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EFFECTS OF HEAT STRESS ON INFLAMMATION, INTESTINAL INTEGRITY, MICROBIOTA, AND PRODUCTIVITY IN DAIRY CALVES

Zhