples with a Qubit fluorometer. Universal primers (U515F/806R) flanked by Illumina adapter sequences (Caporaso et al., 2010) were added to the V4 region of the 16S rRNA gene and sequencing was performed on the Illumina MiSeq platform. The operational taxonomic units (**OTU**) were assigned based on  $\geq 97\%$  nucleotide identity (Caporaso et al., 2010) and annotated against the Silva.v132 database (Quast et al., 2013) using BLAST (McGinnis and Madden, 2004).

### ***Microbiota analysis***

Alpha diversity of rumen fluid and fecal samples was calculated with the Chao1 and Shannon indices and analyzed in SigmaPlot 13.0 (Systat Software Inc., San Jose, CA, USA) using a two-way repeated measures ANOVA. Beta diversity of rumen fluid and fecal samples was evaluated based on Bray-Curtis and Jaccard similarities in Paleontological statistics software (Hammer et al., 2001), and analyzed using a two-way permutational analysis of variance. Principal coordinate analyses (**PCoA**) of rumen and fecal samples were created in paleontological statistics software (**PAST**) after data was fourth root transformed to normalize data. The PCoA was based on Bray-Curtis and Jaccard similarities, and Bray-Curtis distances are reported. Intra-subject variation of rumen fluid and feces was evaluated using Bray-Curtis distances and analyzed using a one-way ANOVA in SigmaPlot 13.0 (Systat Software Inc.). Rumen fluid and feces data were cube root transformed and heat maps were created to display the top 25 most

variable OTU based on hierarchical clustering within treatment over time and to describe composition differences between treatments and days of the experiment (Xia et al., 2009). Venny 2.1 (Oliveros, 2015) was used to determine OTU that were shared between challenge periods of different treatments and between sample types. Temporal profiles of selected phyla and genera were transformed if distribution was non-normal as determined by the Shapiro-Wilk test and visual inspection of Q-Q plots and histograms. Abundances of OTU within phyla and genera were analyzed using ANOVA with repeated measures in PROC GLIMMIX. *Escherichia-Shigella* abundance failed to normalize so data were analyzed using ANOVA with repeated measures and a binary distribution in PROC GLIMMIX. Statistical significance was declared at  $P < 0.05$ . Least squares means  $\pm$  SEM are reported (except where noted).

#### **4.4 RESULTS**

##### ***Overall observations***

All cows ( $n = 18$ ) completed the study and DMI for the PF group was successfully matched to the intake of the HS cows during the challenge and recovery periods (Rodrigues, 2019). There was no evidence of subclinical or clinical mastitis incidence throughout the study as determined by individual cow SCC and foremilk stripping during the milking preparation procedure.

##### ***Sequencing performance***

One rumen fluid sample failed to sequence and was removed from subsequent analyses. Sequencing of the 16S rRNA gene from the rumen fluid samples resulted in a total of 5,564,473 high quality sequences, with an average of  $83,052 \pm 1,366$  sequences

per sample. Fecal samples generated a total of 107,496,677 high quality sequences, with an average of  $74,651 \pm 1,459$  sequences per sample.

### ***Rumen fluid pH***

Rumen fluid pH (Table 4.1) differed between treatments and throughout periods of the study ( $P < 0.05$ ). The CL cows had a higher rumen fluid pH during recovery (d21) compared to all other sampling times ( $P < 0.05$ ), but there was no difference in rumen pH of the HS cows throughout the study ( $P > 0.10$ ). The PF cows had a higher pH on d7 ( $P < 0.05$ ) which was followed by a decline on d13 that continued in recovery. These results indicate that heat stress likely altered the cow's response to feed restriction.

### ***Chao1 richness and Shannon diversity***

Alpha diversity (Figures 4.1 A-D) was measured with Chao1 (richness) and Shannon (evenness) indices to reflect changes in the number of OTU and the evenness of OTU by treatment, time, and treatment by time interaction, respectively. Microbial richness in rumen fluid samples (Figure 4.1 A) was lower in the PF cows compared to the CL cows ( $P < 0.05$ ), but there was no difference in the richness in HS cows compared to either PF or CL cows ( $P > 0.10$ ). The richness in PF cows decreased throughout the feed restriction period, and this downward trend continued into recovery. The CL cows remained relatively stable across time, while the HS cows had a numerically lower richness during the recovery period (d21) compared to the acclimation and heat stress periods. Diversity measures (Figure 4.1 B) differed over time ( $P < 0.05$ ) and between treatments ( $P < 0.05$ ), but their interaction was not significant ( $P > 0.10$ ). Throughout the experiment, diversity was lower during the challenge period compared to pretreatment ( $P < 0.05$ ) and diversity tended to be lower during recovery compared to acclimation ( $P =$

0.052). However, diversity was not different between the challenge period and recovery ( $P > 0.10$ ). Between treatments, PF cows had a lower diversity measure compared to HS and CL cows ( $P < 0.05$ ), while CL and HS cows were not different ( $P > 0.10$ ).

Richness of fecal samples (Figure 4.1 C) differed over time ( $P < 0.05$ ) with higher richness averages across treatments in the late challenge (d13) and recovery (d18 and 21) periods compared to pretreatment and early challenge (d7). There was not a significant interaction between treatment and time, indicating the number of OTU followed a similar pattern in all groups, over time. Fecal diversity (Figure 4.1 D) was not different between treatments or over time ( $P > 0.27$ ).

Overall, richness and diversity measures of rumen fluid were similar between CL and HS cows while PF cows had lower measurements compared to CL. Fecal richness and diversity appeared highly variable over time, regardless of treatment, which indicates that these measures fluctuate over time in feces and were less responsive to the present experimental treatments.

### ***Community composition***

Changes in sample composition were visualized using PCoA. For both rumen fluid and fecal samples, overall bacterial composition differed between treatments and time points. These differences were observed using Bray-Curtis ( $P < 0.001$ ) and Jaccard ( $P < 0.001$ ) similarities with comparable results, and only Bray-Curtis data are reported (Figure 4.2 A-B).

Rumen fluid samples (Figure 4.2 A) clustered together based on community composition during pretreatment (d-3) but shifted markedly during the early challenge period (d7). Then the clusters from the CL and HS cows overlapped during the mid-

challenge period (d13) while the samples from PF cows overlapped less during this period and this lack of overlap continued into recovery (d21).

On d0, feces collected from the CL, HS, and PF samples (Figure 4.2 B) clustered together indicating that sample composition was similar throughout the pretreatment period. During early challenge (d4), the HS fecal samples formed a cluster that barely overlapped with the CL and PF clusters ( $P < 0.05$ ). Then, during mid-heat stress and mid-feed restriction (d7), the HS and PF clusters overlapped while the CL formed a separate cluster ( $P < 0.05$ ). All treatment clusters overlapped by the end of the challenge period (d13;  $P > 0.10$ ), but during recovery (d18 and 21) HS and PF clusters overlapped while the CL cluster remained separate.

When comparing rumen fluid and fecal sample composition (Figure 4.2 A-B) on only the days that rumen fluid was sampled (d-3, 7, 13, and 21), similar shifts in microbiome composition were noted. Within sample type, rumen fluid ( $P > 0.10$ ) and feces ( $P < 0.05$ ) from CL, HS, and PF cows had overlapping clusters on d-3, which indicates that the pretreatment samples within rumen fluid were more similar in composition (Figure 4.2 A-B). However, on d7, rumen fluid samples did not cluster together while feces from PF and HS clustered together but were separate from CL, indicating that rumen composition responded differently to heat stress versus reduced feed intake. Later, on d13, HS and CL rumen fluid clusters overlapped but PF formed a separate cluster, and the fecal clusters all overlapped. Treatment differences continued into recovery when treatment clusters for rumen fluid barely overlapped and the feces from PF and HS cows overlapped while the CL cluster remained separate. These results suggest that for both sample types, the overall bacterial composition of the CL, HS, and

PF cows shifted during the challenge period and the bacterial compositional numerical changes continued into recovery.

### ***Intra-sample similarity***

To evaluate inter-cow variation, intra-sample similarities were calculated. For rumen fluid (Figure 4.3 A) and feces (Figure 4.3 B) comparisons were made between pretreatment and mid-challenge (d-3 versus d7), late-challenge (d-3 versus d13), and recovery (d-3 versus d21). In rumen fluid (Figure 4.3 A), the intra-sample similarity between d-3 and d7 in PF cows was lower than the similarity between the same time points in HS and CL cows ( $P < 0.05$ ). The similarity between d-3 and d13 was also lower in PF cows than HS cows ( $P < 0.05$ ) and tended to differ from CL cows ( $P < 0.08$ ). Again, the similarity between d-3 and d21 was lower in PF cows than HS and CL cows at the same time points ( $P < 0.05$ ).

For feces (Figure 4.3 B), the intra-sample similarity between d-3 and d7 was not different ( $P = 0.743$ ) among treatments and trended towards significance while comparing between d-3 and d13 ( $P = 0.09$ ) because intra-sample similarity from HS cows was numerically lower than CL or PF cows. However, the intra-sample similarity in feces from PF cows was greater than from HS and CL cows when comparing d-3 and d21 ( $P < 0.05$ ).

Together, these data show that feed restriction without heat stress was associated with a significant change in the rumen microbiomes, and this change persisted into recovery. Those changes were quite different from changes in feces. The fecal microbiome only tended to be different when comparing pretreatment to late challenge, and a significant change was detected when comparing pretreatment and recovery. These

data show that the rumen fluid microbiome experienced more rapid changes overall resulting in lower overall intra-sample similarity in PF whereas the fecal microbiome of feed restriction and heat stress groups exhibited higher intra-sample similarity than controls. Clearly, rumen fluid and feces responded very differently.

### ***Shared bacterial populations***

A total of 722 unique OTU were identified in the rumen samples ( $n = 67$ ) and 609 unique OTU were identified in the fecal samples ( $n = 144$ ). Rumen fluid and fecal samples shared 48.2% of OTU with 32.2% belonging exclusively to rumen fluid and 19.6% belonging exclusively to feces.

Within rumen fluid collected during the challenge period (d7 and 13), 639 OTU (88.5%) were shared between all treatments. Only three OTU (0.4%) from rumen fluid were exclusive to HS, and they belonged to the *Clostridiales* and *Bacteroidales* classes. Two OTU (0.3%) were exclusive to PF, and they belonged to the *Bacilli* and *Bacteroidia* classes. Five OTU (0.7%) were exclusive to CL cows during the challenge period. Of the 11.5% of OTU not common to all three groups, rumen fluid from HS and PF cows had 11 OTU (1.5%) in common, HS and CL cows shared 35 OTU (4.8%), and CL and PF cows shared 27 OTU (3.7%). The shared OTU between HS and PF cows consisted of bacteria from the *Actinobacteria* (*Micrococcales* order), *Bacteroidetes* (*Bacteroidia* class), *Firmicutes* (*Bacillales* and *Clostridiales* orders), *Proteobacteria* (*Deltaproteobacteria* class), and *Verrucomicrobia* (*Verrucomicrobiae* class) phyla.

Within the feces collected during the challenge period (d4, 7, 10, and 13) 560 OTU (92%) were common among all treatments. Only one OTU belonged exclusively to HS cows and it was assigned to the *Lachnospiraceae* family. No OTU belonged

singularly to the challenge period of PF cows and only two OTU were restricted to CL cows. Feces from HS and PF cows shared 14 OTU (2.3%) which belonged mostly to the *Clostridia* and *Planctomycetacia* classes. The HS and CL cows shared 19 OTU (3.1%), and PF and CL cows shared 13 OTU (2.1%). These results indicate that heat stress and feed restriction altered the ruminal and fecal microbiomes, but very few OTU were exclusive to one treatment during the challenge periods which suggests that some OTU may be more responsive and serve as a biomarker for each stressor.

#### ***Hierarchical clustering of 25 OTU***

Hierarchical clustering of the 25 most variable OTU from each treatment over time (Figure 4.4 A-F) displays temporal changes in rumen fluid and fecal samples. Hierarchical clustering along with the PCoA further supports the claim of altered composition due to heat stress and pair feeding (full heat maps found in Supplementary Figure 4.6 A-F). Rumen fluid samples (Figure 4.4 A-C) clustered perfectly by day, regardless of treatment (Figure 4.4 A-C). Within the rumen from CL cows, the pretreatment (d-3), late challenge (d13), and recovery (d21) all form one cluster, but for unknown reasons, early challenge (d7) is separate. In HS cows (Figure 4.4 B), samples fell into two main clusters; one including pretreatment (d-3) and early challenge (d7) samples and a separate cluster with late challenge (d13) and recovery samples (d21). In PF cows (Figure 4.4 C), samples appeared to recover quicker than HS cows as shown by the two main clusters. The first cluster contained early challenge (d7) and late challenge (d13), clearly separated from the second cluster, which contained pretreatment (d-3) and recovery (d21), suggesting that these OUT began to return to pretreatment levels during recovery. In CL cows, samples from pretreatment and recovery also clustered together

but separate from d13, which was separate from d7. Reasons for changes over time in CL are unknown but were clearly consistent among cows. The most variable OTU within rumen fluid samples included both Gram-negative (37/75; 49%), Gram-positive (34/75; ~45%), and some OTU with unknown Gram-staining results (4/75; ~5%).

Within feces (Figure 4.4 D-F), the majority (48/75; ~64%) of the most variable OTU were assigned as Gram-positive bacteria. In contrast to rumen fluid, fecal samples did not cluster cleanly by time, however, general patterns could be discerned. Feces from CL cows (Figure 4.4 D) formed some clusters by day but appeared variable over time while the HS cows (Figure 4.4 E) formed two main clusters; one that contained all samples from d-3, 0, 7, and 21 and another that contained all samples from d10, 13, and 18, and all but one sample on d4. Samples from PF cows (figure 4.4 F) also formed two main clusters; one included 18 samples: 5/6 on d-3, 5/6 on d7, but 0/6 from d4, 10, and 18 and only 1/6 from d0 which could indicate that these OTU are more transitional rather than consistent. Notably, samples from the early recovery period (d18) clustered together suggesting that variation on d18 determined OTU selection into the top 25 OTU. This may explain the lack of consistent grouping at other times. Samples from CL cows formed two main clusters; one contained 13 samples including 5/6 on d-3. The other main cluster contained 35 samples and formed 2 secondary clusters, the first contained 12 samples including 6/6 on d4 and 4/6 on d18; the other had 6/6 on d21 and 5/6 on d7. Results from the CL cows appeared to vary and did not display a consistent pattern over time. Many of the OTU that varied most over time within feces belonged to the *Clostridia* class. Of the OTU in the hierarchical clustering analysis, many were the same between treatments but not sample type, suggesting that these OTU may not have directly

responded to the treatments and likely fluctuated over time or may be more sensitive to other factors not accounted for like behavioral changes. Hierarchical clustering of the most variable OTU suggests that rumen fluid was more consistent between cows within treatment while feces exhibited more variation between cows and obscured treatment effects.

We previously reported bacterial OTU from feces that appeared heat stress responsive as shown by hierarchical clustering (Chapter II; unpublished data). The OTU that were most variable differed between the previous study but none of the 25 most variable OTU in the previous study were found in hierarchical clustering of the rumen fluid or feces from the present study. However, in addition to hierarchical clustering, two OTU belonging to the *Paenibacillus* genus and one OTU belonging to the *Mitochondria* family were found to display similar responses in feces between studies. The abundance of one OTU belonging to the *Paenibacillus* genus was numerically higher during the heat stress challenge period in both of our previous study (Chapter II; unpublished data) and in the present study. This OTU was also less abundant in feces from the PF group. The other OTU that belongs to the *Paenibacillus* genus was numerically lower in the previous study and was also lower in feces from HS and PF groups compared to CL in the present study. In this study, that same OTU was also numerically lower in feces from the HS and PF groups but was higher in rumen fluid from heat-stressed cows. The OTU belonging to the *Mitochondria* family was lower in feces of HS cows during the challenge period from the previous study (Chapter II) and the present study. While most of the heat stress responsive OTU were not different in the present study, OTU belonging to the

*Paenibacillus* genera and *Mitochondria* family appeared heat stress and feed restriction responsive.

### ***Phyla level changes***

Overall, there were 25 and 20 bacterial phyla identified in the rumen and fecal samples, respectively. The most abundant phyla within rumen fluid and feces were *Bacteroidetes* and *Firmicutes*, and both were present in 100% of rumen and fecal samples. On average during pretreatment *Bacteroidetes* made up ~62% of rumen fluid OTU and ~45% of feces. Within rumen fluid (Figure 4.5 A), across all times *Bacteroidetes* (59.9%) were the most prevalent on average, followed by *Firmicutes* (26.9%), *Tenericutes* (3.1%), *Proteobacteria* (2.5%), and *Spirochetes* (2.3%). No other phyla comprised more than 2% of rumen fluid OTU. Abundances of *Bacteroidetes*, a phylum of Gram-negative bacteria, differed throughout the experiment (Figure 4.5 A;  $P < 0.05$ ). In general, *Bacteroidetes* in rumen fluid of CL and HS followed a similar pattern but PF differed. Specifically, *Bacteroidetes* was less abundant in PF cows compared to CL and HS cows on d7 ( $P < 0.05$ ). The PF cows had a lower abundance in recovery compared to the pretreatment and challenge periods ( $P < 0.05$ ). Within the CL cows, *Bacteroidetes* were less abundant on d13 compared to pretreatment and recovery ( $P < 0.05$ ). Abundance of *Bacteroidetes* in the rumen fluid of HS cows did not differ over time ( $P > 0.09$ ). We were also interested in *Proteobacteria* (Supplementary Figure 4.7 A), because that phylum contains common pathogens *Enterobacter*, *Escherichia-Shigella*, and *Klebsiella* genera. They were present in 100% of rumen samples but its relative abundance did not differ between treatments or over time ( $P > 0.50$ ).

Within feces (Figure 4.5 B), *Bacteroidetes* were the most prevalent, averaging 45.6%, overall, followed by *Firmicutes* (44.2%), *Spirochaetes* (3.2%), *Proteobacteria* (2.1%), and *Tenericutes* (2.1%). The other phyla found in the feces made up less than 2% of samples. In feces, abundance of *Bacteroidetes* (Figure 4.5 B) differed over time ( $P < 0.05$ ) and was higher in PF compared to CL and HS cows on d4 ( $P < 0.01$ ). Within feces from HS and CL cows, the abundance of *Bacteroidetes* fluctuated throughout the study and was affected by a treatment by time interaction ( $P < 0.05$ ), but no clear pattern emerged. In feces from PF cows, abundance of *Bacteroidetes* was affected by treatment and time ( $P < 0.05$ ) and was lower during late feed restriction (d13) and during recovery (d18 and 21) compared to pretreatment (d0) and early to mid-challenge (d4, 7, and 10;  $P < 0.05$ ). Abundance of *Proteobacteria* in feces (Supplementary Figure 4.7 B) was affected by the interaction of treatment and time ( $P < 0.001$ ). In fecal samples from CL cows, abundance of *Proteobacteria* was higher on d4, but there was no clear pattern throughout the study. In HS cows, fecal abundance of *Proteobacteria* increased during early heat stress (d4 and 7) compared to pretreatment (d-3 and 0), decreased numerically in late heat stress, and did not return to pretreatment levels by the last day of sampling (d21). In PF cows, the abundance of *Proteobacteria* in feces spiked during mid-feed restriction (d7) and again during recovery (d21).

Temporal changes in the relative abundance of dominant phyla differed temporally between rumen fluid and feces. This indicates that even though the rumen and feces shared the same dominant phyla, their abundances were altered during transit through the gastrointestinal tract between the rumen and rectum.

### ***Mastitis pathogens***

Individual genera representing selected mastitis-associated bacteria were evaluated in rumen and fecal samples. The genera *Enterobacter*, *Enterococcus*, *Klebsiella*, *Streptococcus*, and *Staphylococcus* were either not present or were too low in abundance for statistical analysis. The relative abundance of *Escherichia-Shigella* in rumen fluid was not affected by treatment or time (Supplementary Figure 4.8 A;  $P > 0.10$ ).

Of the genera evaluated, in feces, *Escherichia-Shigella* (Supplementary Figure 4.8 B) was affected by the interaction of treatment and time ( $P < 0.05$ ), and was higher on d18 versus d21 in feces collected from CL cows; however, no meaningful pattern was evident ( $P < 0.05$ ). In feces from HS and PF cows, abundance of *Escherichia-Shigella* was higher than in CL cows during the challenge period (d4 and 10) compared to early recovery (d18;  $P < 0.05$ ). Together, these results suggest that *Escherichia-Shigella* abundance in feces appeared heat and feed restriction responsive, suggesting that it is responding to decreased DMI, while *Enterobacter*, *Enterococcus*, *Klebsiella*, *Streptococcus*, and *Staphylococcus* were either not affected by treatment or their abundance was too low to allow for detection of differences.

## **4.5 DISCUSSION**

This study aimed to determine the effects of heat stress on microbiomes and differentiate from those due to reduced feed intake by also comparing to a group of pair-fed cows. The design of this study also allowed for the control of chamber effects on the microbiome with a group of untreated cows. Our results provide evidence that the rumen and fecal microbiomes of lactating dairy cattle were affected by heat stress and feed

restriction. These results support some of the findings from our previous study (Chapter II; unpublished data), however, in that study effects of treatment and time were confounded because one group of cows were followed over time through a period of heat stress and recovery whereas the present study included a group of untreated controls and pair-fed cows to enable separation of effects due to time and reduced DMI. Like our previous study, bacterial richness in feces increased throughout the challenge periods. However, these trends were also observed in the control cows and suggest a temporal pattern that appeared more time-dependent than treatment-dependent; temporal patterns have been reported in rumen fluid and feces previously (Michelland et al., 2009). A prior study speculated that if cows were feed restricted, increased ruminal diversity could be due to decreased passage flow rate, allowing for more slow-growing bacteria to proliferate (McCabe et al., 2015). Results from the present study do not support this hypothesis because the rumen fluid from feed restricted cows displayed richness and diversity measures (Figure 4.1 A-B) that decreased numerically and were lowest during the recovery period. Rumen fluid richness and diversity measures of CL and HS cows displayed similar trends to one another, which suggests that heat stress combined with decreased feed intake and feed restriction alone have different impacts on the rumen and fecal microbiomes.

A previous study (Li et al., 2020) explored the microbiome of feces collected from dairy cows during the spring (not heat stressed) and summer (heat stress) seasons. The present study supports their findings of differences in bacterial community composition of feces during heat stress (Li et al., 2020) and also reports alterations in the feces of feed-restricted cows. Furthermore, this study reports similar changes in

community composition (Figure 4.2 A-B) in rumen fluid of heat-stressed and feed-restricted cows. Hierarchical clustering (Figure 4.4 A-F) of rumen fluid and feces also show that samples clustered by experimental period in HS and PF cows. There were similar numbers of Gram-negative and Gram-positive OTU across all treatments in the rumen fluid, while there were more highly variable Gram-positive OTU in the feces. Of the most variable Gram-positive OTU, the majority belonged to the *Clostridiales* order, but genera within this order responded differently between sample types and treatment. These results indicate that the different stressors (heat stress and feed restriction) alter the overall composition of bacteria in rumen and feces as evidenced by differing results between cows exposed to heat stress combined with the resulting reduced feed intake and cows that were pair-fed without the added heat stressor.

*Firmicutes* and *Bacteroidetes* have been reported as the dominant bacterial phyla in rumen fluid and feces (Ozutsumi et al., 2005; Paz et al., 2016) and during heat stress (Chen et al., 2018; Li et al., 2020). Contrary to our hypothesis, we found no significant interactions of treatment and time in the abundances of *Bacteroidetes* in rumen fluid and feces (Figure 4.5 A-B). Our previous study found that *Bacteroidetes* abundance in feces increased during heat stress (Chapter II; unpublished data) and although the present study also had a time course design, the response of *Bacteroidetes* to heat stress differed between studies. However, we hypothesized that *Proteobacteria* would increase in feces and in support of the previously mentioned hypothesis, *Proteobacteria* in the feces of HS cows did increase during early heat stress and remained numerically higher in recovery (Supplementary Figure 4.7 B). These findings suggest that fecal shedding of *Proteobacteria* increases in response to heat stress which has been reported previously

(Li et al., 2020). *Bacteroidetes* abundance in the feces of PF cows decreased during the challenge period, while *Bacteroidetes* abundance in rumen fluid did not decrease until recovery. Changes in abundance of *Proteobacteria* occurred numerically sooner in feces compared to rumen, which suggests that the microbiome responded to feed restriction and that the abundance of *Proteobacteria* in the rumen fluid was more resistant to heat stress compared to the feces.

Feces can contaminate bedding, which can serve as a reservoir for bacteria that cause mastitis. Coliforms can cause mastitis and coliform counts in bedding have been shown to increase in the summer (Rowbotham and Ruegg, 2016). Most of the mastitis pathogens selected (*Enterococcus*, *Klebsiella*, and *Enterobacter* genera) were present in very few samples (at most 28/67 rumen and 4/144 fecal samples) in agreement with our previous study (Chapter II; unpublished data). The abundances of the *Escherichia-Shigella* genus were only different in the feces; however, this appeared as a slight increase on d10 of heat stress and feed restriction, which is likely due to larger variation at this time point. The relative abundance of *Enterococcus*, *Escherichia-Shigella*, *Streptococcus*, and *Staphylococcus* in the feces of young animals is reported to be low (Alipour et al., 2018) and decreased with increasing age and after weaning (Meale et al., 2016). Overall low abundance could explain why we did not detect these bacterial-associated OTU in sufficient numbers for analysis in rumen and feces and indicates that factors other than fecal shedding must contribute to increased bacterial load in bedding during elevated environmental temperatures. Contrary to one of our hypotheses, there was not an increase in all the evaluated OTU associated with bacteria that commonly cause mastitis in the rumen fluid or feces of heat-stressed cows. While *Escherichia-*

*Shigella* was higher during the challenge period of HS and PF cows, the other selected mastitis-associated OTU did not differ.

The rumen fluid and fecal microbiomes were monitored in untreated control, heat-stressed, and pair-fed cows to distinguish changes in microbial communities due to effects of heat stress or reduced feed intake. Results from the CL cows highlight daily variation that could be due to differences between cows, housing changes, or behavioral stress due to separation from some herd mates. Overall, our results indicate that the ruminal and fecal microbiomes of lactating dairy cows responded to heat stress and feed restriction stressors. We hypothesized that Gram-negative bacteria, *Bacteroidetes* and *Proteobacteria*, would increase in abundance during heat stress and in support of this, these bacterial phyla displayed different temporal responses to heat stress and also to feed restriction.

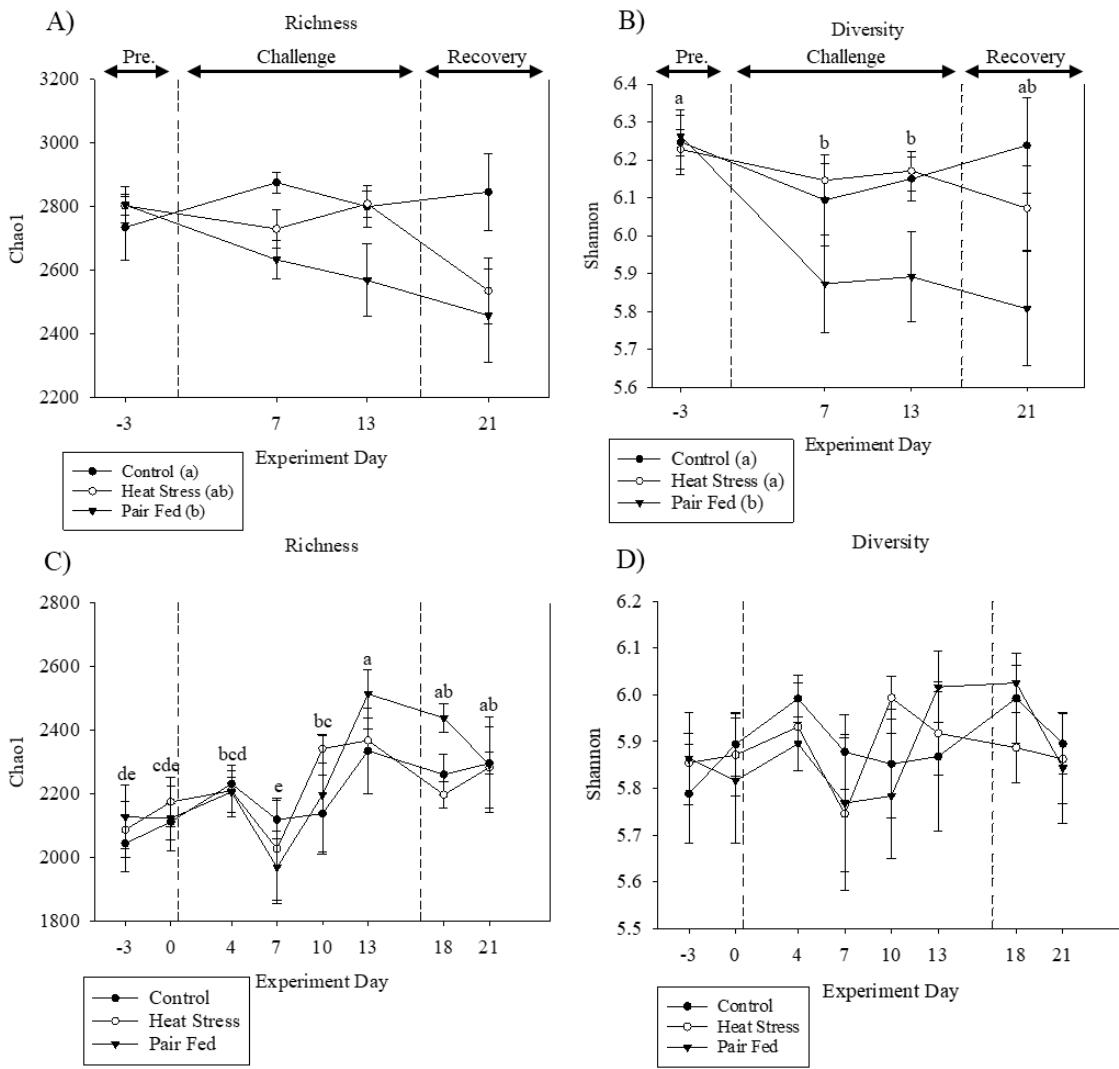
#### **4.6 CONCLUSIONS**

In conclusion, 16d of constant heat stress and feed restriction altered the rumen and fecal microbiomes of lactating dairy cows. Richness and diversity of bacterial populations and individual phyla (*Bacteroidetes* and *Proteobacteria*) and genera (*Escherichia-Shigella*) responded differently to heat stress and feed restriction stress which indicates that bacterial populations are independently altered by heat stress and reduced DMI. These results show that some mastitis-associated bacteria responded to experimental heat stress conditions.

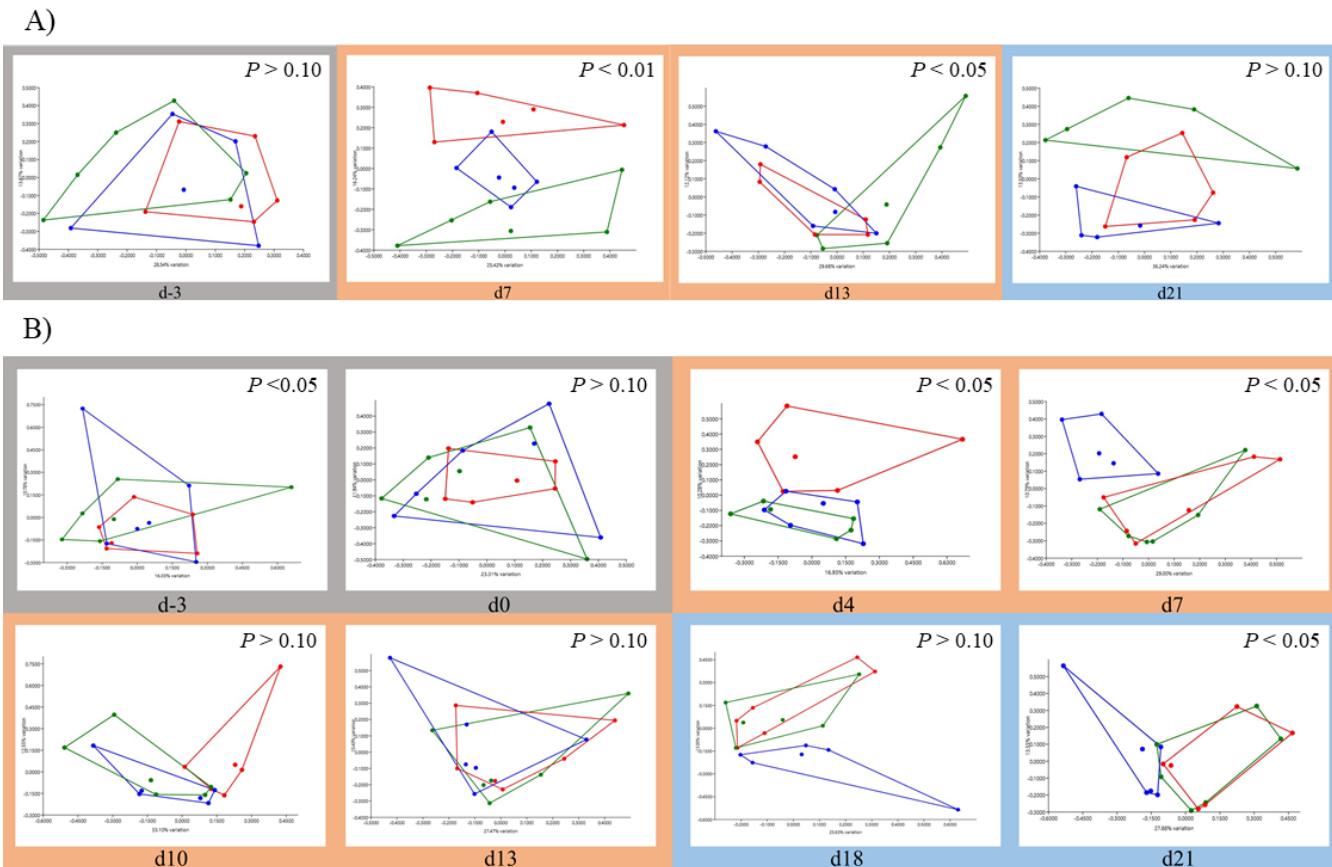
**Table 4.1.** Mean  $\pm$ SEM rumen fluid pH values within treatment and period.

	Pretreatment	Challenge			Recovery	Across period
	d-3	d7	d13	d21		
	Mean $\pm$ SEM	Mean $\pm$ SEM	Mean $\pm$ SEM	Mean $\pm$ SEM		
Control	6.31 $\pm$ 0.21 <sup>b,x</sup>	6.29 $\pm$ 0.30 <sup>b,y</sup>	6.11 $\pm$ 0.30 <sup>b,x</sup>	6.74 $\pm$ 0.09 <sup>a,x</sup>	6.36	
Heat Stress	6.22 $\pm$ 0.15 <sup>a,x</sup>	6.37 $\pm$ 0.18 <sup>a,y</sup>	5.97 $\pm$ 0.15 <sup>a,x</sup>	6.14 $\pm$ 0.32 <sup>a,xy</sup>	6.18	
Pair Fed	6.21 $\pm$ 0.14 <sup>b,x</sup>	6.94 $\pm$ 0.17 <sup>a,x</sup>	6.24 $\pm$ 0.11 <sup>b,x</sup>	5.89 $\pm$ 0.24 <sup>b,y</sup>	6.32	
Across treatment	6.25	6.54	6.11	6.25		

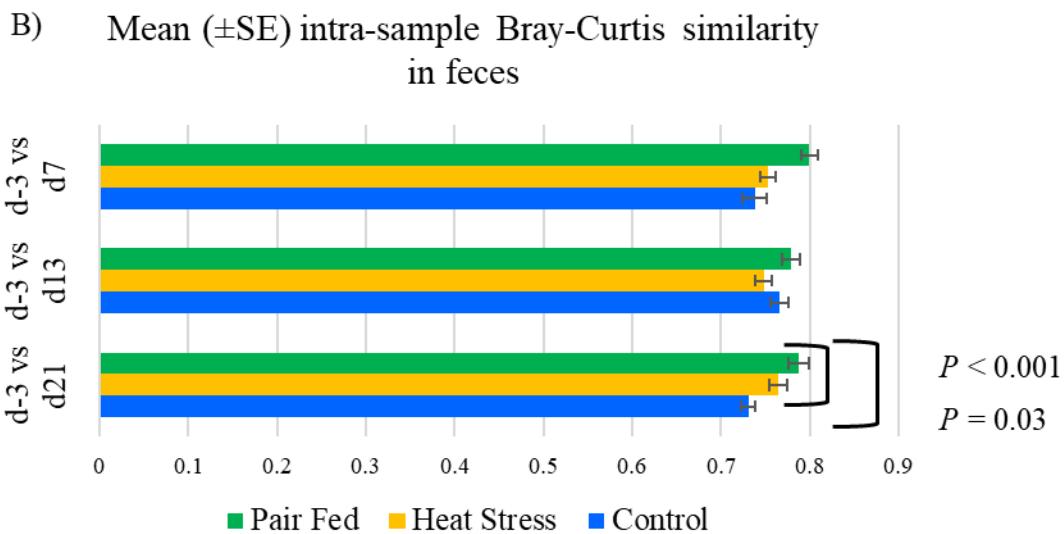
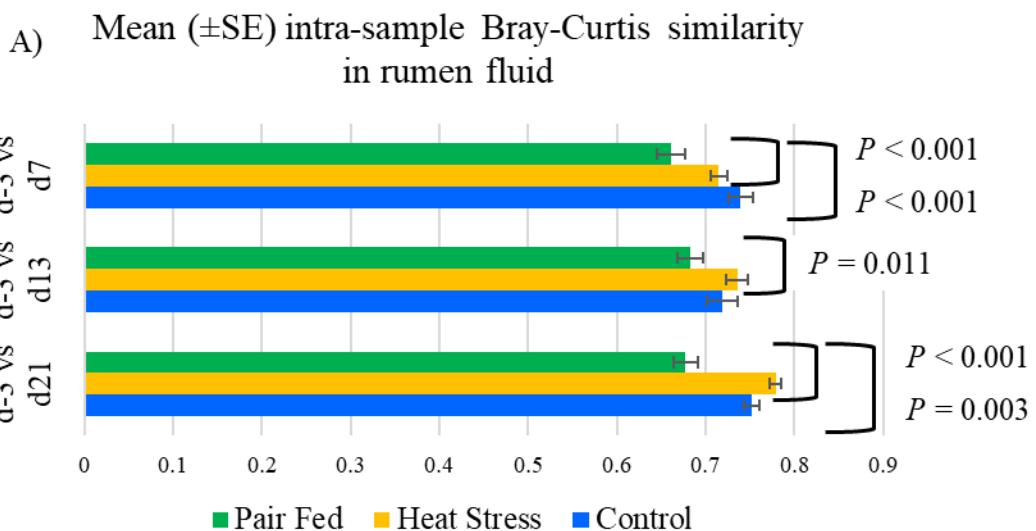
**Table 4.1.** Mean  $\pm$ SEM rumen fluid pH values within treatment and period. Rumen fluid samples were collected during pretreatment (d-3), challenge (d7 and 13), and recovery (d21). <sup>a,b</sup>Means within treatment differ across time. <sup>x,y</sup>Means within timepoint differ between treatments.



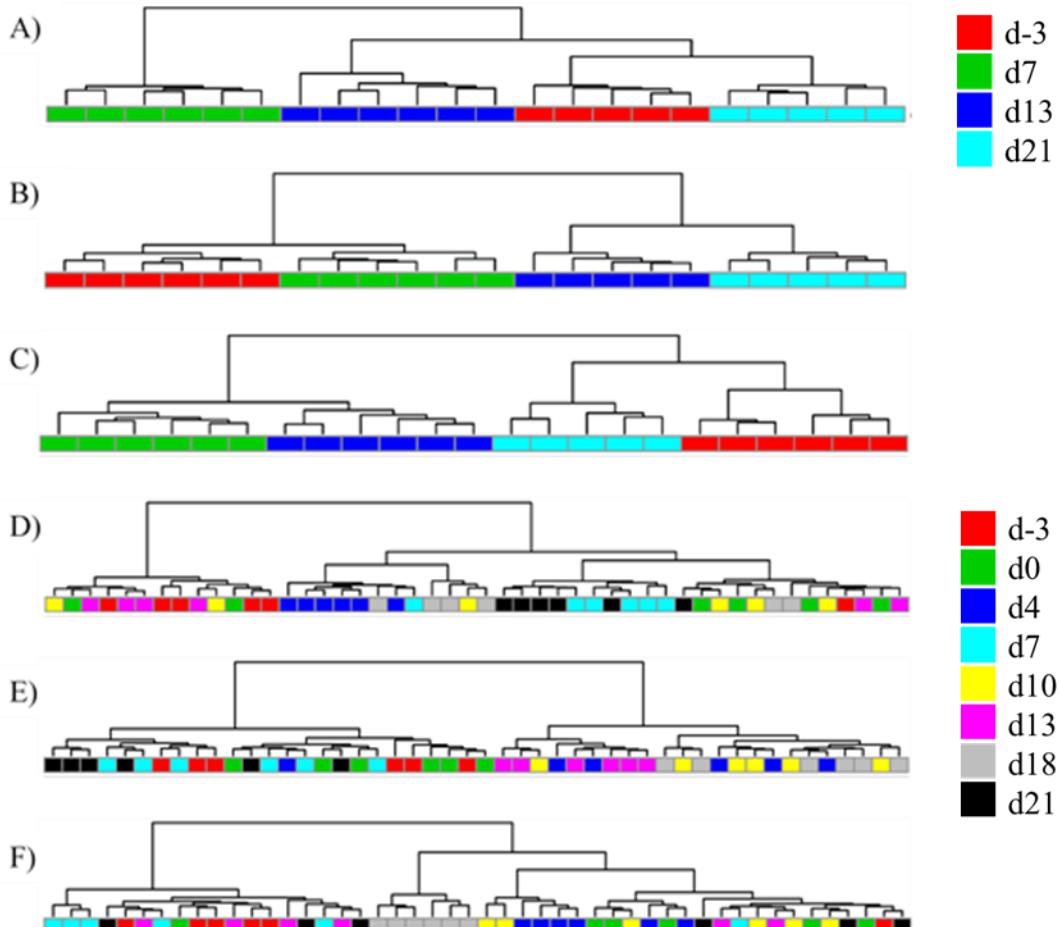
**Figure 4.1.** Changes in rumen fluid A) richness (Chao1) and B) diversity (Shannon Index), and fecal C) richness and D) diversity in dairy cows subjected to heat stress or pair feeding. Rumen samples were collected during pretreatment (Pre.; d-3), challenge (d7 and 13), and recovery (d21). The fecal samples were collected during pretreatment (d-3 and 0), challenge (d4, 7, 10, and 13), and recovery (d18 and 21). <sup>a,b,c,d,e</sup>Within panel, average abundance differed over time at  $P < 0.05$ , <sup>a,b</sup>within legend, average abundance differed between treatments at  $P < 0.05$ .



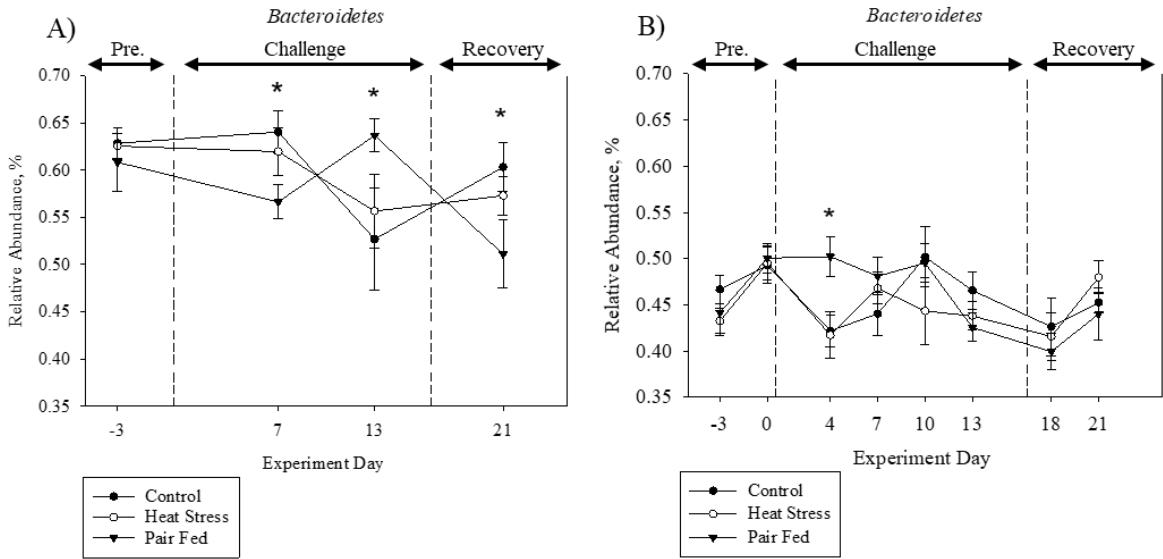
**Figure 4.2.** Principal coordinate analysis (PCoA) based on Bray-Curtis distances in A) rumen fluid and B) feces by treatment and over time. Samples from control cows (CL) are represented by blue, heat-stressed cows (HS) are represented by red, and feed-restricted cows (PF) are represented by green outlines. The rumen samples were collected during pretreatment (grey box; d-3), challenge (orange box; d7 and 13), and recovery (blue box; d21). The fecal samples were collected during pretreatment (grey box; d-3 and 0), challenge (orange box; d4, 7, 10, and 13), and recovery (blue box; d18 and 21).



**Figure 4.3.** Mean  $\pm$ SEM intra-sample similarities based on Bray-Curtis distances in A) rumen fluid and B) feces collected from cows exposed to heat stress or pair-feeding. Comparisons were made between pretreatment and early challenge (d-3 versus d7), between pretreatment and late challenge (d-3 versus d13), and between pretreatment and recovery (d-3 versus d21). Treatments within each panel are indicated by blue (CL), orange (HS), and green (PF). Within comparison differences are noted by brackets with the respective P-value based on the top of each bracket and reading downwards.



**Figure 4.4.** Hierarchical clustering of top 25 most variable OTU in samples from each treatment from rumen fluid (A-C) or feces (D-F) collected from cows exposed to heat stress or pair-feeding. Within the rumen samples treatment differences over time are displayed in A) control cows (CL), B) heat-stressed cows (HS), and C) feed-restricted cows (PF). Rumen samples were collected during pretreatment (d-3), challenge (d7 and 13), and recovery (d21). Within the fecal samples treatment differences over time are displayed in D) control cows (CL), E) heat-stressed cows (HS), and F) feed-restricted cows (PF). The fecal samples were collected during pretreatment (d-3 and 0), challenge (d4, 7, 10, and 13), and recovery (d18 and 21). In the legends on the right side of the figure, sample days are indicated by color.



**Figure 4.5.** Changes in relative abundance of Bacteroidetes in A) rumen fluid and B) feces of dairy cows exposed to heat stress or pair-feeding. Rumen samples were collected during pretreatment (Pre.; d-3), challenge (d7 and 13), and recovery (d21). Fecal samples were collected during pretreatment (d-3 and 0), challenge (d4, 7, 10, and 13), and recovery (d18 and 21). \*Within panel, means within day differed between treatments as the result of a treatment by time interaction at  $P < 0.05$ .

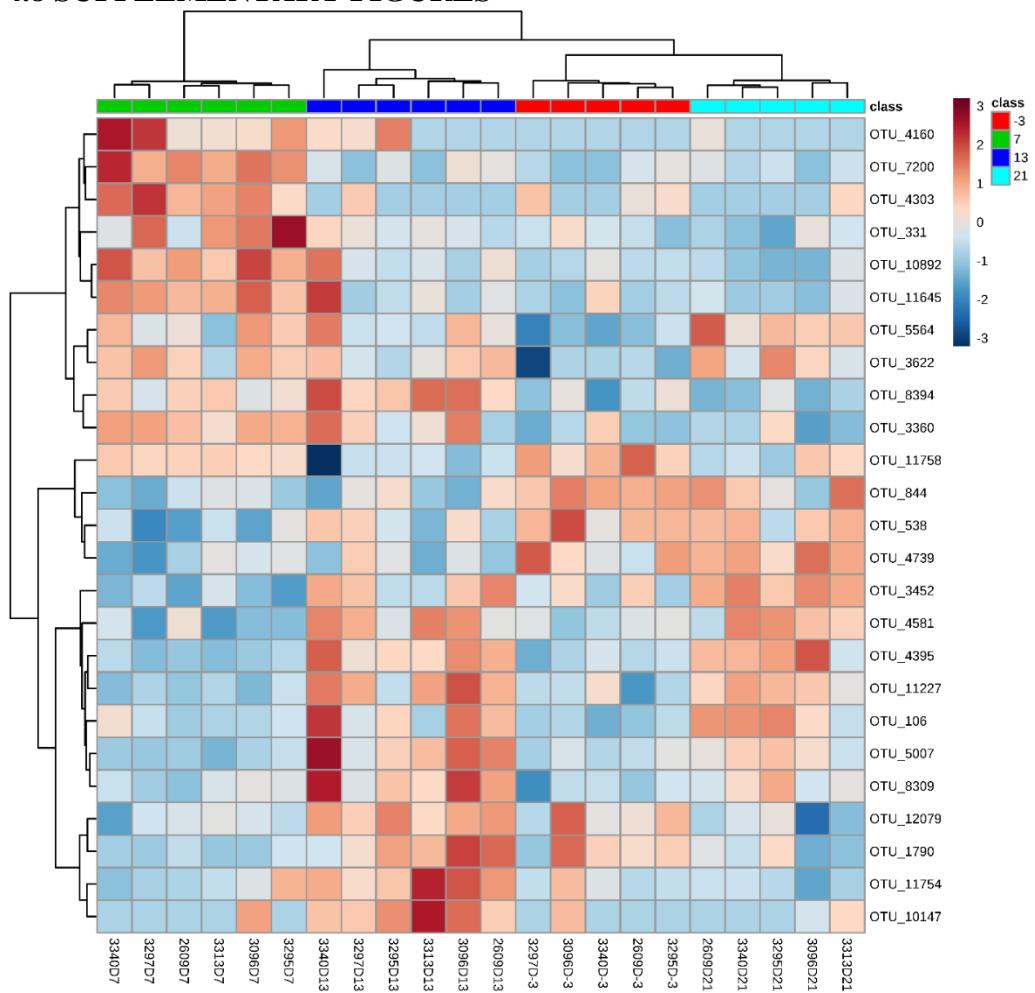
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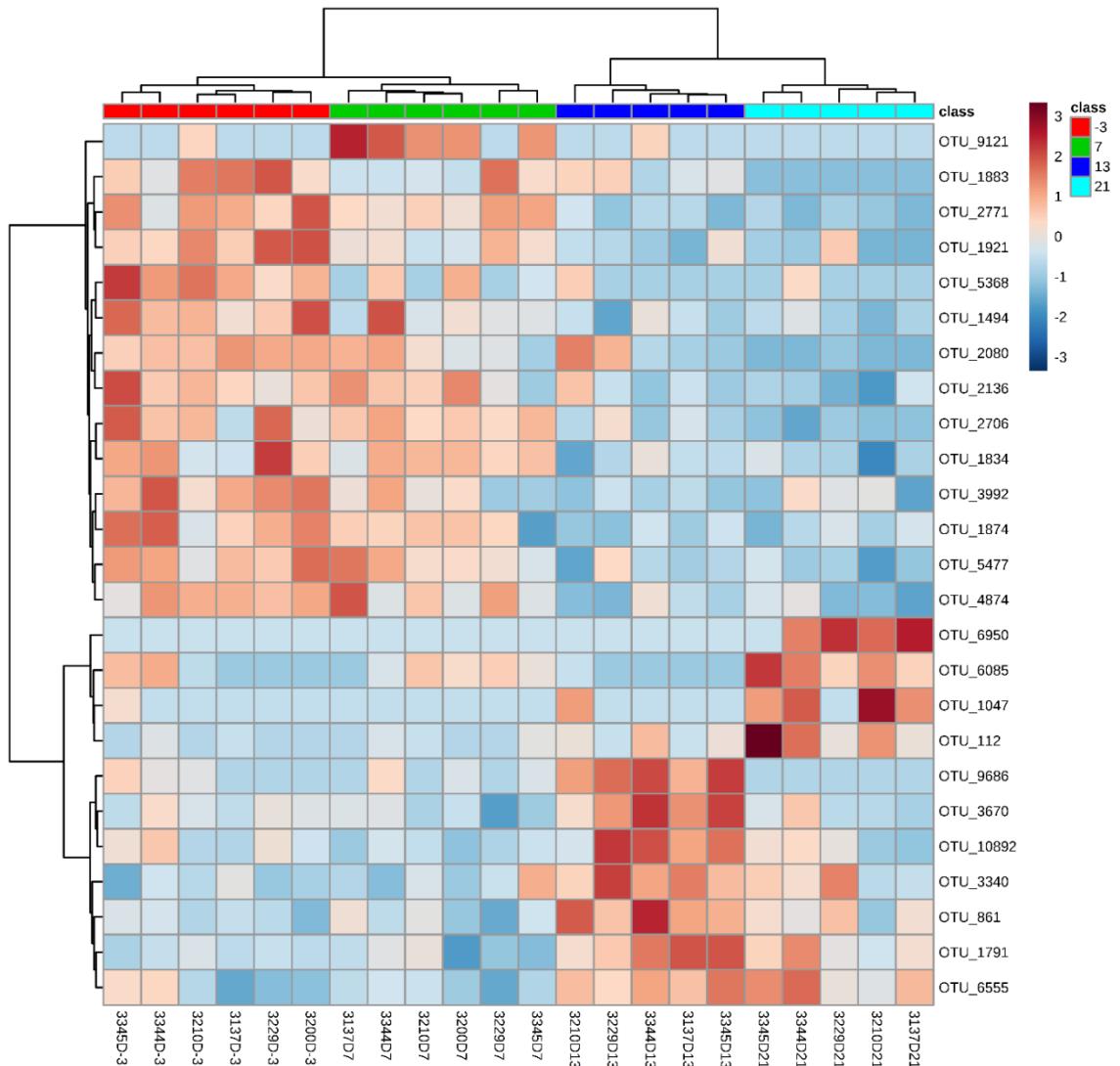
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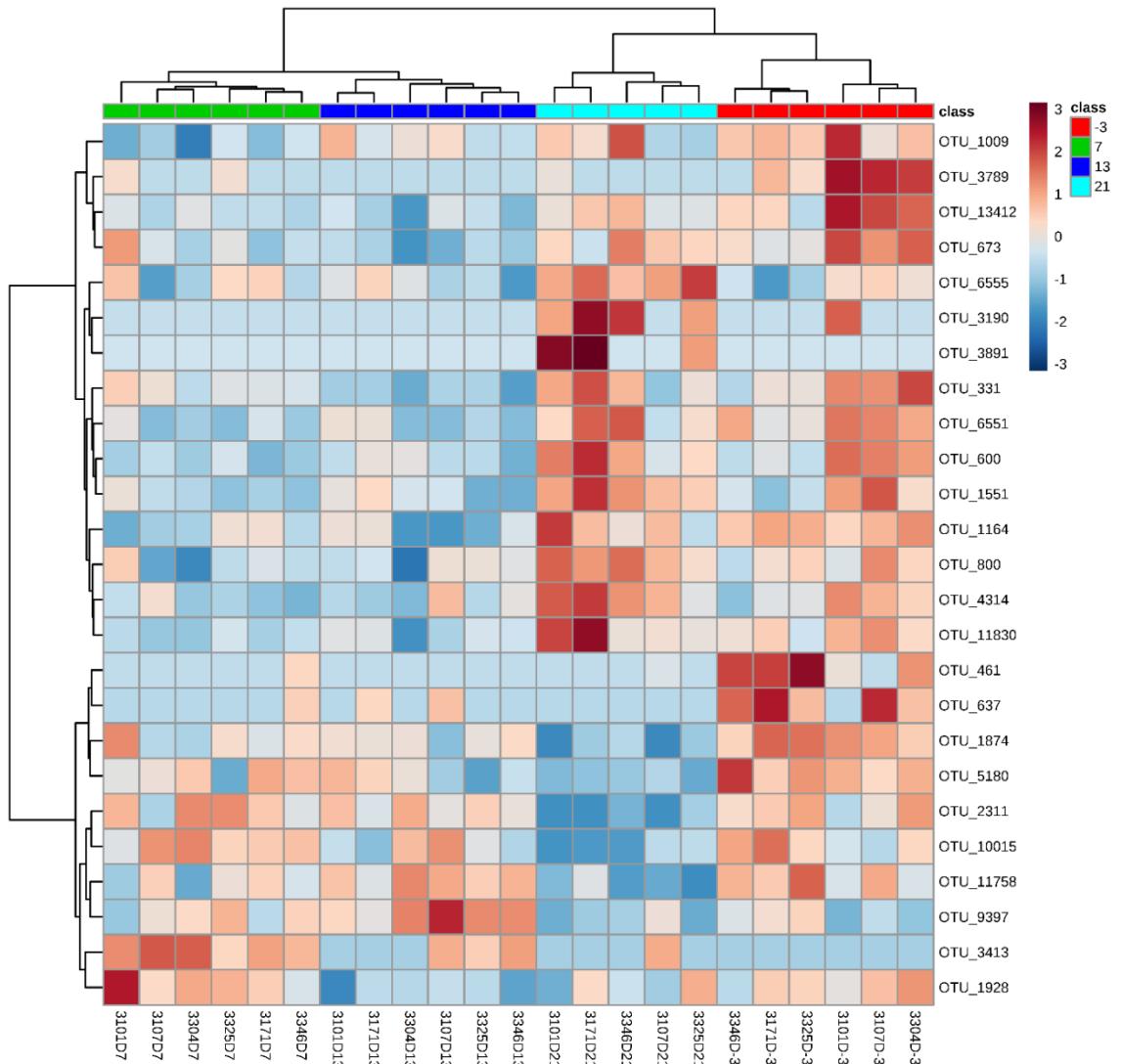
## 4.8 SUPPLEMENTARY FIGURES



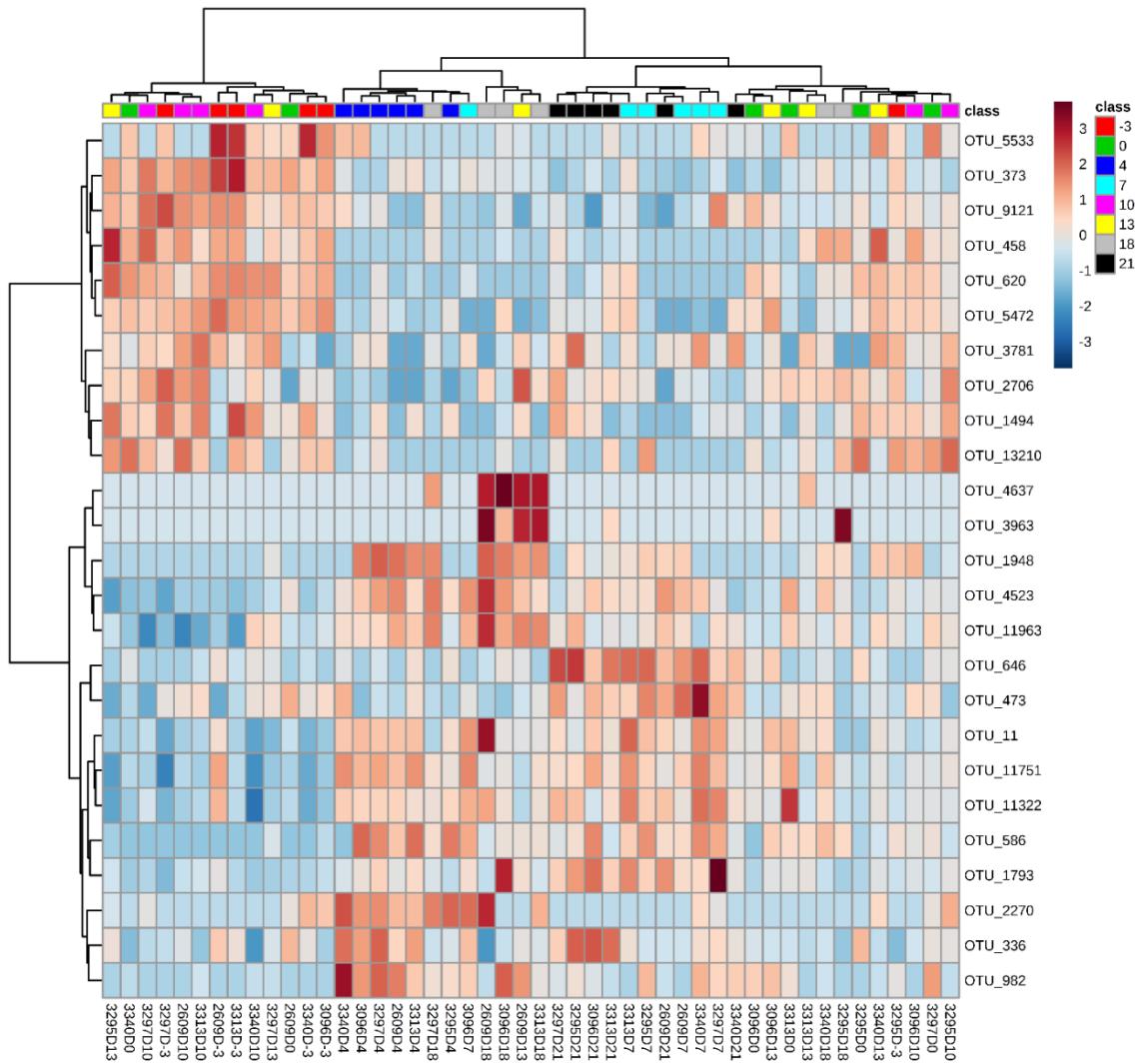
**Supplementary Figure 4.6 A.** Hierarchical clustering of the 25 most variable OTU from the rumen fluid of control cows (CL). Red to blue key on the right signifies an OTU is more abundant (red) or less abundant (blue) at a given timepoint represented by the class key on the far right. Pretreatment is represented by d-3, challenge by d7 and 13, and recovery by d21. Bottom numbers are individual cows and sample day.



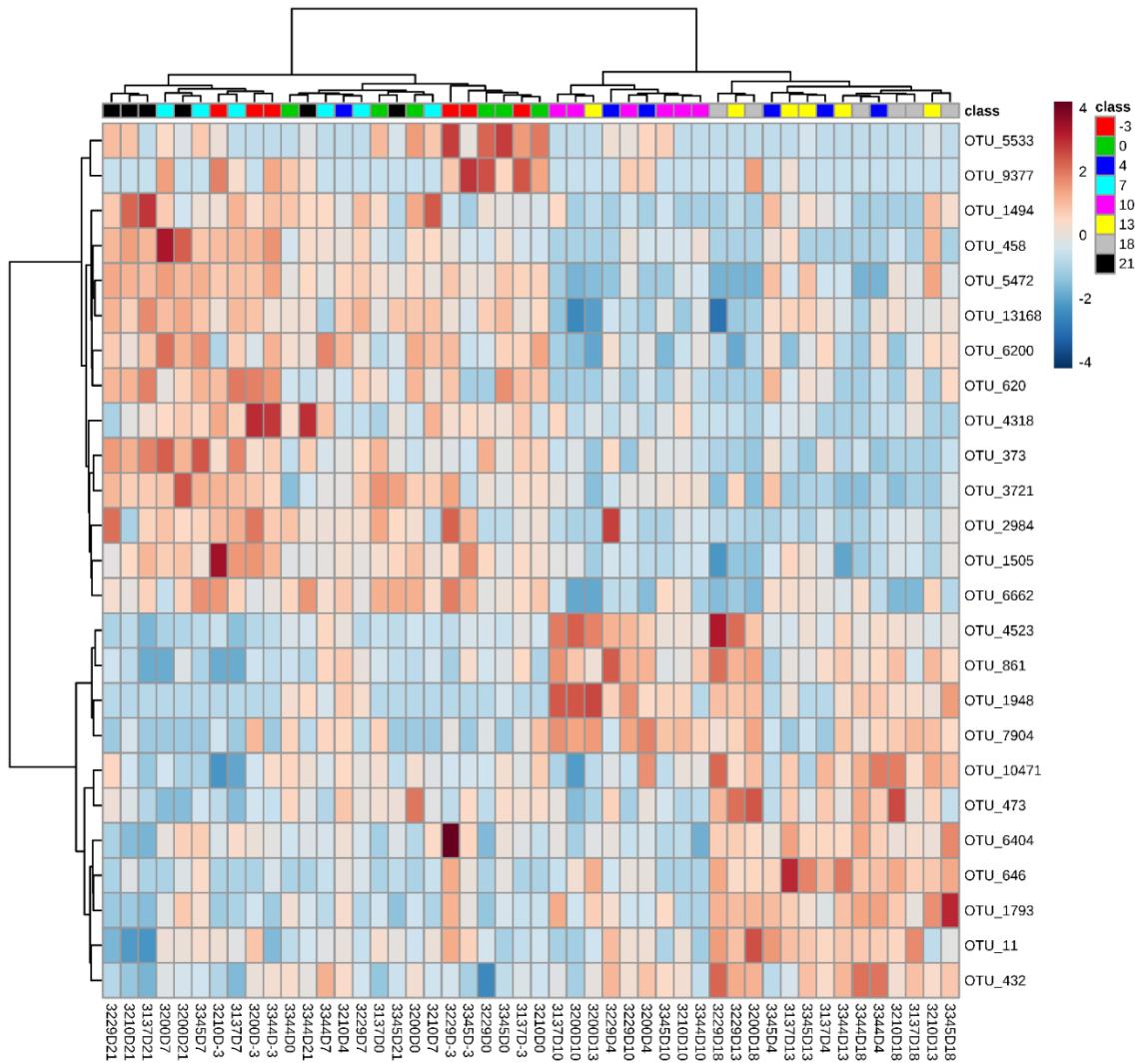
**Supplementary Figure 4.6 B.** Hierarchical clustering of the 25 most variable OTU from the rumen fluid of heat-stressed cows (HS). Red to blue key on the right signifies an OTU is more abundant (red) or less abundant (blue) at a given timepoint represented by the class key on the far right. Pretreatment is represented by d-3, challenge by d7 and 13, and recovery by d21. Bottom numbers are individual cows and sample day.



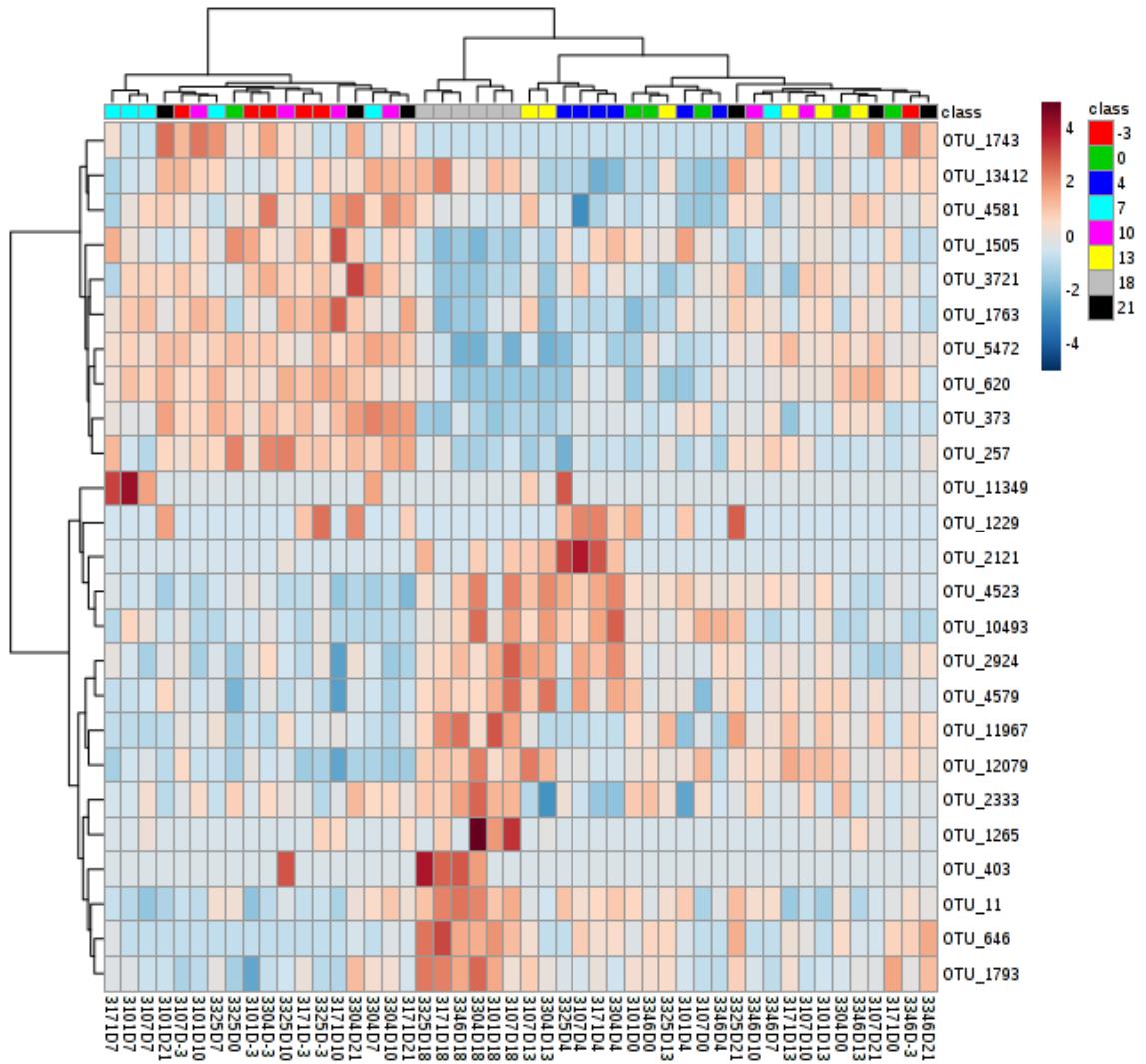
**Supplementary Figure 4.6 C.** Hierarchical clustering of the 25 most variable OTU from the rumen fluid of pair-fed cows (PF). Red to blue key on the right signifies an OTU is more abundant (red) or less abundant (blue) at a given timepoint represented by the class key on the far right. Pretreatment is represented by d-3, challenge by d7 and 13, and recovery by d21. Bottom numbers are individual cows and sample day.

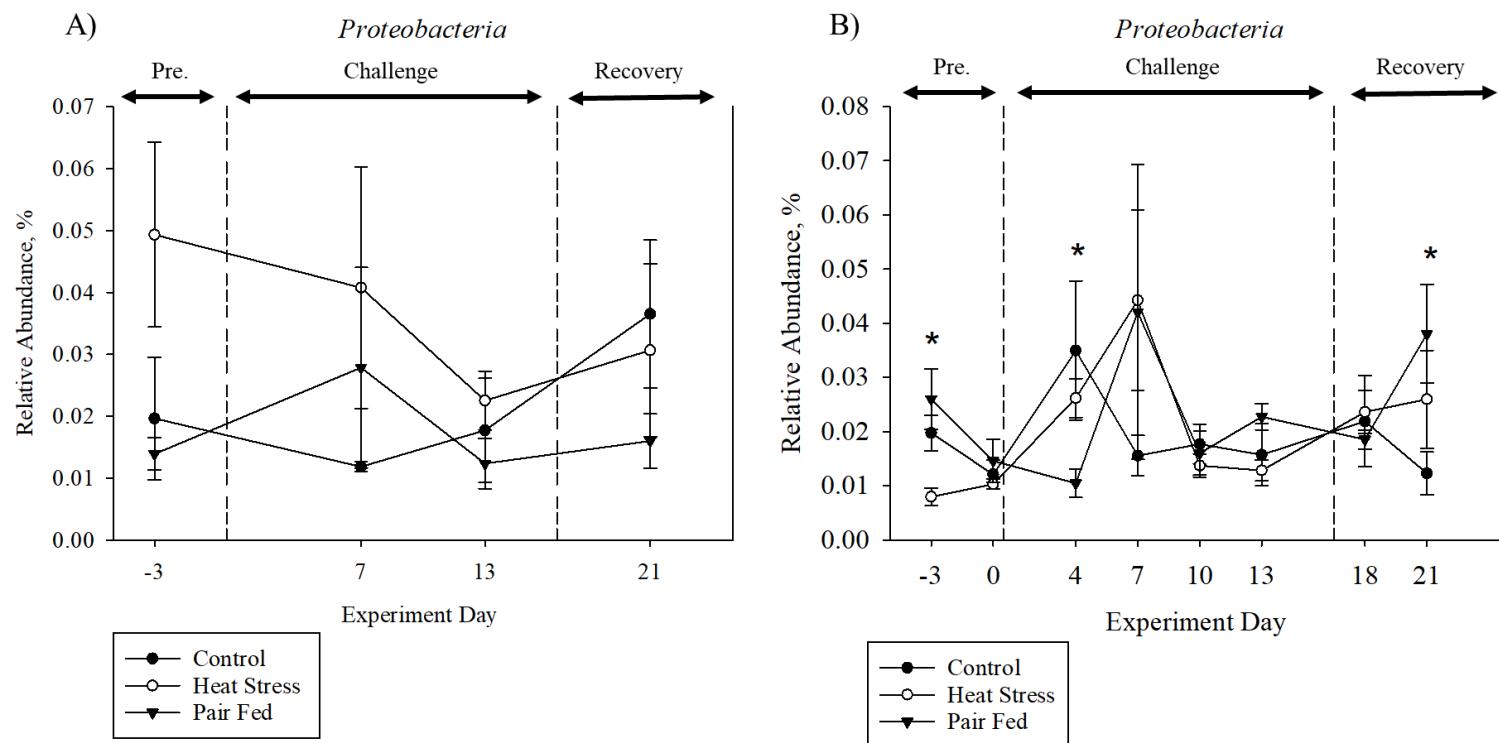


**Supplementary Figure 4.6 D.** Hierarchical clustering of the 25 most variable OTU from feces of control cows (CL). Red to blue key on the right signifies an OTU is more abundant (red) or less abundant (blue) at a given timepoint represented by the class key on the far right. Pretreatment is represented by d-3 and 0, challenge by d4, 7, 10, and 13, and recovery by d21. Bottom numbers are individual cows and sample day.

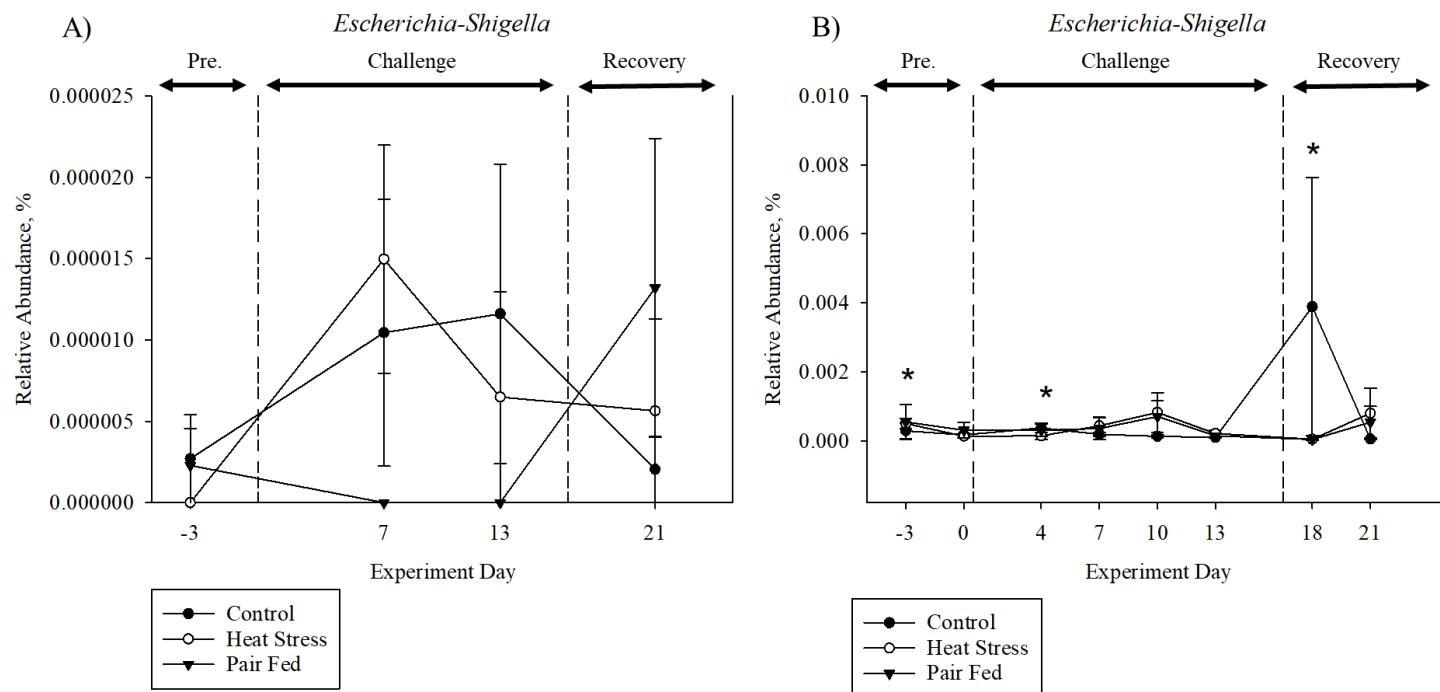


**Supplementary Figure 4.6 E.** Hierarchical clustering of the 25 most variable OTU from feces of heat-stressed cows (HS). Red to blue key on the right signifies an OTU is more abundant (red) or less abundant (blue) at a given timepoint represented by the class key on the far right. Pretreatment is represented by d-3 and 0, challenge by d4, 7, 10, and 13, and recovery by d21. Bottom numbers are individual cows and sample day.





**Supplementary Figure 4.7 A-B.** Relative abundance of *Proteobacteria* phyla from A) rumen fluid and B) feces collected from cows exposed to heat stress or pair-feeding. The rumen samples were collected during pretreatment (Pre.; d-3), challenge (d7 and 13), and recovery (d21). The fecal samples were collected during pretreatment (d-3 and 0), challenge (d4, 7, 10, and 13), and recovery (d18 and 21). \*Within panel indicates significant time effect at that time ( $P < 0.05$ ).



**Supplementary Figure 4.8 A-B.** Relative abundance of *Escherichia-Shigella* genus from A) rumen fluid and B) feces collected from cows exposed to heat stress or pair-feeding. The rumen samples were collected during pretreatment (Pre.; d-3), challenge (d7 and 13), and recovery (d21). The fecal samples were collected during pretreatment (d-3 and 0), challenge (d4, 7, 10, and 13), and recovery (d18 and 21). \*Within panel indicates significant effect of time at that time ( $P < 0.05$ ).

## **CHAPTER V**

### ***HOT TOPIC: HEAT STRESS CONDITIONS INCREASE ABUNDANCE OF ENTEROCOCCUS IN BEDDING AND ON TEAT AND INGUINAL SKIN OF LACTATING COWS***

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## **HOT TOPIC: HEAT STRESS CONDITIONS INCREASE ABUNDANCE OF ENTEROCOCCUS IN BEDDING AND ON TEAT AND INGUINAL SKIN OF LACTATING COWS**

### **5.1 ABSTRACT**

The objective of this study was to evaluate the effects of heat stress or pair-feeding on the bedding, inguinal skin, teat skin, and milk microbiomes of lactating dairy cows. We hypothesized that cows exposed to an increased temperature-humidity index (**THI**) would have an increased abundance of environmental mastitis-causing pathogens in bedding, leading to an increased abundance on teat skin, and resulting in an increased abundance of these pathogens in milk. Holstein dairy cows ( $n = 18$ ) were housed in Brody Environmental Units for 25 days and subjected to one of three treatments: control (**CL**), heat stressed (**HS**), or pair fed (**PF**). The CL cows were kept at thermoneutral conditions and received feed ad libitum for the duration of the study. The HS cows were kept at thermoneutral conditions during a 5-day pretreatment and an 8-day recovery period but during the challenge period were subjected to constantly elevated THI to induce heat stress. These cows were fed ad libitum for the duration of the study. To separate the effects of elevated THI from those of reduced feed intake, alone, the PF cows were fed to match the reduced intake of the HS cows during the challenge and recovery periods. Cows on PF were kept at thermoneutral conditions for the duration of the study and received feed ad libitum during the pretreatment period. Bedding, inguinal skin, teat skin, and milk samples were collected during the pretreatment (d-3 and 0), challenge (d4, 7, 10, and 13), and recovery (d18 and 21) periods from all cows. From samples, DNA was subjected to 16S rRNA sequencing to evaluate the abundance of

selected environmental mastitis-causing pathogens including *Enterobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, and *Streptococcus*. Most of the genera evaluated varied by treatment and over time but did not appear to respond to elevated THI, except for *Enterococcus* which appeared heat-responsive as evidenced by an increase in abundance in bedding and on inguinal and teat skin samples beginning early (d4) and continuing throughout the challenge period in HS cows. Additionally, *Enterococcus* displayed similar patterns of abundance in bedding and on inguinal and teat skin. Inguinal skin should be less exposed to the bedding compared to teat skin and suggests that teat end exposure to bacteria may not be entirely dependent on bedding. Our results illustrate that some environmental pathogens responded to elevated THI. Further studies are needed to determine if these changes persist outside of a controlled environment.

## 5.2 HOT TOPIC

Elevated environmental temperature and relative humidity can result in heat stress conditions for dairy cattle (Kadzere et al., 2002). One common result of heat stress is decreased milk production which is partially attributed to decreased feed intake (Rhoads et al., 2009; Baumgard et al., 2011). Additional factors that could account for the remainder of lost milk production remain unknown.

Another outcome often associated with heat stress is increased disease incidence, especially mastitis. Indeed, an association between increased linear score of bulk tank milk and summer season has been described (Zucali et al., 2011) and bulk tank SCC (BTSCC) has been reported to peak in July and August (Bodoh et al., 1975; Olde Riekerink et al., 2006). When bacteria on the teat end invade the mammary gland through the streak canal, IMIs commonly occur resulting in subclinical or clinical mastitis. Bedding can contaminate teat ends, and increased teat end exposure to pathogens can

increase the likelihood of an IMI (Rowbotham and Ruegg, 2016; Rowe et al., 2019). Periods of elevated environmental temperature and humidity are associated with increased Gram-negative bacterial counts in bedding (Hogan et al., 1989; Rowbotham and Ruegg, 2016) and isolation of Gram-negative pathogens (*Escherichia coli* and *Klebsiella* species) from milk (Gao et al., 2017). It has been suggested that elevated temperature and humidity may increase growth of bacteria in the environment, including in bedding (Hogan and Smith, 2012). In contrast, Gram-negative bacterial counts on teat skin have been reported to be lower during summer and fall except for *Klebsiella* which was higher in summer compared to spring (Rowbotham and Ruegg, 2016). Conversely, Gram-positive bacterial counts in bedding and on teat skin vary during elevated environmental temperature and humidity. *Streptococcus* counts in bedding were unchanged across seasons (Hogan et al., 1989; Rowbotham and Ruegg, 2016). On the contrary, Rowbotham and Ruegg (2016) reported fewer *Streptococcus*-like organisms on teat ends during the summer and fall. The previously mentioned studies used traditional bacterial culturing methods to enumerate bacterial counts while the present study used 16S rRNA gene sequencing.

The impact of elevated heat and humidity on the microbial populations of the dairy cow has recently been evaluated. Changes in the cow's bedding microbiome throughout the year (Nguyen et al., 2019a; Nguyen et al., 2019b) and the effects of heat stress on the rumen (Zhao et al., 2019) and fecal microbiota (Chen et al., 2018; Li et al., 2020) have been assessed, while the effects of acute heat stress on the skin and bedding microbiomes and their potential interaction has not been reported. Changes in the environmental and skin microbiota of cattle exposed to elevated environmental

temperatures could help explain the higher BTSCC and milk loss that occur during times of heat stress. The objective of this study was to evaluate the effects of experimentally induced heat stress and feed restriction on the microbiome of mid-lactation Holstein cows. We hypothesized that increased THI and rectal temperature would increase the abundance of environmental mastitis-causing pathogens—*Enterobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, and *Streptococcus* species—in bedding, resulting in an increased abundance on teat skin and in milk. We also hypothesized that inguinal skin in all treatment groups would not display the same changes as teat skin due to less exposure to bedding.

To evaluate these hypotheses, mid-lactation Holstein cows ( $n = 18$ ) from the University of Missouri Foremost Dairy Research and Teaching farm were blocked based on DIM, parity, milk yield, and SCC. Cows within blocks were assigned to one of three treatments: control (**CL**,  $n = 6$ ), heat stress (**HS**,  $n = 6$ ), or pair fed (**PF**,  $n = 6$ ) and housed in tie stalls on crushed soyhull bedding in separate Brody Environmental Units at the University of Missouri. The CL cows were kept at thermoneutral conditions (temperature-humidity index (**THI**) ~ 66) and fed ad libitum for the duration of the experiment (27 days). The HS cows were kept at thermoneutral conditions (**THI** ~ 66) during the pretreatment (d-5 to 0) and recovery (d15 to 22) periods. During the challenge period (d0 to 14), HS cows were subjected to constant heat stress (**THI** ~80) and received feed ad libitum. The PF cows were kept at thermoneutral conditions (**THI** ~ 66) for the duration of the experiment but during the challenge (d0 to 14) and recovery (d15 to 22) periods, PF cows were pair-fed to match the reduced intake of HS cows. Pair-feeding was used to separate effects of increased environmental temperatures from those due to

reduced feed intake, alone. All cows were milked twice daily, fed a TMR formulated to meet or exceed nutrient requirements (NRC, 2001), and had unlimited access to water.

All samples were collected approximately three hours after morning milking (~9AM) during the pretreatment (d-3 and 0), challenge (d4, 7, 10, and 13), and recovery (d18 and 21) periods. For crushed soyhull bedding samples, a 12-square grid was placed in the back third of each individual cow's stall, and five samples were randomly collected from the grid spaces selected by a random number generator. Disposable latex gloves (Latex Exam Gloves; AmerisourceBergen; Oceanside, CA) were used, and samples were immediately placed into sterile Whirl-paks (Nasco; Fort Atkinson, WI). Within each chamber, bedding samples from three cows were pooled, resulting in two pooled samples per room per time point. Additionally, one sample was collected from the stockpile of unused bedding, at each sampling time. Inguinal and teat skin samples were collected using one-half of an autoclaved electrostatic duster (Swiffer<sup>TM</sup>; Procter and Gamble, Cincinnati, OH). Wearing a new pair of disposable gloves (Latex Exam Gloves; AmerisourceBergen) for each cow, inguinal samples were collected by swiping one-half of an electrostatic duster (Swiffer<sup>TM</sup>; Procter and Gamble) up the inguinal region from both inguinal locations and pooled as one sample per cow per sampling time. To collect teat skin samples, a new pair of disposable gloves (Latex Exam Gloves; AmerisourceBergen) were worn for each cow and autoclaved electrostatic dusters (Swiffer<sup>TM</sup>; Procter and Gamble) were used to sample around the barrel and across the base of the teat. Samples from two teats of each cow were pooled, resulting in one teat skin sample per cow per time point. All milk samples were collected at the quarter level and analyzed individually. Milk samples were aseptically collected into 5 mL round

bottom tubes (Falcon; ThermoFisher Scientific; Waltham, MA). The aseptic technique that used was recommended by the National Mastitis Council (Middleton et al., 2017).

All samples were immediately frozen and stored at -20°C prior to DNA extraction.

Bedding (n = 55), inguinal skin (n = 144), teat skin (n = 144), and milk (n = 288) were thawed at room temperature prior to sample processing for DNA extraction.

Bedding samples were vortexed to mix and approximately 0.25 g of bedding and 10 mL of Mili-Q water was added to a 50 mL conical tube (Falcon; ThermoFisher Scientific; Waltham, MA). The bedding samples were agitated on a shaker (Stovall Belly Dancer; ThermoFisher Scientific) for 10 minutes prior to removing 750 µL of liquid as the starting material for DNA extraction. To remove DNA from inguinal and teat skin electrostatic dusters, 15 mL of Mili-Q water was added to each Whirl-pak and samples were agitated for 10 minutes on a shaker (Stovall Belly Dancer). Like bedding samples, 750 µL of liquid from each inguinal and teat sample was used as the starting material for DNA extraction. From milk samples, 1,500 µL was added to a sterile 2 mL microcentrifuge tube (ThermoFisher Scientific). Milk samples were centrifuged for 20 minutes at 4700 RPM and afterwards, the fat layer was removed with an autoclaved Q tip and the sample was vortexed to mix. From milk samples, 800 µL of liquid was used as the starting material for DNA extraction.

DNA was extracted using QIAamp PowerFecal DNA kits (Qiagen; Germantown, MD), following the manufacturer's protocol with a minor alteration. Instead of using the vortex adapter to lyse cells, a TissueLyser II (Qiagen) was used for 10 minutes at 30 Hz. Quantification of DNA was determined using a Qubit 2.0 fluorometer (ThermoFisher Scientific) with the quant-iT BR dsDNA reagent kit (Invitrogen; Carlsbad, CA) for

bedding, inguinal skin, and teat skin or the quant-iT HS dsDNA reagent kit (Invitrogen) for milk. The V4 region of the 16S rRNA gene was amplified with universal primers (U515/806R) and flanked by Illumina standard adapter sequences (Caporaso et al., 2011) to construct the libraries. At the University of Missouri DNA Core Facility, the Illumina MiSeq platform was used to sequence the 16S rRNA libraries. Operational taxonomic units (**OTU**) were assigned based on  $\geq 97\%$  nucleotide similarity (Caporaso et al., 2010) and were annotated against the Silva.v132 database using NCBI BLAST (McGinnis and Madden, 2004).

All individual genera identified from bedding, inguinal skin, and teat skin, and *Staphylococcus* and *Streptococcus* from milk, were analyzed using SAS (SAS Institute Inc. Cary, NC). Data were rank transformed due to lack of normality as determined by Shapiro-Wilk test and visual inspection of Q-Q plots and histograms. In milk, *Enterobacter*, *Enterococcus*, and *Klebsiella* individual genera were analyzed in a binary model due to lack of normality that could not be resolved with transformations. A repeated measures analysis with randomized complete block design in PROC GLIMMIX was used to determine changes in individual genera. Fixed effects included treatment, time, and their interaction. All results are reported as least squares means  $\pm$  standard error of the mean and statistical significance was declared at  $P < 0.05$ .

Of the environmental mastitis pathogens evaluated, all were present in at least 89%, 33%, 63%, and 5% of bedding, inguinal skin, teat skin, and milk samples, respectively. The average relative abundance for most genera varied between treatment and sample type (Table 5.1). *Enterobacter*, *Enterococcus*, *Klebsiella*, and *Streptococcus*

abundance varied across sample types ( $P < 0.05$ ). *Escherichia* abundance was not different in any of the sample types across treatment or over time ( $P > 0.10$ ).

*Enterobacter* abundance was not different in bedding ( $P > 0.10$ ), inguinal skin ( $P > 0.70$ ), and milk ( $P > 0.90$ ) but had a significant interaction on teat skin samples ( $P < 0.05$ ). *Enterobacter* abundance on teat skin samples from HS cows was lower on d4 versus d10, and from PF cows, was lower on d13 and 18 compared to d-3 and 4. *Enterobacter* abundance on teat skin appeared slightly responsive to heat stress and feed restriction over time.

Abundance of *Klebsiella* was not different in bedding ( $P > 0.10$ ), teat skin ( $P > 0.10$ ), and milk ( $P > 0.40$ ), but was affected by treatment only in inguinal samples ( $P < 0.05$ ). Inguinal samples from HS and PF cows had a slightly greater abundance of *Klebsiella* compared to CL cows. While the effect of time was not significant ( $P = 0.10$ ), *Klebsiella* abundance in HS cows increased numerically throughout the heat stress period, declined during early recover, and fully recovered by the last day of sampling.

Abundance of *Streptococcus* was not different in bedding ( $P > 0.10$ ), which appeared due to large amounts of variation in this genus over time. Abundance of *Streptococcus* on inguinal and teat skin was affected by significant treatment by time interactions ( $P < 0.05$ ) and displayed similar patterns of abundance across treatments. On both, abundance of *Streptococcus* in CL was low and changed little through the study. The abundance of *Streptococcus* on inguinal skin from HS cows increased during the challenge (d7), remained elevated during the challenge period, and did not fully return to pretreatment levels by the last day of sampling. Similarly, abundance of *Streptococcus* on teat skin from HS cows increased during early heat stress (d4), increased further

throughout the challenge period, and did not fully recover by d21. *Streptococcus* abundance on inguinal skin and teat skin from PF cows also changed markedly over time and remained more abundant in recovery (d21) compared to pretreatment. The abundance of *Streptococcus* in milk displayed a significant time effect ( $P < 0.05$ ) but no difference was noticeable.

*Enterococcus* abundance (Table 5.1; Figure 5.1 A-D) was not different in milk ( $P > 0.10$ ), however abundance in bedding was affected by treatment ( $P < 0.05$ ), and there was a significant interaction of treatment and time for inguinal and teat skin samples ( $P < 0.05$ ). In bedding (Figure 5.1 A), samples from the heat-stressed cows contained a greater relative abundance of *Enterococcus* compared to bedding collected from control cows, pair fed cows, or unused bedding ( $P < 0.05$ ). These data display a marked increase in *Enterococcus* in the bedding of HS cows reflecting a clear response to the increased environmental temperature of the experimental chamber independent of any changes in DMI.

Like bedding samples, inguinal skin samples (Figure 5.1 B) also displayed a rise in abundance of *Enterococcus* in the samples from heat-stressed cows during the challenge period that peaked on d10, then declined during late challenge, and into recovery. Inguinal skin samples from HS cows contained a greater abundance of *Enterococcus* than samples from CL cows at all time points except d0, and a greater abundance than PF cows on d7, 10, 13, and 18. Inguinal samples from PF cows contained a greater abundance of *Enterococcus* than CL cows at d10, 13, 18, and 21 ( $P < 0.05$ ). However, the abundance of *Enterococcus* on inguinal samples from both PF and CL cows had a slightly higher abundance of *Enterococcus* on the last sampling day (d21)

compared to the first sampling day (d-3;  $P < 0.05$ ). Nevertheless, the abundance of *Enterococcus* on inguinal samples from PF and CL cows was relatively stable over time.

Like bedding and inguinal samples, teat skin samples from heat-stressed cows (Figure 5.1 C) also displayed a rise in abundance of *Enterococcus* that peaked during the challenge period on d10. Teat skin samples from HS cows contained a greater relative abundance of *Enterococcus* than CL cows during the challenge and recovery periods, and a greater abundance compared to PF cows during pretreatment, and at d4, 7, 13, and 21. Teat skin samples from PF cows, compared to CL cows, contained a greater abundance of *Enterococcus* at every time point except d0. Similar to the inguinal samples, the abundance of *Enterococcus* was slightly higher on the last day (d21) of sampling compared to the first day (d-3) in the PF and CL cows, even though *Enterococcus* abundance on teat skin from PF and CL cows remained relatively stable throughout the study.

Conversely, abundance of *Enterococcus* in the milk samples did not differ statistically significantly due to treatment (Figure 5.1 D;  $P > 0.10$ ). Abundance of *Enterococcus* in milk of CL appeared variable over time except for d21 where the abundance numerically increases.

Overall, although the relative abundance is low, the abundance of *Enterococcus* was clearly increased in HS indicated by the rise in abundance in bedding and skin samples from the HS cows during the periods of increased environmental temperatures and humidity and this was independent of effects due to PF. While the abundance of *Enterococcus* in milk was not statistically significant, numerically there was an increase in abundance during the heat stress challenge.

Relative abundances of most of the environmental mastitis-causing pathogens differed across the various sample types. In particular, *Enterococcus* appeared heat responsive. Abundance of *Enterobacter*, *Escherichia*, and *Klebsiella*, Gram-negative bacteria, remained relatively unchanged across samples throughout the elevated temperature challenge. Gram-negative bacterial counts in bedding have previously been reported to increase during summer months (Hogan et al., 1989) which is contrary to the present bacterial relative abundance findings. Gao et al. (2017) reported increased isolation of *Escherichia* and *Klebsiella* in milk from cows with clinical mastitis during summer, but those previous studies were uncontrolled field studies and utilized traditional culture. In contrast, the present study was conducted under carefully controlled conditions and used 16S rRNA sequencing both of which may account for the different results. Additionally, within the present study, no clinical mastitis cases occurred, and IMI incidence evaluated with traditional culture did not differ.

*Enterococcus* can cause subclinical mastitis or less commonly, clinical mastitis (Gianneechini et al., 2002), and has been estimated to account for 8-21% of IMIs that are found in clinical and subclinical mastitis cases (Aleksieva and Todorov, 1981; Gianneechini et al., 2002; Różańska et al., 2019). While Todhunter et al. (1995) reported a higher prevalence of new IMIs during summer due to environmental Streptococci, including *Enterococcus*, the present study reported numerical increases in *Enterococcus* abundance in milk. *Enterococcus* species can be found in the gastrointestinal tract, manure, feed, and bedding (Petersson-Wolfe et al., 2008) and have been identified in over 88% of fecal samples from dairy cattle (Jackson et al., 2011). Of the *Enterococcus* species isolated from bovine feces using traditional culture, *E. faecalis*, *E. hirae*, and *E.*

*faeicum* are the most common (Jackson et al., 2011; Różańska et al., 2019). A previous study reported that Enterococci isolates from feces were different than those found in milk, and the authors speculated that feces were not contaminating milk with *Enterococcus* (Kagkli et al., 2007). Because the present study evaluated *Enterococcus* using 16S rRNA sequencing methods, we did not have isolates to speciate or strain type. Therefore, it is unknown if the *Enterococcus* species or strains that increased in abundance during the heat stress challenge are the same across sample types. Intuitively, inguinal skin should have less exposure to bedding compared to teat skin, but we observed similar responses to heat stress in these sample types, suggesting that other factors than bedding alone may alter the skin microbiota. Because bedding, inguinal skin, and teat skin samples all displayed similar patterns of increased *Enterococcus* abundance in response to increased temperature, we cannot determine if the *Enterococcus* found in bedding was transferred to skin or if increased abundance on the shed bacteria into bedding, or if both occurred. Regardless, there was a clear response during elevated THI resulting in increased *Enterococcus* in bedding and on both skin samples. Although not significant, there was a numerical increase in *Enterococcus* abundance in milk of HS cows during elevated THI. Recently, the majority of OTU found on teat skin were also found in milk, which highlights the important role of teat skin as a potential reservoir for possibly introducing bacteria to the mammary gland (Fretin et al., 2018). Additionally, a study comparing the effects of indoor to outdoor housing and inclusion versus exclusion of teat preparation reported similarities between the microbiome of teat skin and bulk tank milk samples (Doyle et al., 2017). While those studies did not evaluate mastitis pathogens or milk from individual quarters, the results highlight the interconnectedness

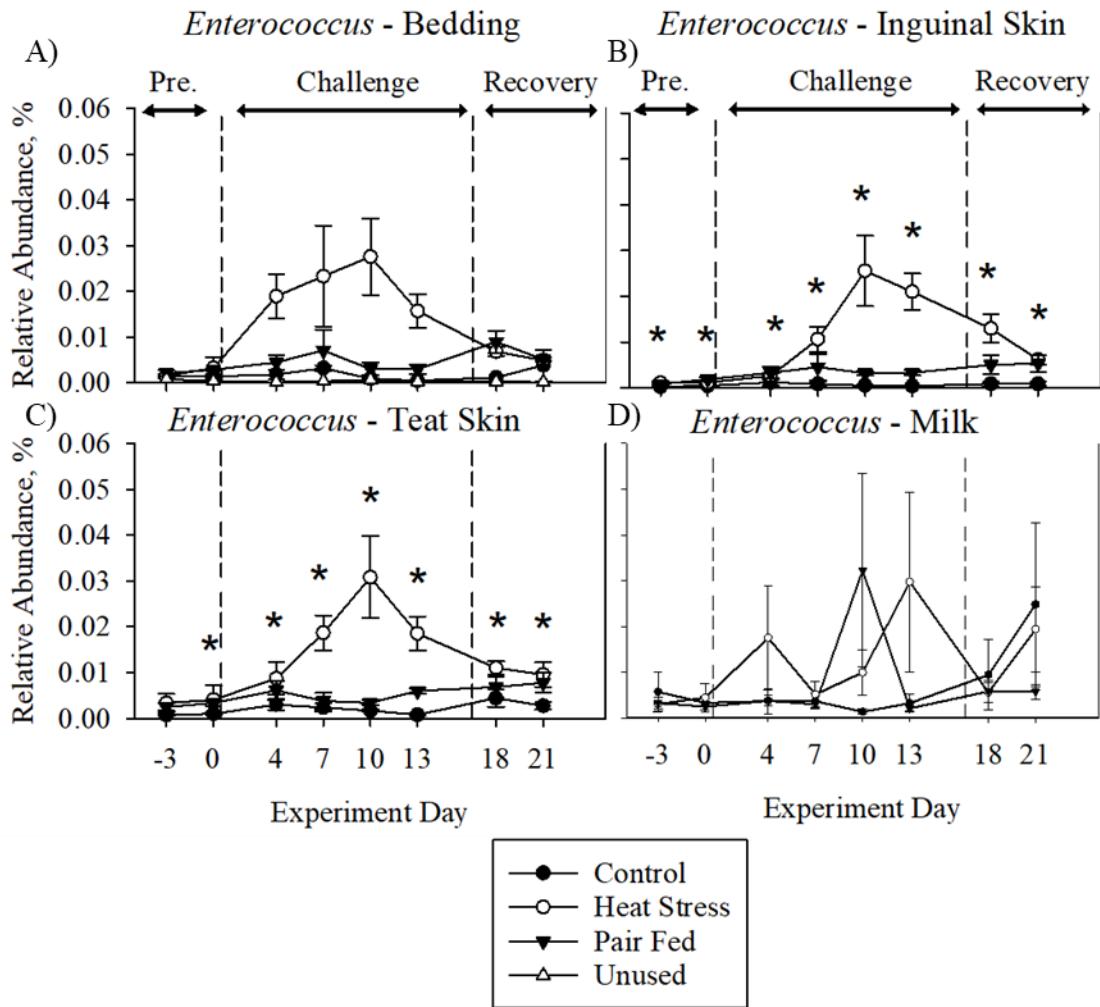
of the dairy cow microbiome which extends to alterations induced by elevated environmental temperatures.

The present study design included both a control group and a pair-fed group, which enabled us to conclude that effects were due to elevated THI, independent of associated depression of DMI. Sampling unused bedding demonstrated that changes occurred between treatments. Our study provides novel insights into effects of heat stress and feed restriction on mastitis pathogen prevalence in bedding, on skin, and in milk. Notably, abundance of *Enterococcus* in bedding, on inguinal skin, and on teat skin, was clearly increased during HS. Future studies are needed to determine if alterations in *Enterococcus* abundance during heat stress could predispose the cow to mastitis or if this is part of maintaining udder homeostasis.

**Table 5.1.** Relative abundance of environmental mastitis pathogens.

Sample Type	Enterobacter			Enterococcus			Escherichia			Klebsiella			Streptococcus		
	Pre <sup>1</sup>	Challenge <sup>2</sup>	Recovery <sup>3</sup>	Pre <sup>1</sup>	Challenge <sup>2</sup>	Recovery <sup>3</sup>	Pre <sup>1</sup>	Challenge <sup>2</sup>	Recovery <sup>3</sup>	Pre <sup>1</sup>	Challenge <sup>2</sup>	Recovery <sup>3</sup>	Pre <sup>1</sup>	Challenge <sup>2</sup>	Recovery <sup>3</sup>
Bedding															
CL	0.02%	0.02%	0.02%	0.14%	0.16%	0.29%	0.22%	0.14%	0.02%	0.40%	0.39%	0.22%	0.04%	0.01%	0.07%
HS	0.01%	0.01%	0.01%	0.24%	2.14%	0.59%	0.21%	0.23%	0.53%	0.24%	0.17%	0.17%	0.15%	1.45%	0.29%
PF	0.05%	0.07%	0.03%	0.25%	0.44%	0.70%	0.11%	0.03%	0.02%	0.77%	1.07%	0.33%	0.10%	0.52%	0.20%
UN	0.16%	0.05%	0.03%	0.06%	0.04%	0.02%	0.01%	0.03%	0.01%	3.79%	0.84%	0.64%	0.00%	0.00%	0.00%
Inguinal Skin															
CL	0.00%	0.00%	0.00%	0.04%	0.08%	0.09%	0.07%	0.03%	0.06%	0.00%	0.01%	0.00%	0.01%	0.01%	0.02%
HS	0.00%	0.00%	0.00%	0.11%	1.50%	0.96%	0.04%	0.10%	0.08%	0.01%	0.03%	0.03%	0.01%	0.51%	0.41%
PF	0.00%	0.00%	0.00%	0.14%	0.36%	0.52%	0.04%	0.05%	0.04%	0.03%	0.03%	0.02%	0.03%	0.23%	0.28%
Teat Skin															
CL	0.00%	0.00%	0.00%	0.09%	0.20%	0.36%	0.14%	0.11%	0.10%	0.09%	0.06%	0.05%	0.08%	0.02%	0.03%
HS	0.01%	0.01%	0.00%	0.37%	1.92%	1.03%	0.06%	0.12%	0.16%	0.19%	0.15%	0.13%	0.09%	0.78%	1.40%
PF	0.01%	0.00%	0.00%	0.29%	0.48%	0.73%	0.15%	0.04%	0.07%	0.16%	0.11%	0.10%	0.07%	0.23%	0.28%
Milk															
CL	0.00%	0.00%	0.00%	0.46%	0.30%	1.71%	0.13%	0.07%	0.09%	0.07%	0.04%	0.02%	1.20%	1.30%	0.66%
HS	0.00%	0.00%	0.00%	0.39%	1.56%	1.25%	0.05%	0.11%	0.02%	0.04%	0.08%	0.04%	1.15%	1.26%	0.60%
PF	0.00%	0.00%	0.00%	0.29%	1.03%	0.58%	0.12%	0.04%	0.01%	0.15%	0.05%	0.07%	1.57%	0.81%	0.33%

**Table 5.1.** Relative abundance of selected environmental mastitis pathogens in samples of bedding, inguinal skin, teat skin, and milk from cows exposed to heat stress or pair-feeding. CL = control group, HS = heat-stressed group, PF = pair-fed group, and UN = unused bedding, when applicable. Results are recorded as the average within treatment and experimental period. All samples were collected from control cows, heat-stressed cows, and pair fed cows during <sup>1</sup>pretreatment (d-3 and 0), <sup>2</sup>challenge (d4, 7, 10, and 13), and <sup>3</sup>recovery (d18 and 21).



**Figure 5.1.** *Enterococcus* relative abundance in A) bedding, on B) inguinal skin, C) teat skin, and D) in milk from cows exposed to heat stress or pair-feeding. The samples were collected in the pretreatment period (Pre. d-3 and 0), challenge (d4, 7, 10, and 13), and the recovery period (d18 and 21). \*Within panel indicates significant treatment by time interaction within day ( $P < 0.05$ ).

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## **CHAPTER VI**

### **THE IMPACT OF STRESS ON THE MICROBIOME OF LACTATING DAIRY COWS: III. HEAT STRESS AND FEED RESTRICTION MODIFIES BACTERIAL COMMUNITIES IN BEDDING, SKIN, AND MILK OF LACTATING COWS**

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## HEAT STRESS AND FEED RESTRICTION MODIFIES BACTERIAL COMMUNITIES IN BEDDING, SKIN, AND MILK

### 6.1 ABSTRACT

Heat stress increase incidence of disease in dairy cattle but potential effects of heat stress and the resulting decreased feed intake on the microbiota of dairy cattle remain unknown. The objectives of this study were to evaluate the microbiomes of bedding, inguinal skin, teat skin, and milk during a period of heat stress or pair-feeding to determine changes associated with these stressors. We hypothesized that mastitis-causing pathogens would increase in abundance in bedding during increased environmental temperature and humidity and result in an increased abundance on teat skin and in the milk microbiome. Eighteen mid-lactation Holstein cows were blocked by MY, DIM, and parity and randomly assigned, within block, to one of three treatments in separate environmental chambers: untreated control (**CL**), heat stress (**HS**), or pair fed (**PF**). All cows were given a 5-day pretreatment period, under thermoneutrality (temperature-humidity index (**THI**) ~66) and ad libitum feeding. Then, during a 14-day challenge period, CL and PF cows were kept at thermo-neutral conditions (**THI** ~66) but HS cows were subjected to constant, non-cyclical heat stress (**THI** ~80). To isolate the effects of reduced feed intake on the microbiomes, PF cows were feed-restricted during the challenge period to match the intake of the HS cows. The CL cows were fed ad libitum at all times. Finally, during tan 8-day recovery period, HS cows were returned to normothermic conditions (**THI** ~66). The PF cows continued to be pair-fed during recovery. Bedding, inguinal skin samples, teat skin samples, and milk samples were collected and 16S rRNA sequencing of isolated, amplified DNA was performed to evaluate the

response of the microbiomes to heat stress and feed restriction stressors. Abundance of individual bacterial phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*) and families (*Enterobacteriaceae*, *Enterococcaceae*, *Staphylococcaceae*, and *Streptococcaceae*) changed throughout the study. Abundance of *Actinobacteria* and *Enterococcaceae* was higher during heat stress in bedding which could have caused the increased abundance also seen on teat skin or the skin could have caused an increased abundance in bedding. Also, *Proteobacteria* and *Enterobacteriaceae* abundance was higher on inguinal and teat skin of HS cows but not in bedding from HS cows, highlighting that pathogen load on skin is not entirely dependent on the bedding exposure. The milk microbiome exhibited some responses of heat stress and feed restriction; however, it was highly variable over time. The findings from the present study reveal that the bedding and skin microbiomes are responsive to elevated THI as evidenced by increased abundance of some mastitis-associated bacteria, *Enterobacteriaceae* and *Enterococcaceae*.

## 6.2 INTRODUCTION

Heat stress in dairy cattle results in increased risk of disease, reduced feed intake, and reduced milk production (Kadzere et al., 2002; Collier et al., 2012). Annually, heat stress costs the dairy industry over \$1.2 billion (Key et al., 2014); therefore, a better understanding of the effects of heat stress on the cow and her environment is of utmost importance to dairy producers.

Next-generation sequencing technologies have allowed a more in-depth look into the bacterial communities that reside within and on cattle. Investigation into these bacterial populations could allow us to better understand the impact of changes of

environmental conditions on bacterial communities and could identify a role of the microbiome in the increased incidence of diseases such as metritis (Kadzere et al., 2002) and mastitis (Hogan et al., 1989a; Hogan et al., 1989b; Gao et al., 2017) during periods of high environmental temperatures. While the milk and tissues of the bovine mammary gland have long been considered sterile, recent advances in next generation sequencing technologies have brought this into question. The milk microbiome has been evaluated to attempt to explain differences between the microbiota of healthy and mastitis quarters (Oikonomou et al., 2014; Andrews et al., 2019), effects of antimicrobial use on the mammary microbiota (Bonsaglia et al., 2017; Derakhshani et al., 2018b), possible internal transfer routes of bacteria into milk (Young et al., 2015), and environmental sources of bacteria in milk (Wu et al., 2019).

Previously it has been reported that the mammary microbiota differs throughout the year (Li et al., 2018; Andrews et al., 2019) and between summer and winter seasons (Nguyen et al., 2019b). Bedding, teat skin, and airborne dust microbiotas have also exhibited seasonal variation (Nguyen et al., 2019a; Nguyen et al., 2019b). Furthermore, it has been reported that the fecal, teat (Doyle et al., 2017; Derakhshani et al., 2018a), and bedding microbiotas (Nguyen et al., 2019b; Wu et al., 2019) are related to the milk microbiota which could indicate that these are potential sources of bacteria in milk. Bedding has been proposed as a source of teat end contamination in microbiome (Derakhshani et al., 2018b; Nguyen et al., 2019b) and traditional culture-based studies (Rowbotham and Ruegg, 2016; Patel et al., 2019). Additionally, previous studies have evaluated the relationship between colonization of the teat end with bacteria and the likelihood of an IMI (da Costa et al., 2014; Svennesen et al., 2019) and found an

increased risk of IMI when the same bacteria was found on the teat end. Therefore, changes in the bacterial communities that reside within bedding could lead to increased exposure of the teat end to bacteria which could increase the odds of bacteria entering the mammary gland and altering the milk microbiome. Such alterations to the milk microbiota may increase the incidence of mastitis. While it is known that periods of increased environmental temperature coincide with increased subclinical mastitis, as demonstrated by elevated SCC (Bodoh et al., 1976; Olde Riekerink et al., 2007), bulk tank SCC (**BTSCC**; Olde Riekerink et al., 2007), and SCS (Guinn et al., 2019), and clinical mastitis (Hogan et al., 1989b), the potential link between bedding, skin, and milk microbiomes especially during periods of elevated environmental temperatures have not been established.

The objectives of this study were to evaluate the bedding, inguinal skin, teat skin, and milk microbiomes during a period of heat stress and reduced feed intake or a period of feed restriction alone to determine changes associated with these stressors. We hypothesized that the dominant phyla and operational taxonomic units (**OTU**) associated with environmental mastitis-associated bacteria in the bedding microbiome would be increased by elevated environmental temperatures leading to consequential changes in teat skin and milk microbiomes due to increased teat end exposure. We also hypothesized that the environmental mastitis-causing bacteria on the inguinal skin would remain unchanged due to minimal contact with bedding.

## 6.3 MATERIALS AND METHODS

### *Design of the study*

The University of Missouri Animal Care and Use Committee approved this study (Protocol Number 9283). Mid-lactation Holstein cows ( $n = 18$ ) from the University of Missouri Foremost Dairy Research and Teaching farm were blocked based on parity, days pregnant, milk production, DIM, and low SCC from the previous month of testing (< 200,000 cells/mL). Cows were housed in tie stalls on rubber mats topped with crushed soyhulls as bedding in the Body Environmental Unit at the University of Missouri. The experiment was conducted over 27 days (d-5 to 0, pretreatment; d0 to 14, challenge; d14 to 22, recovery), and the treatments included control (**CL**;  $n = 6$ ), heat-stressed (**HS**;  $n = 6$ ), or pair fed (**PF**;  $n = 6$ ) groups. Cows were randomly allocated, within block, to one of these treatments. During the pretreatment period, CL, HS, and PF cows received feed ad libitum and were kept at thermoneutral conditions (temperature-humidity index (**THI**) ~66) to maintain rumen temperature at approximately 39.5°C. During the challenge period, CL cows and PF cows were kept at thermoneutral conditions, but PF cows were feed restricted to match the intake of HS cows. During the challenge period, HS cows were fed ad libitum and subjected to constant, non-cyclical, heat stress (THI ~80; rumen temperature ~40.6°C). The THI was adjusted to induce and maintain heat stress, which was defined as a body temperature 1°C above normal (Rodrigues, 2019). During recovery, CL, HS, and PF cows were kept at thermoneutral conditions (THI ~ 66) and CL and HS cows received feed ad libitum. The PF cows continued to be pair-fed to HS cows throughout recovery (Rodrigues et al., 2019). Feed was TMR formulated to meet or exceed predicted nutrient requirements (NRC, 2001); cows were fed and milked twice

daily. Further details on cow performance and physiological responses are reported in Rodrigues (2019).

### ***Microbiome sampling***

All samples were collected approximately three hours after morning milking (~9AM) during the pretreatment (d-3 and 0), challenge (d4, 7, 10, and 13), and recovery (d18 and 21) periods. Wearing disposable gloves (Latex Exam Gloves; AmerisourceBergen; Oceanside, CA), bedding samples ( $n = 55$ ) were collected from each chamber and the unused stockpile of bedding into sterile Whirl-paks (Nasco; Fort Atkinson, WI). Two bedding samples were collected from each chamber; samples from three adjacent stalls were combined into one bag and the samples from the remaining three stalls were collected into a separate bag. Bedding samples from the same three stalls were combined at every sample collection timepoint. To collect samples from each stall, a grid was placed in the back 1/3<sup>rd</sup> of the cow's stall and five samples were collected from randomly selected squares from the grids within the panel using a random number generator. From the stockpile of unused bedding, five samples were collected into one bag to represent unused bedding (UN). Dry, autoclaved electrostatic dusters (Swiffers™; Procter and Gamble, Cincinnati, OH) were used to collect inguinal ( $n = 144$ ) and teat ( $n = 144$ ) skin samples. Disposable gloves (Latex Exam Gloves; AmerisourceBergen) were worn for each cow and sample. Individual inguinal samples from each cow were collected by using a dry, autoclaved electrostatic duster (Swiffers™; Procter and Gamble) to swipe twice up both inguinal locations on an individual cow and placed in the same Whirl-pak (Nasco) for one composite sample per cow. Teat skin samples were collected from each cow by swabbing around the barrel and across the base of two teats per cow.

Teats for sampling were selected at the beginning of the study and the same teats from each cow were sampled throughout. Both teat skin samples from each cow were combined into one Whirl-pak (Nasco) as one composite sample per cow. Milk samples were collected from the same two quarters that were chosen for teat skin samples of every cow for sequencing ( $n = 277$ ) and traditional culture ( $n = 288$ ). Milk samples were collected aseptically as recommended by the National Mastitis Council (Middleton et al., 2017) into 5 mL round bottom tubes (Falcon; ThermoFisher Scientific; Waltham, MA). All samples were frozen immediately and stored at -20°C prior to DNA extraction.

### ***Sample preparation and processing***

All samples were thawed at room temperature prior to preparation for DNA extraction. Bedding samples were vortexed to mix and approximately 0.25 g of previously ground soyhull bedding was collected with a sterile spoon (Sterile Spoon 1/4tsp; ThermoFisher Scientific) and placed into a sterile 50 mL conical tube (Falcon; ThermoFisher Scientific). Then, 10 mL of Mili-Q water was added to each conical tube and bedding samples were agitated on a shaker (Stovall Belly Dancer; ThermoFisher Scientific) for 10 minutes without filtration or centrifugation. Of each bedding sample, 750 µl of liquid was used as the starting material for DNA extraction. Inguinal and teat skin samples were agitated on a shaker (Stovall Belly Dancer; ThermoFisher Scientific) for 10 minutes after the addition of 15 mL Sterile MiliQ water. Of each inguinal and teat skin Swiffer™ duster, 750 µl of liquid was used as the starting material for DNA extraction. For milk samples, 1.5 mL of milk was placed in a sterile 2 mL microcentrifuge tube (Falcon; ThermoFisher Scientific) and centrifuged for 20 minutes at 4700 RPM. After centrifugation, the fat layer was removed with a sterile Q tip and the

sample was vortexed to mix. Of the milk samples, 800 µl was used as the starting material for DNA extraction.

QIAmp PowerFecal DNA kits (Qiagen; Germantown, MD) were used to extract DNA from all samples. The manufacturer's instructions were followed with one alteration. Instead of using the vortex adapter to lyse cells, a Tissue Lyser II (Qiagen) was used. Qubit dsDNA BR Assay kits (Invitrogen; Carlsbad, CA) were used to measure the concentration of DNA. After extraction, DNA was amplified by PCR using universal primers (U515/806R) flanked by Illumina standard adapter sequences (Caporaso et al., 2010). The PCR amplification steps used for bedding and inguinal and teat skin samples were: initial denaturation for 3 minutes at 98°C, denaturation, amplification, and annealing for 25 cycles of 15 seconds at 98°C, 30 seconds at 50°C, and 30 seconds at 72°C, respectively, followed by a final extension for 7 minutes at 72°C. Milk samples were subjected to the previously mentioned PCR steps with the exception of using 40 cycles instead of 25 cycles which has been validated in our lab (Chapter III; Witzke et al., submitted). Sequencing of the 16S rRNA gene amplicon library was performed at the University of Missouri DNA Core Facility using the Illumina MiSeq platform. Sequences were assigned to Operational Taxonomic Units (OTUs) based on 97% nucleotide identity (Caporaso et al., 2010) and annotated against the Silva.v132 database (Quast et al., 2013) using BLAST (McGinnis and Madden, 2004).

### ***Data analysis***

Chao1 (richness) and Shannon indices were evaluated and analyzed in SigmaPlot 13.0 (Systat Software Inc.; San Jose, CA) using a two-way repeated measures ANOVA. Intra-sample similarity was based on Bray-Curtis distances and analyzed using a one-way

ANOVA in SigmaPlot 13.0 (Systat Software Inc.). Hierarchical clustering to evaluate the most variable operational taxonomic units (**OTU**) was performed in MetaboAnalyst (Xia et al., 2009). The SCC of individual quarter-level samples and selected OTU representing phyla and families were analyzed to evaluate temporal profiles. Data were transformed, rank or squared, if normality failed as determined by the Shapiro-Wilk test ( $P > 0.05$ ) and visual inspection of Q-Q plots and histograms. Individual phyla and family OTU were analyzed with a randomized complete block design in SAS using ANOVA with repeated measures in PROC GLIMMIX (SAS Institute Inc.; Cary, NC SAS, 2010). Fixed effects included treatment, time and treatment by time interaction. Results are presented as least squares means  $\pm$ SEM and statistical significance was declared at  $P < 0.05$ .

## 6.4 RESULTS

### *General observations and sequencing performance*

All the cows ( $n = 18$ ) completed the study. A total of 55 bedding, 144 inguinal skin, 144 teat skin, and 277 milk samples were sequenced successfully with an average read count of 4,456,080, 12,636,185, 11,474,823, and 45,074, respectively. In addition, 288 milk samples were analyzed for SCC and incidence of new intramammary infections based on milk culturing on days of sample collection did not differ by treatment, time, or their interaction ( $P > 0.10$ ). Furthermore, there were no clinical cases of mastitis during the experiment.

### *Alpha diversity*

Alpha diversity was measured with Chao1 richness (Supplementary Figure 6.5 A-D) and Shannon diversity indices (Supplementary Figure 6.6 A-D). Richness of bedding

samples was affected by a significant treatment by time interaction ( $P < 0.05$ ). Richness (Supplementary Figure 6.5 A) and diversity (Supplementary Figure 6.6 A) were higher in used (CL, HS, and PF) compared to unused bedding. While there were differences in richness and diversity over time, these measures varied with no clear treatment response.

Richness and diversity of inguinal skin samples were affected by interactions of treatment and time ( $P < 0.05$ ). Richness (Supplementary Figure 6.5 B) and diversity (Supplementary Figure 6.6 B) of inguinal skin samples from the CL cows did not differ over time while HS and PF cows displayed similar increases in richness during the challenge period. Diversity also increased on inguinal skin from HS in mid-challenge and remained higher into recovery, whereas the response in PF was more variable.

There was also a significant interaction between treatment and time for teat skin sample richness (Supplementary Figure 6.5 C;  $P < 0.01$ ), while diversity (Supplementary Figure 6.6 C) had a treatment effect ( $P < 0.05$ ) and interaction trend ( $P = 0.06$ ). Within CL, richness and diversity of teat skin samples remained stable over time except for d0 when both measures were lower. Compared to pretreatment, teat skin sample richness from HS cows increased during early and mid-challenge, peaked during late challenge (d13;  $P < 0.05$ ), and did not recover fully by the last day of sampling (d21). Richness in PF cows was higher during late challenge (d10 and 13) compared to pretreatment (d-3 and 0) and early challenge (d4 and 7) and did not recover by the end of sampling (d21). Within treatment, diversity was numerically lower on teat skin from HS cows compared to CL and PF cows on d0, 4, and 10.

In milk samples, richness (Supplementary Figure 6.5 D) and diversity (Supplementary Figures 6.6 D) were affected by time ( $P < 0.01$ ). While richness in milk

from HS and CL cows appeared to follow a similar pattern over time, PF had a different pattern. overall richness of milk samples appeared highly variable while diversity was fairly stable.

Overall, richness and diversity of the skin microbiome appear responsive to the treatments as evidenced by elevations in HS and PF samples, relative to CL, suggesting an effect of decreased DMI. Richness and diversity in bedding did not appear treatment responsive but were greater in used bedding versus unused bedding.

### ***Changes in community composition***

Intra-sample similarity measures (Figure 6.1 A-D) within sample type were compared between pretreatment and mid-challenge (d-3 versus d7), late challenge (d-3 versus d13), and recovery (d-3 versus d21). In bedding (Figure 6.1 A), intra-sample similarity was higher ( $P < 0.05$ ) in PF compared to HS (d-3 vs 7) suggesting a greater change in HS over this period. No other treatment differences were observed for bedding. Bedding from PF cows had a higher ( $P < 0.05$ ) intra-sample similarity compared to HS cows (d-3 vs 7). Numerically, the intra-sample similarity was higher in UN compared to all other treatments, at all times, indicating less change if unused. Intra-sample similarity of inguinal samples from HS cows (Figure 6.1 B) was lower ( $P < 0.05$ ) than CL and PF cows when comparing d-3 to d7 or d13. No other comparisons within inguinal skin differed. Teat skin intra-sample similarities of HS and PF cows (Figure 6.1 C) were lower ( $P < 0.05$ ) than CL cows when comparing pretreatment (d-3) to challenge (d7 and 13) and recovery (d21). In milk samples (Figure 6.1 D), intra-sample similarity of CL cows was higher ( $P < 0.05$ ) than HS and PF cows when comparing d-3 to d7. There were no differences when comparing d-3 to 13 ( $P = 0.31$ ). However, when comparing d-3 to 21,

milk samples from PF cows had a higher intra-sample similarity than CL cows ( $P < 0.05$ ). Overall, intra-sample similarity differed more between treatments in early challenge (d7) compared to late challenge and most, aside from teat skin, returned to pretreatment levels by the last day of sampling (d21). These data display changes in overall bacterial composition over time due to treatment. The differences in intra-sample similarity of HS, PF, and CL cows and UN, in bedding comparisons when applicable, emphasizes that multiple OTU are altered by heat and feed restriction stress.

### ***Most variable OTU***

Hierarchical clustering was used to determine individual OTU that varied most in response to challenge and recovery, but, none of the most variable OTU were the same between HS and PF groups across sample types, including inguinal skin, teat skin, and milk. Due to the unbalanced and small sample size of bedding, hierarchical clustering was unable to be performed to make these comparisons. Nevertheless, in the present study, among HS cows, inguinal and teat skin from HS cows had OTU corresponding to *Methanobrevibacter* species in common, and inguinal skin and milk from HS cows had one OTU corresponding to a *Lactococcus* species in common. The *Methanobrevibacter* species, common to inguinal and teat skin had a similar temporal profile in that abundance decreased numerically during the challenge period and remained low during recovery. In inguinal skin the *Lactococcus* species increased numerically in abundance during the heat stress period and remained elevated in recovery while the abundance in milk appeared variable over time. The lack of commonality among the most variable OTU between most sample types, suggests that sample types represent distinct and

largely independent niches that differ in response to elevated environmental temperature or feed restriction. But this is limited to the most changeable OTU.

### ***Changes in phyla abundance***

The most prevalent phyla in unused and used bedding throughout the experiment included: *Proteobacteria*, *Cyanobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* with average relative abundances of 32.35%, 27.82%, 21.88%, 10.3%, and 6.34%, respectively. *Actinobacteria* (Figure 6.2 A-D), *Bacteroidetes* (Supplementary Figure 6.6 A-D), *Firmicutes* (Supplementary Figure 6.7 A-D), and *Proteobacteria* (Supplementary Figure 6.8 A-D) were the dominant phyla in all samples and were subjected to further analysis. Inguinal skin samples contained the following dominant phyla: *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* with average relative abundances of 51.47%, 28.04%, 11.51%, and 6.00%, respectively. Teat skin samples contained dominant phyla that included: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* with average relative abundances of 55.27%, 15.86%, 13.74%, and 8.22%, respectively. The dominant phyla in milk samples included: *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* with average relative abundances of 41.26%, 29.71%, 12.75%, and 10.41%, respectively.

In bedding, *Actinobacteria* (Figure 6.2 A) relative abundance had a time effect ( $P < 0.05$ ) and an interaction trend ( $P = 0.06$ ). Over all treatments, average *Actinobacteria* relative abundance in bedding from HS cows was higher during the challenge period but was not different to pretreatment levels by the last day of sampling (d21). *Actinobacteria* in bedding from HS cows increased numerically in early challenge and remained high throughout the rest of the study. Bedding from CL cows increased numerically during the

early challenge period on d10 and remained slightly higher during recovery, while bedding from PF cows was mostly stable and UN did not change throughout the study.

Inguinal skin samples *Actinobacteria* abundance displayed a treatment by time interaction ( $P < 0.02$ ; Figure 6.2 B). While PF cows had a lower abundance of *Actinobacteria* compared to HS and CL, inguinal skin from HS and CL followed a similar pattern throughout the challenge period until recovery when CL abundance was numerically higher than HS. Abundance of *Actinobacteria* on teat samples (Figure 6.2 C) had a significant interaction ( $P < 0.01$ ) being markedly higher on samples from HS cows during the challenge period while its abundance on CL and PF cows appeared relatively stable. Like skin samples, *Actinobacteria* abundance also had a significant interaction in milk samples (Figure 6.2 D;  $P < 0.01$ ). Abundance of *Actinobacteria* in milk from HS cows increased in early challenge (d4 and 7) and returned to pre-challenge levels by d21. Abundance of *Actinobacteria* in PF and CL milk was relatively stable aside from a peak on d13. In general, *Actinobacteria* abundance clearly increased during the challenge period in bedding, teat skin, and milk of HS cows and perhaps on inguinal skin. These results indicate that increased abundance in HS samples was driven by temperature, not reduced DMI. Moreover, changes in bedding may increase teat end exposure to *Actinobacteria* and influence abundance in milk.

*Bacteroidetes* relative abundance in bedding (Supplementary Figure 6.6 A) displayed a trend towards a treatment effect. Bedding from HS and PF cows tended to have a greater abundance of *Bacteroidetes* than UN ( $P = 0.05$ ) but were not different than CL. *Bacteroidetes* abundance did not differ over time or by treatment on inguinal skin (Supplementary Figure 6.6 B;  $P > 0.10$ ). Teat skin abundance of *Bacteroidetes*

(Supplementary Figure 6.6 C) had a significant interaction ( $P < 0.01$ ); in HS, *Bacteroidetes* abundance was greater in recovery compared to pretreatment. Like teat skin, the abundance of *Bacteroidetes* had a significant interaction in milk samples (Supplementary Figure 6.6 D;  $P < 0.02$ ). In milk from CL cows, a spike in abundance of *Bacteroidetes* on d10 followed by a decrease on d13 occurred and was otherwise stable over time. *Bacteroidetes* in milk from PF cows was stable throughout the experiment, aside from a transient decline on d13. Milk from HS cows displayed a variable abundance of *Bacteroidetes* during pretreatment but was mostly stable thereafter. Overall, *Bacteroidetes* abundance appeared mostly stable across sample types from HS cows and did not appear heat responsive, in stark contrast to *Actinobacteria* abundance.

*Firmicutes* relative abundance in bedding (Supplementary Figure 6.7 A) differed over time ( $P < 0.05$ ) and a treatment trend was present ( $P = 0.06$ ). Over time, *Firmicutes* abundance was lower during the early challenge period compared to pretreatment (d-3) but fully recovered by the last day of sampling (d21). Nevertheless, the time effect appeared due to single high means on d-3 and 13 but was stable in general. *Firmicutes* abundance in bedding was affected by a trend towards a treatment by time interaction in which CL, HS, and PF cows tended to have a greater *Firmicutes* abundance than UN ( $P = 0.06$ ). On inguinal skin, *Firmicutes* (Supplementary Figure 6.7 B) had a significant interaction ( $P < 0.01$ ) such that PF cows had a greater abundance on d13, 18, and 21 compared to HS and CL cows ( $P < 0.05$ ) because CL and HS cows decreased during the challenge period and remained lower than pretreatment (d-3 and 0) in the recovery period (d18;  $P < 0.05$ ). While differences occurred in inguinal skin from PF cows, the abundance of *Firmicutes* appeared to have variation over time. The abundance of

*Firmicutes* within teat skin samples (Supplementary Figure 6.7 C) had a significant interaction ( $P < 0.01$ ). Like inguinal skin, CL and HS abundance declined during challenge while PF was more stable. The abundance of *Firmicutes* within teat skin samples from CL cows was lower in late challenge (d10 and 13) compared to pretreatment but returned to pretreatment levels by the end of sampling. Teat skin samples from PF cows exhibited higher abundance of *Firmicutes* on d-3 and lower abundance on d13 but no persisting changes. In HS, abundance of *Firmicutes* on teat skin decreased during the challenge period and remained lower in recovery compared to pretreatment. The abundance of *Firmicutes* in milk samples (Supplementary Figure 6.7 D) trended towards a significant interaction ( $P = 0.08$ ), but not meaningful patterns were apparent. Based on the changes exhibited by skin samples and a lack of treatment responsive changes in bedding and milk, *Firmicutes* abundance appears to vary on skin without accompanying changes in bedding or milk.

*Proteobacteria* abundance in bedding (Supplementary Figure 6.8 A) was higher than in other samples and did not differ over time or by treatment ( $P > 0.10$ ). Notably, relative abundance was similar in used and unused bedding, suggesting the source of *Proteobacteria* is bedding and not the cow. *Proteobacteria* on inguinal samples (Supplementary Figure 6.8 B) differed over time and by treatment ( $P < 0.05$ ). No differences were detected in bedding from CL or PF cows over time ( $P > 0.10$ ). However, *Proteobacteria* abundance on inguinal samples from HS cows increased during the challenge period, peaked at d10, and declined throughout the rest of the challenge period and recovery to return to pretreatment levels. *Proteobacteria* abundance in teat skin (Supplementary Figure 6.8 C) had a treatment effect ( $P < 0.05$ ) in which HS cows

contained a greater abundance of this phyla compared to CL. The abundance of *Proteobacteria* in HS cows increased numerically on d0 and remained higher during the challenge period before decreasing in recovery. *Proteobacteria* abundance in milk differed over time (Supplementary Figure 6.8 D;  $P < 0.01$ ) as shown by a higher abundance in pretreatment (d-3 and 0) compared to challenge (d7 and 13) and recovery (d18 and 21). Overall, *Proteobacteria* abundance on skin appeared to increase in response to elevated environmental temperatures as shown by the effects of heat stress on its abundance on inguinal and teat skin but not in bedding or milk. This indicates that *Proteobacteria* abundance on the teat was independent of bacterial abundance in bedding.

### ***Changes in family abundance***

Changes in relative abundance of OTU corresponding to bacterial families associated with mastitis, including *Enterobacteriaceae* (Supplementary Figure 6.9 A-D), *Enterococcaceae* (Figure 6.3 A-D), *Staphylococcaceae* (Supplementary Figure 6.10 A-D), and *Streptococcaceae* (Figure 6.4 A-D), were evaluated. In bedding, *Enterobacteriaceae*, *Enterococcaceae*, *Staphylococcaceae*, and *Streptococcaceae* were present in all samples with average relative abundances of 2.44%, < 1%, 2.23%, and < 1%, respectively. In inguinal skin, *Enterobacteriaceae*, *Enterococcaceae*, *Staphylococcaceae*, and *Streptococcaceae* were present in at least 95.1% of samples. These families had an average relative abundance of <1% in inguinal skin except for *Staphylococcaceae* which had an average relative abundance of 4.07%. In teat skin, *Enterobacteriaceae*, *Enterococcaceae*, *Staphylococcaceae*, and *Streptococcaceae* were present in at least 99.3% of samples. Like on inguinal skin, these families had an average relative abundance of <1%, aside from *Staphylococcaceae* which had an average

abundance of 3.78%. In milk samples, *Enterobacteriaceae*, *Enterococcaceae*, *Staphylococcaceae*, and *Streptococcaceae* were present in at least 62.1% of samples; and the average relative abundances were <1%, < 1%, 4.10%, and 2.09%, respectively.

*Enterobacteriaceae* (Supplementary Figure 6.9 A) abundance displayed a treatment trend with higher abundance in UN compared to bedding from CL and HS cows ( $P = 0.09$ ) during the pretreatment and challenge period. In inguinal skin (Supplementary Figure 6.9 B), *Enterobacteriaceae* had a treatment trend ( $P = 0.06$ ). During the challenge and recovery periods, inguinal skin samples from PF and especially HS cows had a numerically greater abundance of *Enterobacteriaceae* than CL. In teat skin samples (Supplementary Figure 6.9 C), *Enterobacteriaceae* displayed an interaction ( $P < 0.02$ ). During the challenge period, abundance of *Enterobacteriaceae* on teat skin from HS cows increased whereas CL and PF cows remained stable throughout the study aside from d0 and d18, respectively. In milk samples (Supplementary Figure 6.9 D), *Enterobacteriaceae* abundance differed over time ( $P < 0.01$ ). During the pretreatment period the abundance of *Enterobacteriaceae* was greater than d7 and recovery (d18 and 21). However, no clear response to treatment emerged. *Enterobacteriaceae* abundance differed on skin but not in milk and bedding. Bedding abundance of *Enterobacteriaceae* did not appear to be treatment responsive but was higher in unused bedding during pretreatment and challenge and therefore, with the addition of new, unused bedding, could have predisposed teat end exposure to this bacterial family. These data suggest that abundance on inguinal and teat skin was increased by heat stress and not decreased DMI.

Abundance of *Enterococcaceae* in bedding was affected by treatment with markedly greater abundance in HS cows compared to CL cows and PF ( $P < 0.02$ ; Figure

6.3 A). In bedding from HS cows, *Enterococcaceae* abundance increased numerically during heat stress, peaked at d10, and began declining which continued into recovery. In inguinal skin (Figure 6.3 B), *Enterococcaceae* abundance had a significant interaction ( $P < 0.01$ ) with samples from HS cows increasing during the early challenge period (d4 and 7), peaking on d10, then declining in late challenge, and into recovery, compared to pretreatment. Abundance of *Enterococcaceae* on inguinal skin from PF cows increased slightly throughout the experiment and was greater in recovery compared to pretreatment while the abundance on CL cows remained stable throughout time. Like inguinal skin, *Enterococcaceae* abundance on teat skin was affected by interaction of treatment and time (Figure 6.3 C;  $P < 0.01$ ). Abundance of *Enterococcaceae* on teat skin from CL cows remained low throughout the study while abundance on PF cows gradually increased throughout and was greater in recovery compared to pretreatment. The main difference was on teat skin from HS cows, *Enterococcaceae* abundance displayed the same pattern as inguinal skin where the abundance peaked during mid-challenge (d10), declined thereafter, but remained higher in recovery compared to pretreatment. In milk samples (Figure 6.3 D), *Enterococcaceae* abundance exhibited a treatment trend ( $P = 0.08$ ) where HS samples had a greater abundance than CL cows, although there was considerable variation. Overall, abundance of *Enterococcaceae* was greater during the heat stress challenge in bedding and on both skin sites which suggests that bedding may transfer *Enterococcaceae* to skin. Alternatively, increased abundance on skin may lead to shedding onto bedding. Although there were indications of increased abundance in PC, suggesting influence of DMI, by far the greatest effect was clearly due to heat.

In bedding (Supplementary Figure 6.10 A), *Staphylococcaceae* abundance had time ( $P < 0.01$ ) and treatment effects ( $P < 0.01$ ) increasing in early challenge, and remaining higher throughout the challenge period and until the last day of sampling (d21). *Staphylococcaceae* abundance was higher in bedding from HS and CL cows, which displayed similar temporal patterns, than in PF cows, implying that reduced DMI lowered *Staphylococcaceae* in bedding. In inguinal skin, *Staphylococcaceae* abundance did not differ (Supplementary Figure 6.10 B;  $P > 0.10$ ). In teat skin (Supplementary Figure 6.10 C), *Staphylococcaceae* abundance displayed a significant interaction ( $P < 0.05$ ). However, this appeared due to a single high mean on d0 and all treatments were otherwise stable throughout. In milk samples (Supplementary Figure 6.10 D), *Staphylococcaceae* abundance did not differ ( $P > 0.10$ ). These data suggest that used bedding contained greater abundance of *Staphylococcaceae*, but it was diminished by pair-feeding. This family did not appear temperature responsive and could suggest the cow's skin as a source of exposure.

*Streptococcaceae* abundance in bedding was numerically higher in PF and HS but was not different ( $P > 0.10$ ) due to high variation (Figure 6.4 A). In inguinal skin (Figure 6.4 B), *Streptococcaceae* abundance had a significant interaction ( $P < 0.01$ ). Inguinal skin from CL cows was low in abundance of *Streptococcaceae*, while the abundance on samples from HS cows increased in early challenge (d4), remained elevated during the challenge period, then declined by the last day of sampling (d21) but did not fully recover to pretreatment levels. Abundance of *Streptococcaceae* on inguinal skin from PF cows was lower in abundance than HS on d10, 13, and 18 but was numerically higher than CL during the challenge and recovery periods compared to pretreatment. Like inguinal skin,

*Streptococcaceae* abundance on teat skin (Figure 6.4 C) was affected by the interaction of treatment and time ( $P < 0.02$ ). *Streptococcaceae* relative abundance on teat skin from CL was low and stable, whereas PF was numerically higher during challenge and recovery compared to pretreatment. Abundance of *Streptococcaceae* on teat skin of HS cows increased during the challenge period and peaked on d18. In milk (Figure 6.4 D) samples, the abundance of *Streptococcaceae* differed by time ( $P < 0.01$ ) and treatment ( $P < 0.05$ ). *Streptococcaceae* abundance in milk was greater in HS cows compared to CL cows and is likely attributed to increased variation within the HS cows on d-3 and 18. The variable abundance of *Streptococcaceae* in milk during the heat stress challenge appeared independent of bedding exposure and is potentially related to the increased inguinal and teat skin abundance.

## 6.5 DISCUSSION

The findings from the present study indicate that bedding, inguinal skin, teat skin, and milk microbiomes of lactating dairy cows responded to heat and feed restriction stressors. Elevated THI results in alterations to bedding, teat (Nguyen et al., 2019b), and milk (Li et al., 2018) microbiotas, and it has been proposed that as temperatures increase, pathogen load would also be expected to increase (Hogan et al., 1989; Dahl et al., 2020). Gram-negative bacteria have been shown to increase in bedding during elevated environmental temperatures (Hogan et al., 1989; Rowbotham and Ruegg, 2016), and bacterial counts in bedding have been associated with bacterial counts on teat skin (Zdanowicz et al., 2004). By subjecting lactating dairy cows to constant, non-cyclical, heat stress for 16-d, we hypothesized that bacteria and specific mastitis pathogens increase in bedding which could in-turn increase the exposure of the teat end to bacterial

changes and result in changes to the milk microbiota. Inguinal skin should have limited contact with bedding and was evaluated to determine if there were differences in pathogen load between inguinal and teat skin due to differences in exposure. A group of pair-fed cows was also evaluated to attempt to control for the effects of reduced feed intake that commonly result when cattle are heat stressed. Furthermore, a group of untreated control cows were included to control for the effects of the chamber and time on these microbiomes.

Consistent with the present study, but using traditional culture methods, it has been previously established that bacterial populations in bedding increase after use (Patel et al., 2019; Robles et al., 2019). In the present study, bedding richness and diversity measures did not display treatment effects but were higher in used bedding compared to unused bedding. Studies have proposed bedding as the primary source of teat skin colonization (Derakhshani et al., 2018b) and as a possible source of bacteria in milk (Metzger et al., 2018). Additionally, it has been reported that teat skin is a source of raw milk contamination (Doyle et al., 2017). Richness and diversity of teat end samples from cows with subclinical or clinical mastitis were lower in infected than non-infected quarters (Braem et al., 2012). A longitudinal study over 12 months (Li et al., 2018) and a study evaluating cows from dry off to 150 DIM (Metzger et al., 2018) reported that Chao (richness) and Shannon (diversity) indices in milk were higher in the spring and summer compared to winter. The previously mentioned studies were longitudinal, whereas the present study used a timecourse design, so, other factors like the cold stress or the more gradual changes in temperature when comparing summer to winter may have influenced the results. Another study failed to find changes in alpha diversity of milk from cows

housed either indoors during the winter or outdoors during the summer (Doyle et al., 2017). Aside from season, alpha diversity has been reported to differ based on infection status and is higher in milk from healthy quarters compared to those with subclinical (Pang et al., 2018) or a history of clinical (Falentin et al., 2016) mastitis.

The predominant bacterial phyla found in bedding (Wu et al., 2019), on teat ends and in the teat canal (Braem et al., 2013; Derakhshani et al., 2018a), and in milk (Pang et al., 2018) include *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*. These phyla remain dominant regardless of quarter infection status (Braem et al., 2012; Braem et al., 2013), use of antimicrobial therapy during lactation (Ganda et al., 2016), and at time of dry off (Bonsaglia et al., 2017; Cremonesi et al., 2018). The current study found differences in *Proteobacteria* abundance which appeared responsive to heat stress. Changes in phyla abundance could be due to increased exposure to these bacteria from bedding and skin or due to independent microbiome variability. The concept of altered exposure of the teat end is supported by increased *Actinobacteria* abundance during heat stress in bedding, teat skin, and milk and a lack of difference in inguinal skin. Similarly, *Firmicutes* and *Proteobacteria* also appeared responsive to elevated environmental temperatures and humidity but these changes were not noted in bedding and milk which suggests bedding is not the only factor influencing the skin microbiome or the skin microbiome could have more variability, partially independent of exposure from bedding. *Firmicutes* abundance on inguinal and teat skin was lower during the heat stress challenge which suggests that the skins response to heat stress is not fully dependent on exposure to bedding. Andrews et al. (2019) compared bulk tank milk samples to quarter level milk samples and reported differences between the microbiotas which may be due

to the milking units and pipeline. Therefore, the correlations between bacterial phyla in bulk tank milk and season, reported by Li et al. (2018), does not accurately represent changes in the microbiota of individual cows housed in a controlled environment, like in the present study.

Individual mastitis-causing pathogens, *Enterobacteriaceae*, *Enterococcaceae*, *Staphylococcaceae*, and *Streptococcaceae*, were evaluated over time through a period of heat stress or pair-feeding to determine if increased abundance of these pathogens in bedding influenced the teat skin and milk abundances. We focused on the bacterial families that are commonly found in milk samples (Rodrigues et al., 2017; Taponen et al., 2019) and are associated with mastitis genera. A higher abundance of *Streptococcaceae* and *Enterobacteriaceae* have been reported in bulk tank milk samples with SCC >200,000 (Rodrigues et al., 2017). In milk, *Staphylococcaceae* have been reported to be higher during summer months while *Streptococcaceae* were lower and *Enterobacteriaceae* tended to be lower in winter months (Nguyen et al., 2019b). Additionally, the previously mentioned study evaluated composite milk samples from cows one and two months postpartum which might account for the higher abundance of *Staphylococcaceae* that was reported in their study compared to the present study that evaluated quarter-level milk samples from mid-lactation cows. Another potential reason for *Staphylococcaceae* counts to be low on skin samples in the present study could be due to the Swiffer<sup>TM</sup> duster that was used to collect the samples. During sample preparation, sterile water was added to the Swiffer<sup>TM</sup> and could have diluted the already low abundance of the mastitis associated bacterial OTU. A recent study evaluated aseptically collected milk samples over a period of two years (Andrews et al., 2019), and reported

higher abundances of *Staphylococcaceae* in milk and on teat ends collected from cows with infected mammary glands. However, the authors did not account for weather-related variation like differences in temperature or humidity which could be confounded with infection status over time. This could indicate that changes to the microbiome of milk is dependent on other environmental factors that can cause heat stress aside from temperature and humidity and were not included in our temperature-controlled chambers such as air movement and solar radiation.

While it has been well established that individual SCC and BTSCC increase in summer months (Olde Riekerink et al., 2007; Tao et al., 2018), SCC did not differ in the present study which could be due to the use of an acute heat stress period versus multiple summer months. However, based on the elevated abundance on inguinal and teat skin during heat stress, *Enterobacteriaceae* abundance appeared temperature-responsive on skin due to while bedding and milk appeared relatively unaffected. Similarly, *Enterococcaceae* displayed an increased abundance in bedding and on skin during the heat stress period and tended to be higher in milk from HS cows compared to CL cows. Previous work by this group reported similar changes in the *Enterococcus* genera from the same bedding, skin, and milk samples (Chapter V; unpublished data). The *Enterococcaceae* family includes the *Enterococcus*, *Melissococcus*, *Pilibacter*, *Tetragenococcus*, *Catellicoccus*, and *Vagococcus* genera (Švec and Franz, 2014). Due to very similar relative abundances of *Enterococcaceae* and *Enterococcus*, the *Enterococcaceae* family is likely made up of mostly *Enterococcus* in this study which could explain the similarity between this family and genus. The results from this study support the concept that bedding may alter the exposure of the teat end to different

bacterial populations, but, clearly, skin could also change independently of bedding as was the case of *Enterococcaceae* abundance on inguinal skin. These results combined with the lack of change in *Staphylococcaceae* indicate that not all mastitis-associated bacteria in bedding, on skin, and in milk have similar responses to elevated temperature and humidity.

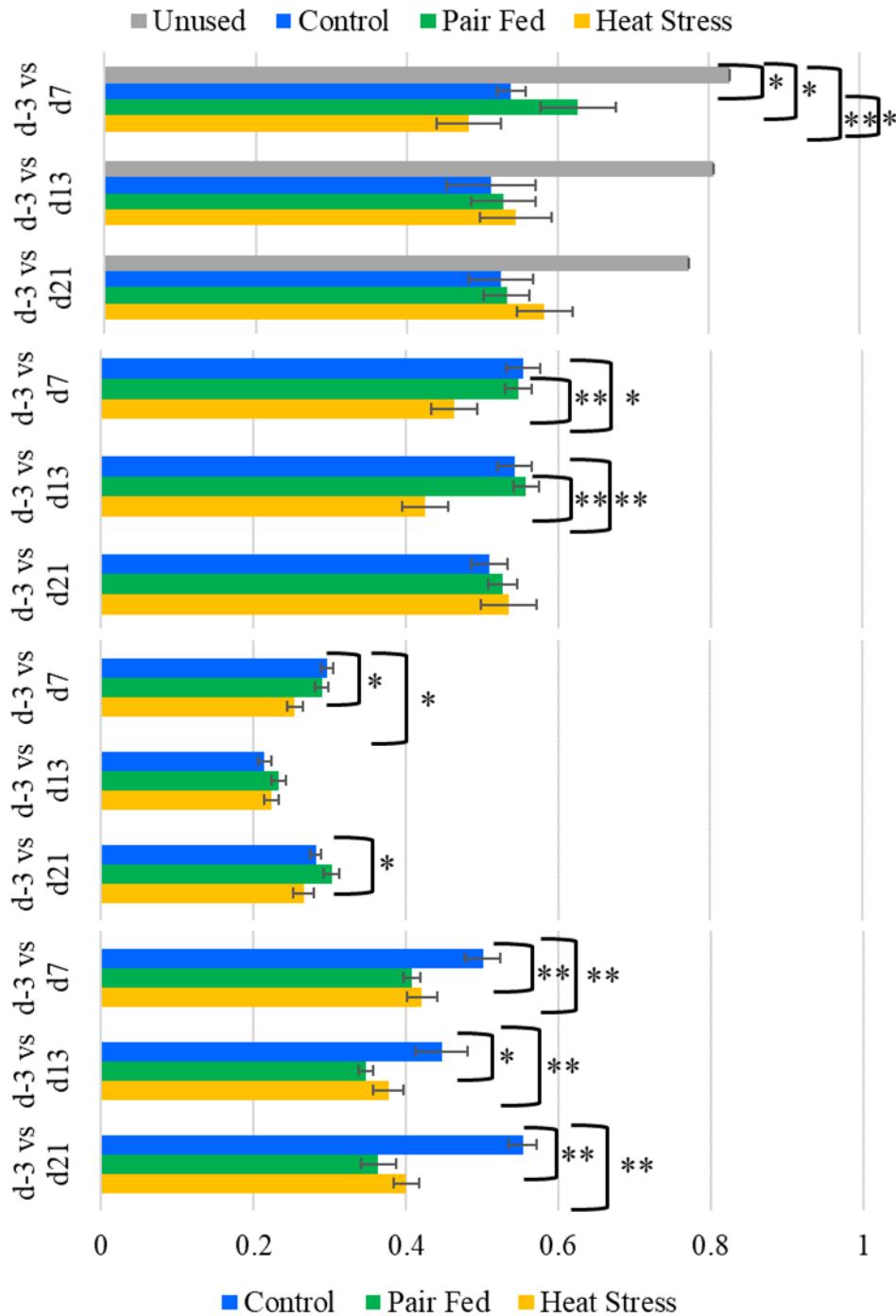
In a previous work by this group, we investigated the effects of heat and feed restriction stress on the rumen and fecal microbiomes of dairy cattle (Chapter IV; unpublished data) and found these microbiomes to be altered by heat stress and reduced feed intake and feed restriction stress alone. Even though none of the most variable OTU were the same between HS and PF cows across sample types in the present study, within HS cows, *Methanobrevibacter* species was found to be among the most variable on both inguinal and teat skin samples from HS cows. Additionally, one *Lactococcus* species was among the most variable OTU found on inguinal skin and in milk from HS cows. *Lactococcus* have been previously reported as a common genus that varies throughout the year in milk (Li et al., 2018). Results from the present study and our previous study highlight different effects of heat stress and feed restriction across microbial niches.

In the current study, the effects of elevated temperature and humidity and reduced feed intake on the bedding, inguinal skin, teat skin, and milk microbiomes of lactating dairy cows was evaluated. Our study design allowed for a comparison group of untreated control animals and a group of cows that were feed restricted to account for microbiome variation over time and to differentiate between effects of elevated temperature and reduced feed intake on the microbiome. The untreated control cows displayed changes over time which highlights the fluidity of the microbiome over time and the necessity of

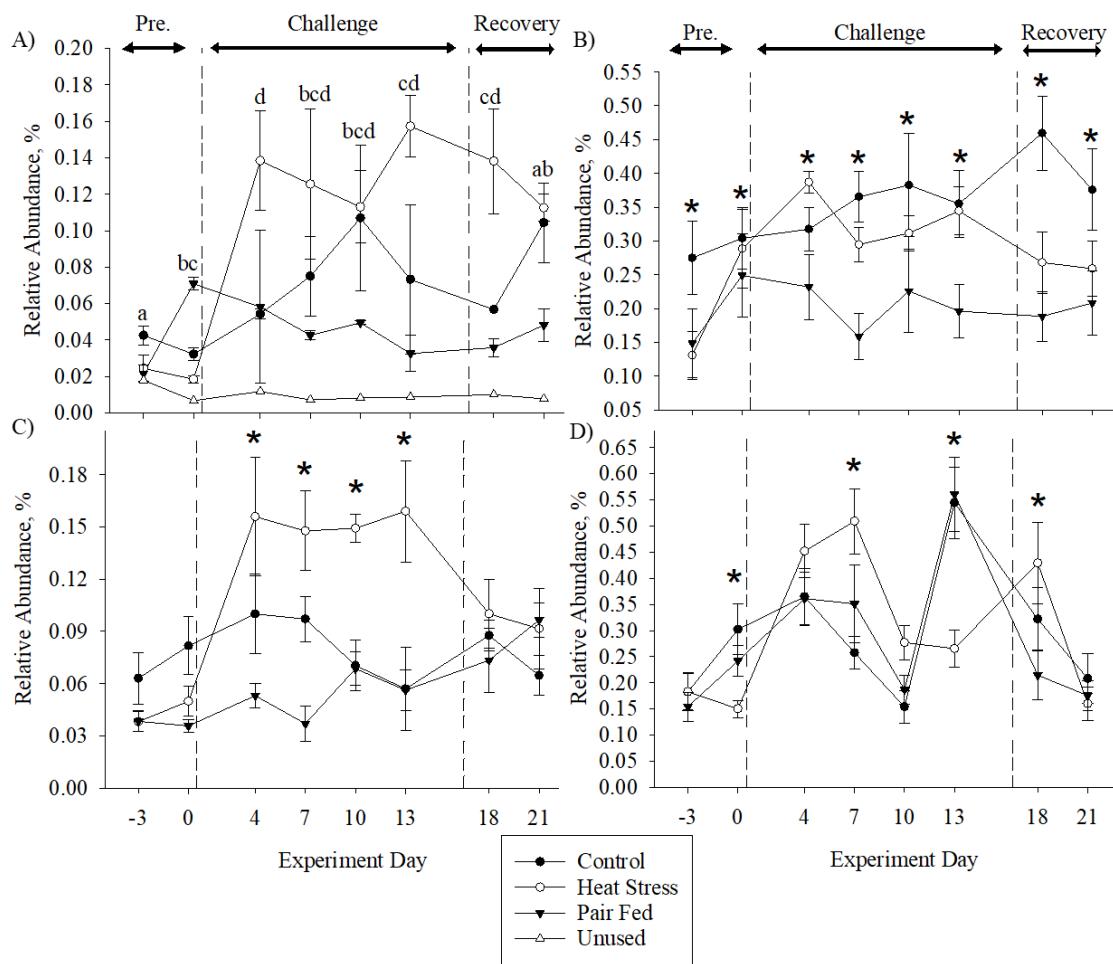
sampling the microbiomes multiple times. Nevertheless, this time course study allowed us to compare changes in the microbiomes of heat-stressed, feed-restricted, and control cows and revealed changes in individual bacterial phyla and families that responded to experimentally induced heat stress.

## 6.6 CONCLUSION

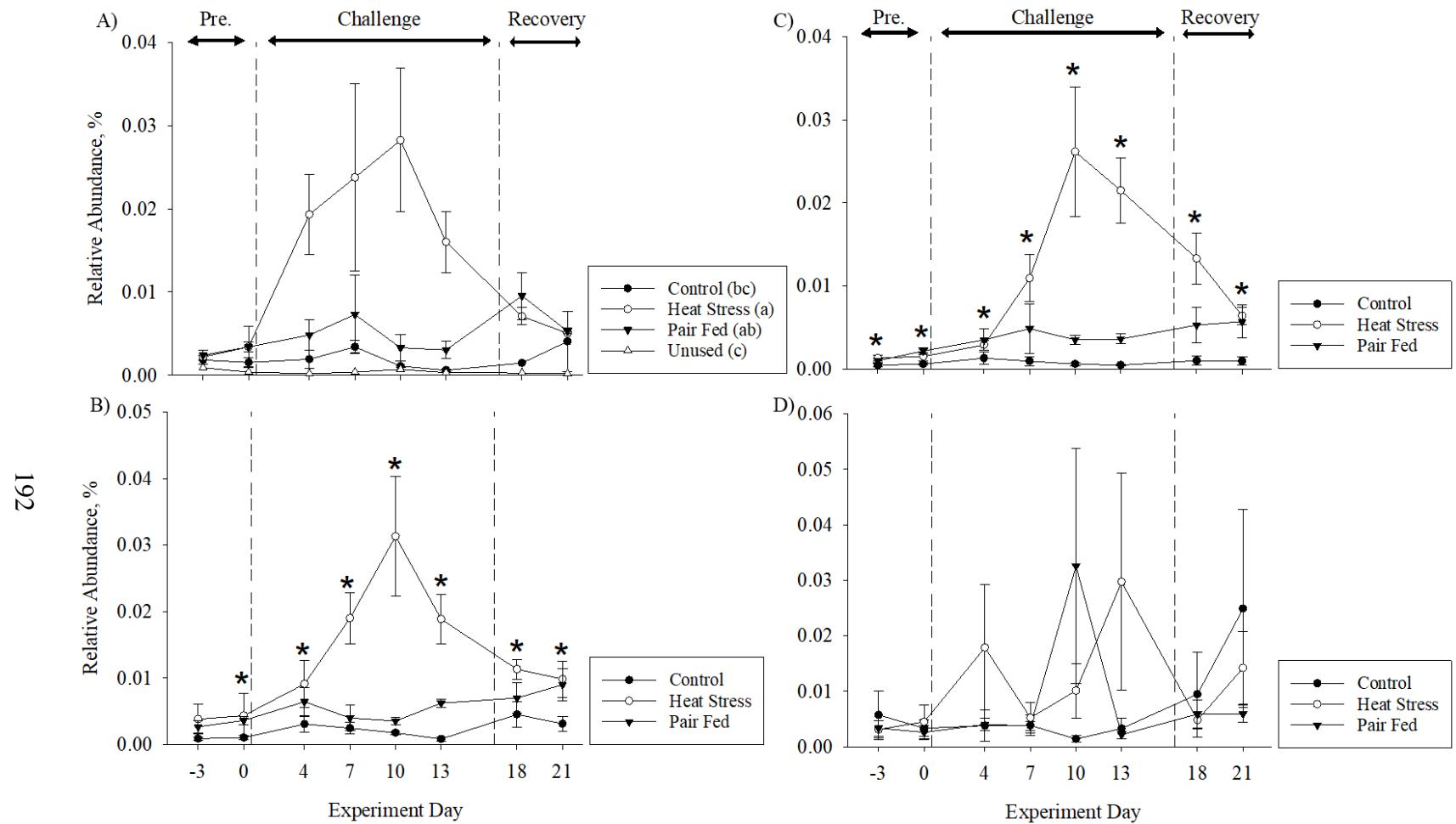
The present study identified changes in the microbiome in response to heat and feed restriction stressors. Specifically, changes in abundance of *Actinobacteria* and families belonging to *Firmicutes*, *Enterococcaceae* and *Streptococcaceae*, increased in bedding and on inguinal and teat skin during a period of heat stress, suggesting that these bacteria respond similarly to elevated environmental temperatures in bedding and on skin. In contrast, *Proteobacteria* abundance increased during the heat stress challenge on inguinal and teat skin but not in bedding, indicating that composition of teat skin communities is not entirely dependent on exposure from bedding. The findings from this study discovered changes in *Enterococcaceae* and *Streptococcaceae* in bedding and on skin that may predispose the dairy cow to higher incidence of disease during times of heat stress.



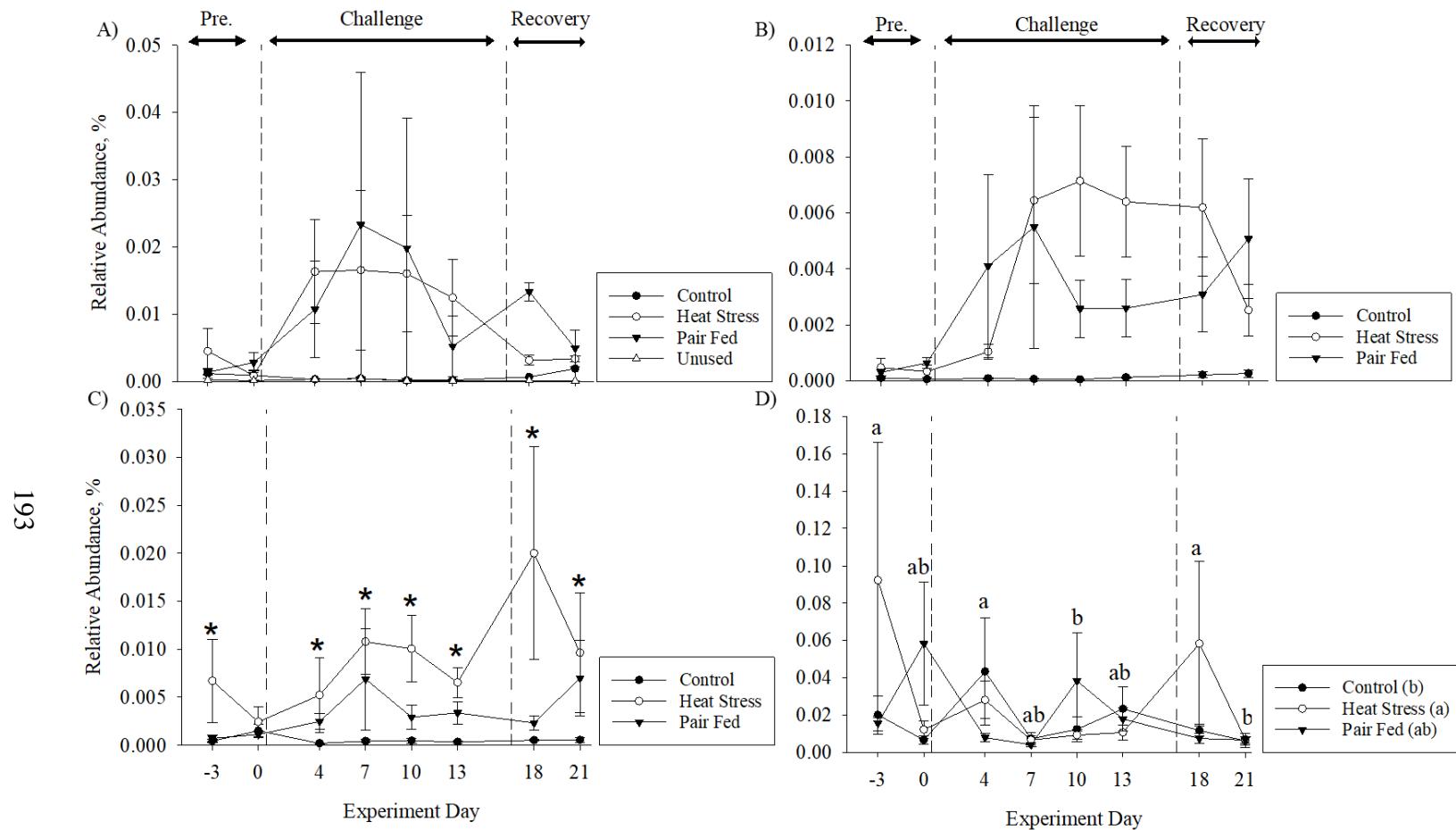
**Figure 6.1 A-D.** Intra-sample similarity for A) bedding, B) inguinal skin, C) teat skin, and D) milk samples from cows exposed to heat stress or pair feeding. Comparisons were made between pretreatment and early challenge (d-3 vs 7), pretreatment and late challenge (d-3 vs d13), and pretreatment and recovery (d-3 vs 21). \*Within panel means differ within comparison at  $P < 0.05$ . \*\*Within panel means differ within comparison at  $P < 0.01$ .



**Figure 6.2 A-D.** Relative abundance of *Actinobacteria* phyla in A) bedding, on B) inguinal skin, on C) teat skin, and in D) milk from cows exposed to heat stress or pair-feeding. <sup>a,b,c,d</sup>Within panel indicate significant effects of time ( $P < 0.05$ ). \*Within panel indicate significant interaction within the respective timepoint ( $P < 0.05$ ).



**Figure 6.3 A-D.** Relative abundance of *Enterococcaceae* for A) bedding, B) inguinal skin, C) teat skin, and D) milk samples from cows exposed to heat stress or pair-feeding. <sup>a,b</sup>Within legend indicate significant effects of treatment ( $P < 0.05$ ). \*Within panel indicate significant time effect within the respective timepoint ( $P < 0.05$ ).



**Figure 6.4 A-D.** Relative abundance of *Streptococcaceae* for A) bedding, B) inguinal skin, C) teat skin, and D) milk samples from cows exposed to heat stress or pair-feeding. <sup>a,b</sup>Within panel indicate significant effects of time ( $P < 0.05$ ). \*Within panel indicate significant difference in treatment means within the respective timepoint ( $P < 0.05$ ). <sup>a,b</sup>Within legend indicate significant effects of treatment ( $P < 0.05$ ).

## 6.7 LITERATURE CITED

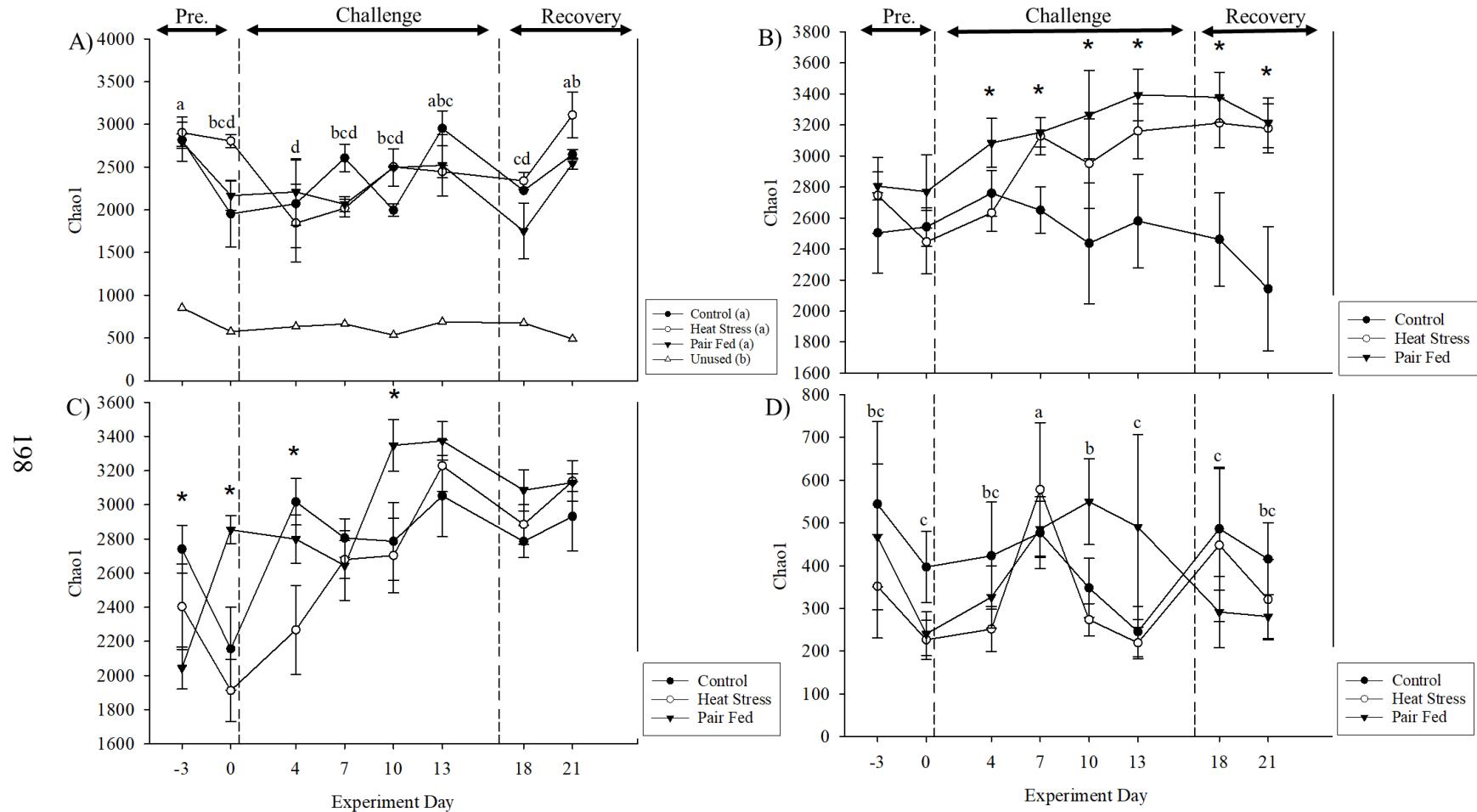
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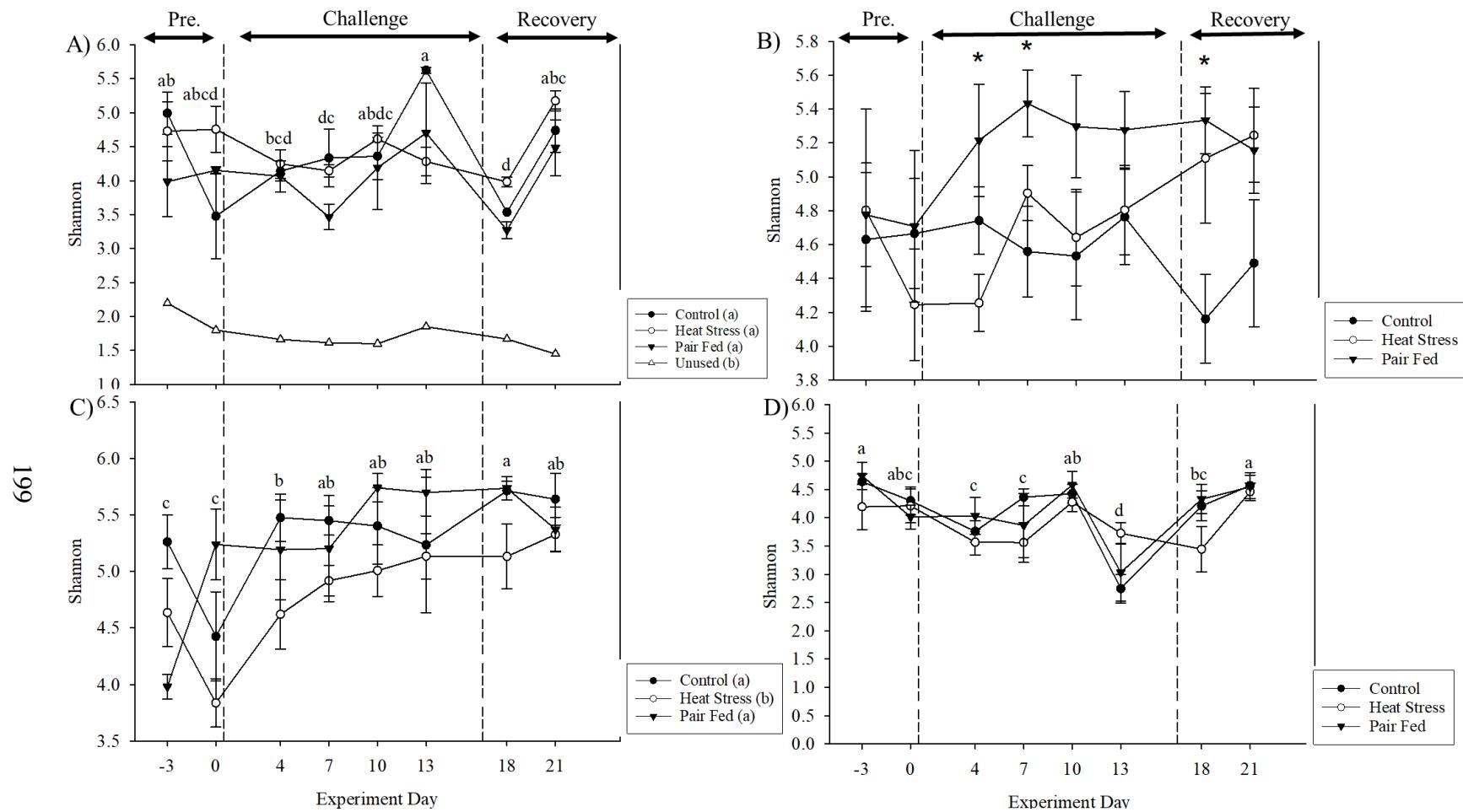
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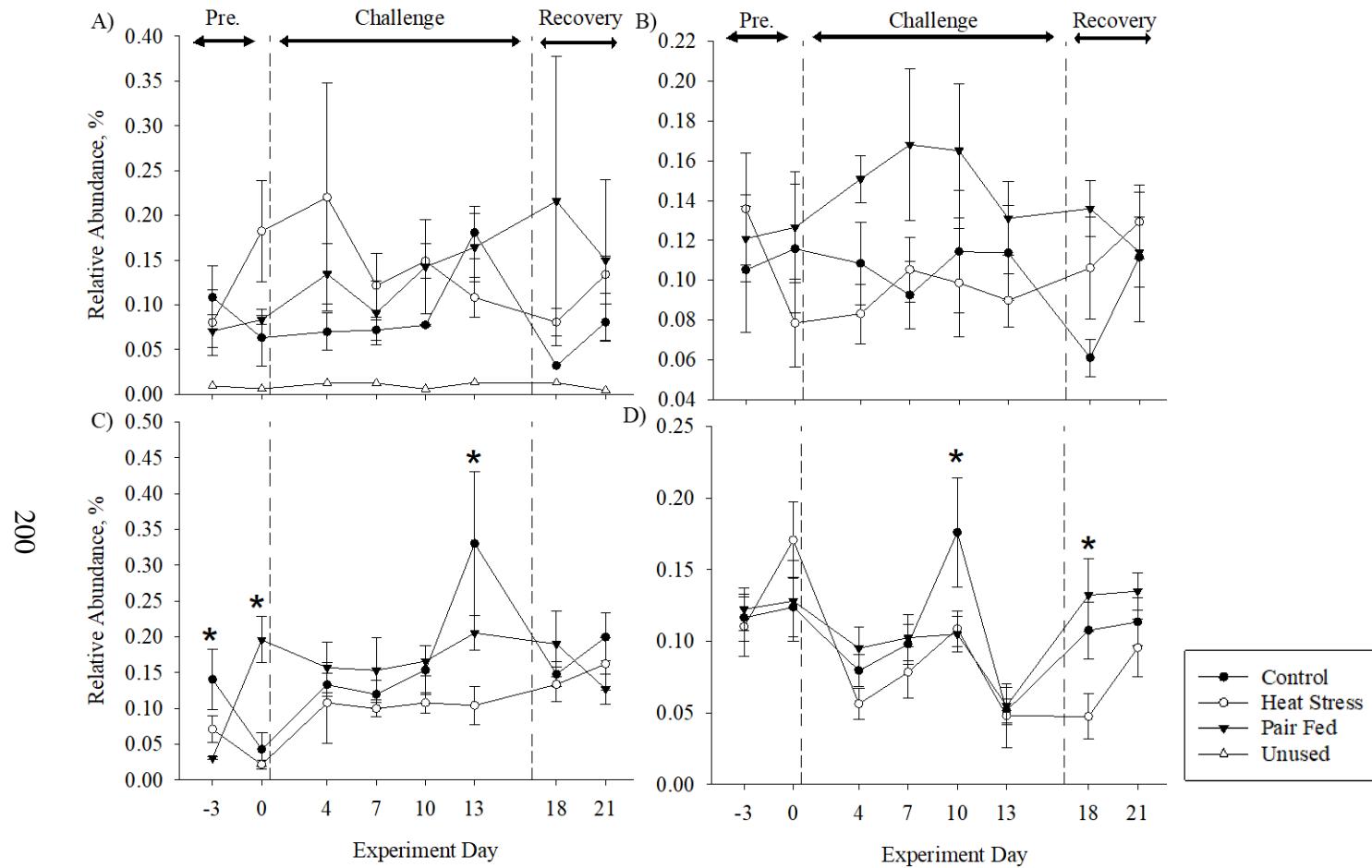
## 6.8 SUPPLEMENTARY MATERIAL



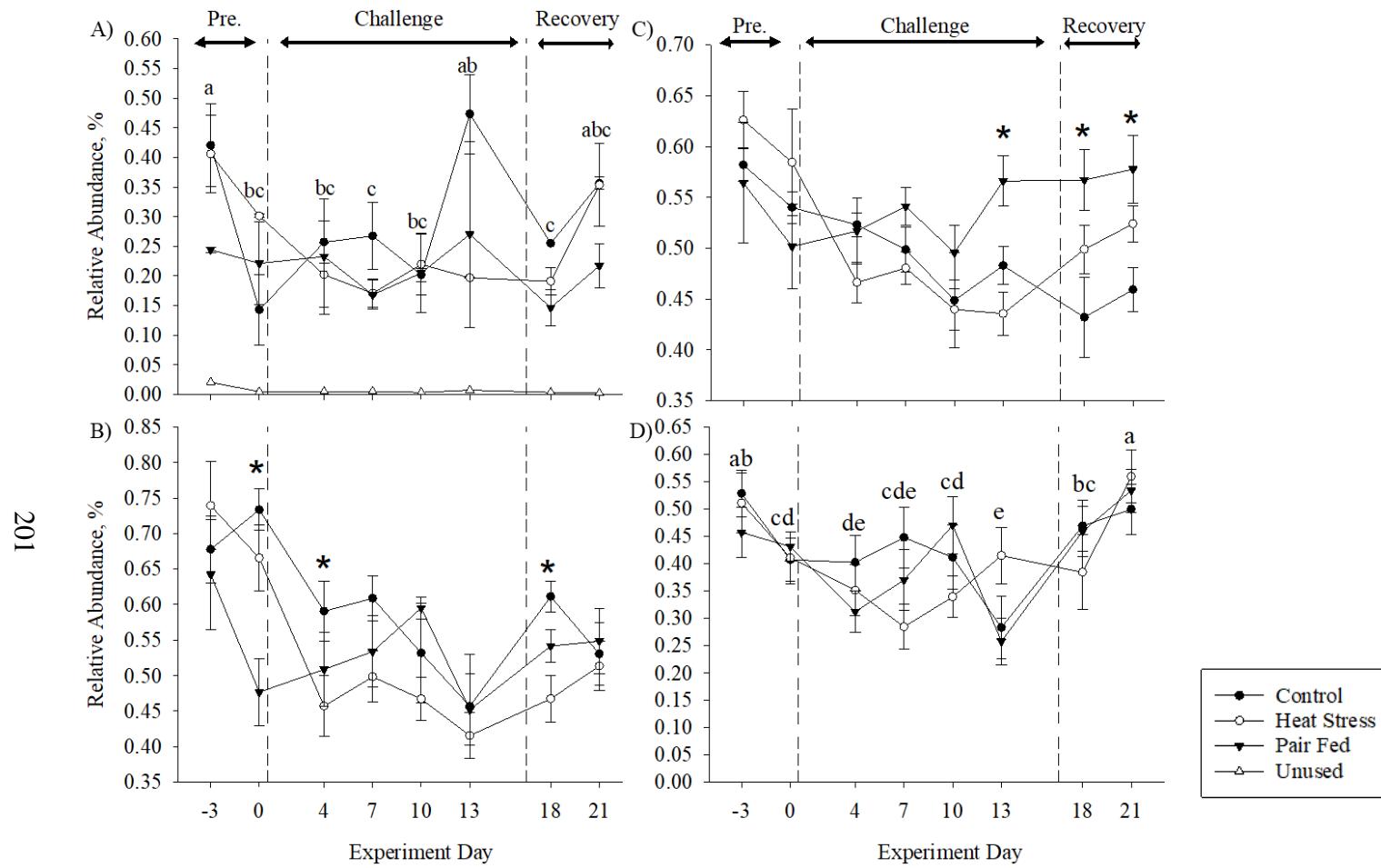
**Supplementary Figure 6.5 A-D.** Richness (Chao1) measures for A) bedding, B) inguinal skin, C) teat skin, and D) milk samples from cows exposed to heat stress or pair-feeding. <sup>a,b,c,d</sup>Within panel indicate significant effects of treatment within each timepoint ( $P < 0.05$ ). <sup>a,b</sup>Within legend indicate significant effects of treatment ( $P < 0.05$ ).



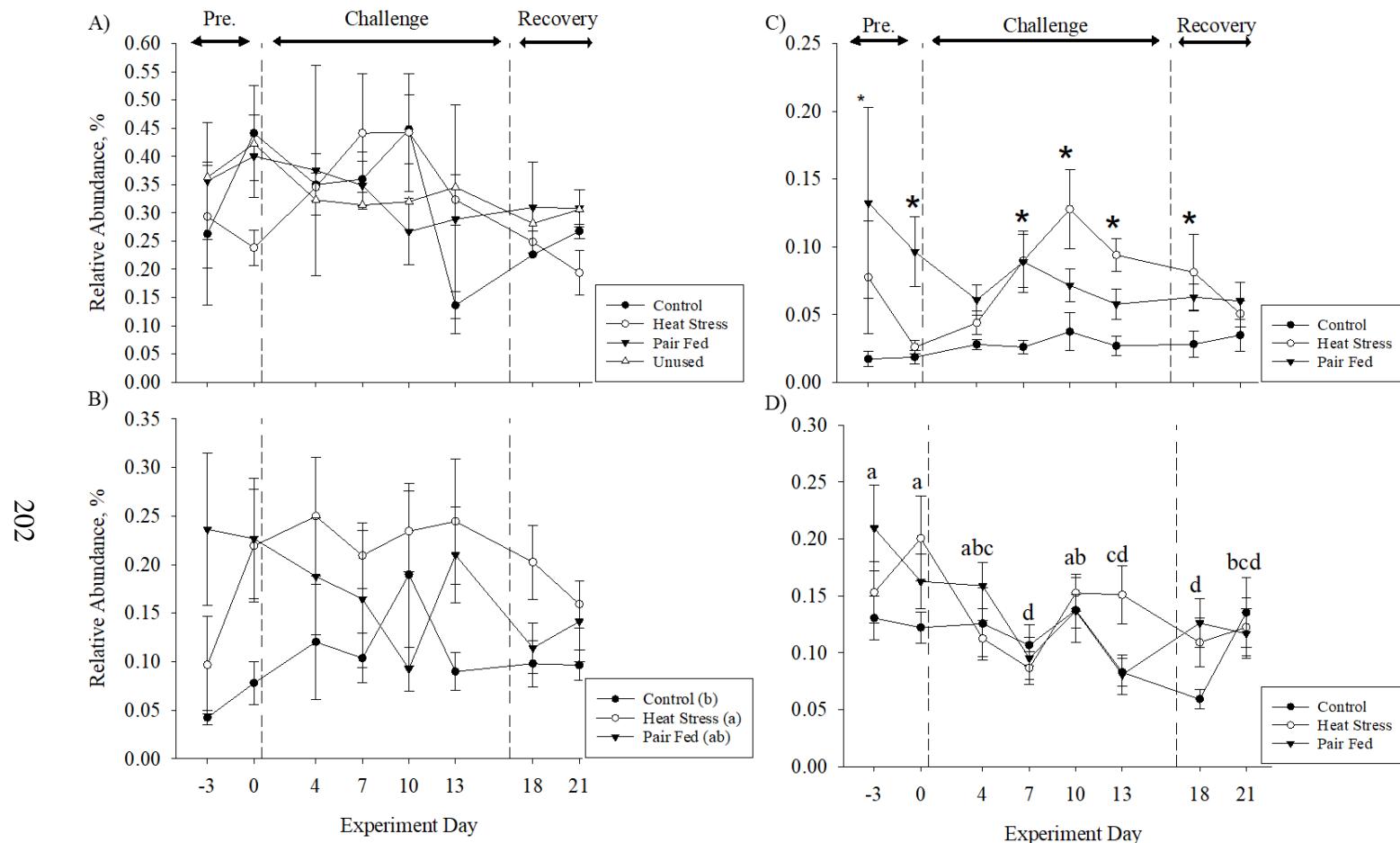
**Supplementary Figure 6.6 A-D.** Diversity (Shannon) measures for A) bedding, B) inguinal skin, C) teat skin, and D) milk samples from cows exposed to heat stress or pair-feeding. <sup>a,b,c,d</sup>Within panel indicate significant effects of treatment within each timepoint ( $P < 0.05$ ). <sup>a,b</sup>Within legend indicate significant effects of treatment ( $P < 0.05$ ). <sup>\*</sup>Within panel indicate significant difference between treatment means within the respective timepoint ( $P < 0.05$ ). Lack of identifier within panel or legend indicate no significant interaction ( $P > 0.10$ ).



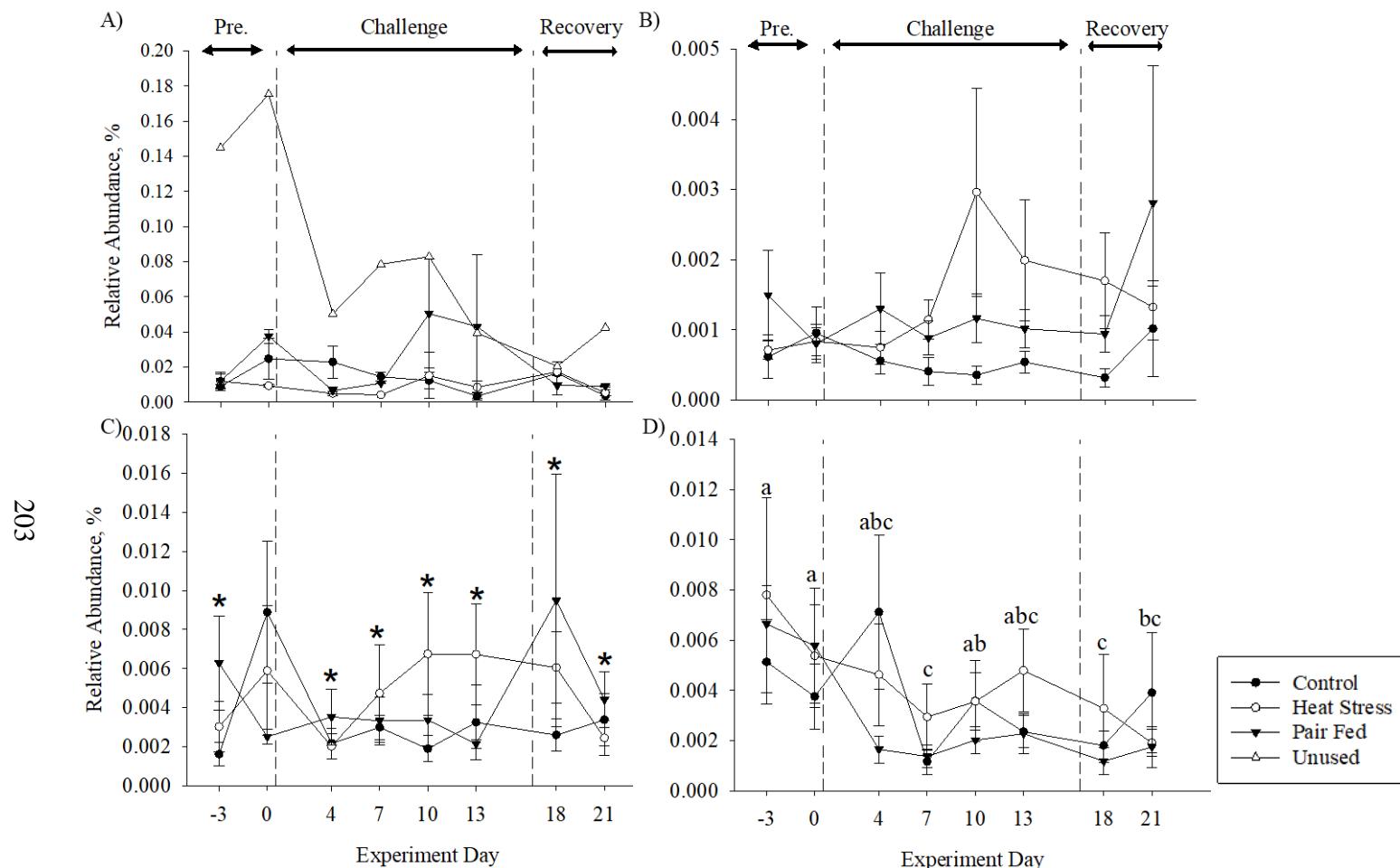
**Supplementary Figure 6.7 A-D.** Relative abundance of *Bacteroidetes* phyla in A) bedding, on B) inguinal skin, on C) teat skin, and in D) milk from cows exposed to heat stress or pair-feeding. \*Within panel indicate significant difference in treatment means within the respective timepoint ( $P < 0.05$ ).



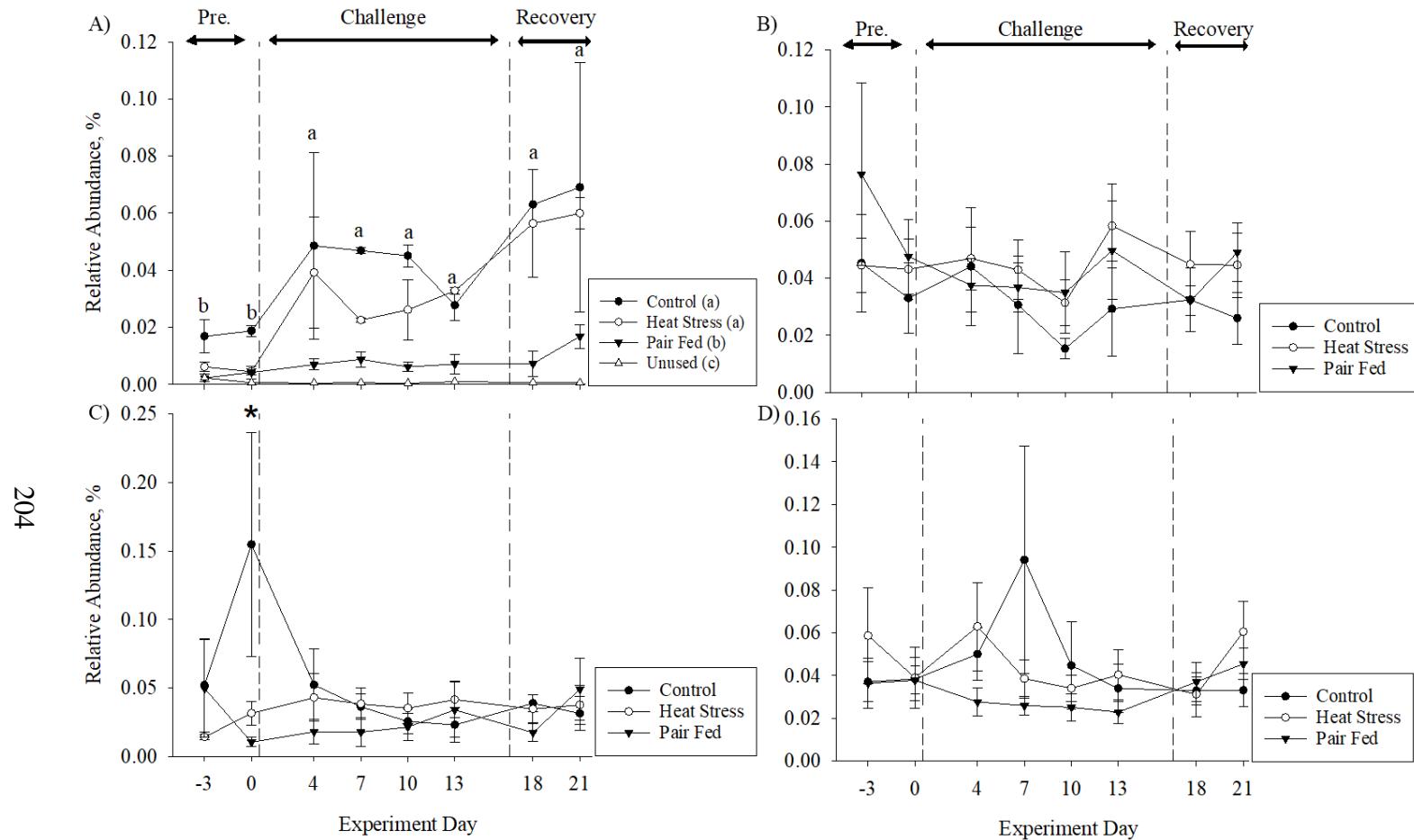
**Supplementary Figure 6.8 A-D.** Relative abundance of *Firmicutes* phyla in A) bedding, on B) inguinal skin, on C) teat skin, and in D) milk from cows exposed to heat stress or pair-feeding. <sup>a,b,c,d,e</sup>Within panel indicate significant differences in average abundance across treatments over time ( $P < 0.05$ ). \*Within panel indicate significant interaction within the respective timepoint ( $P < 0.05$ ).



**Supplementary Figure 6.9 A-D.** Relative abundance of *Proteobacteria* phyla in A) bedding, on B) inguinal skin, on C) teat skin, and in D) milk from cows exposed to heat stress or pair-feeding. <sup>a,b,c,d</sup>Within panel indicate significant effects of time ( $P < 0.05$ ). \*Within panel indicate significant difference in treatment means within the respective timepoint ( $P < 0.05$ ). <sup>a,b</sup>Within legend indicate significant main effects of treatment ( $P < 0.05$ ).



**Supplementary Figure 6.10 A-D.** Relative abundance of *Enterobacteriaceae* family in A) bedding, on B) inguinal skin, on C) teat skin, and in D) milk from cows exposed to heat stress or pair-feeding. <sup>a,b,c</sup>Within panel indicate significant effects of time ( $P < 0.05$ ). \*Within panel indicate significant differences in treatment means within the respective timepoint ( $P < 0.05$ ).



**Supplementary Figure 6.11 A-D.** Relative abundance of *Staphylococcaceae* family in A) bedding, on B) inguinal skin, on C) teat skin, and in D) milk from cows exposed to heat stress or pair-feeding. <sup>a,b</sup>Within panel indicate significant effects of time ( $P < 0.05$ ). <sup>\*</sup>Within panel indicate significant difference in treatment means within the respective timepoint ( $P < 0.05$ ). <sup>a,b</sup>Within legend indicate significant main effects of treatment ( $P < 0.05$ ).

## CHAPTER VII

### GENERAL DISCUSSION

Each of the previous heat stress studies reported differing conclusions to the question of how elevated temperature impacts the dairy cow microbiome. These studies aimed to determine if fecal shedding of bacterial operational taxonomic units (**OTU**) and growth of bacteria in bedding responded to elevated temperature-humidity index (**THI**) and could increase the exposure of the teat end to bacteria. Cows shed bacteria into their environment via feces and could alter bedding bacterial counts and teat end exposure to bacteria. This, in turn, could alter the milk microbiome and dysbiosis may partially explain the elevated rise in bulk tank SCC (**BTSCC**) and disease incidence during periods of elevated THI.

In the first study (Chapter II), the effects of elevated environmental temperature and relative humidity on the fecal microbiome were investigated in a single group of heat-stressed cows. A follow-up study (Chapters IV-VI) also investigated the effects of elevated environmental temperature and relative humidity on the fecal microbiome and the rumen fluid, bedding, inguinal skin, teat skin, and milk to determine if alterations in one microbial niche could be found in other microbiomes. This study also incorporated a group of control cows to evaluate microbiome stability over time. A third group of cows was pair fed to match the intake of the heat-stressed cows to differentiate between the combined effects of heat stress and reduced feed intake and the effects of feed restriction alone. The purpose of these studies was to evaluate the effects of heat stress and pair-feeding on the rumen, fecal, bedding, inguinal skin, teat skin, and milk microbiomes over time. Our second study also investigated various mastitis-associated bacteria to determine

if these OTU increase in abundance during heat stress and could partially explain the increase in BTSCC and mastitis incidence in the summer.

Overall, we found bacterial community composition was altered within each heat stress study (Chapters II, IV-VI) and these results combined with hierarchical clustering and intra-sample similarity analyses indicate that the dairy cow microbiome was altered by elevated environmental temperatures and feed restriction. However, individual phyla, families, and genera did not display the same effects between studies or sample types. Within the heat stress and feed restriction study, the multiple sample types contained different abundances of mastitis associated bacterial OTU which is likely due to different sample composition and nutrient availability of niches. Within the bedding, inguinal skin, and teat skin, some OTU—*Actinobacteria*, *Enterococcaceae*, and *Enterococcus*—displayed similar responses to the induced stressors. Inguinal skin should be less exposed to bedding and therefore, these results suggest that skin also responds to heat stress and is at least partially independent of bedding exposure. Furthermore, we cannot determine if the bedding altered the teat skin or if the teat skin altered the bedding.

The results from these studies indicate that the dairy cow microbiome is altered by environmental changes and reduced feed intake. While most mastitis-associated bacteria corresponding to OTU were not consistently higher throughout the elevated environmental temperature challenge or across sample types, the abundance of other bacterial OTU were likely altered and could partially explain the increased BTSCC and disease incidence that is witnessed during the summer. Investigation into additional, more rare, bacterial populations that are altered by environmental stress and their functional capabilities could help explain the role of bacterial populations in dairy cow production.

A better understanding of stress-responsive bacteria and their interaction with other microbes is warranted to understand the complex relationship between the dairy cow and her microbes as each respond to heat stress. These studies have highlighted the importance of untreated control animals and future studies should incorporate controls to evaluate treatment effects on microbial populations and attempt to minimize microbiome variability over time with aseptic sample collection. Overall, these studies identified multiple bacterial OTU that responded to elevated THI in the various sample types. In support of our hypotheses, *Bacteroidetes* abundance increased during late heat stress in feces but these changes were not consistent between studies or across multiple sample types. Additionally, *Enterobacteriaceae*, *Enterococcaceae*, and *Streptococcaceae* displayed increased abundance in response to elevated THI in bedding and on skin which supports the concept of an interaction between bedding and skin. Together, these results highlight changes to various microbiomes of lactating dairy cattle and may partially explain the rise in BTSCC during summer months.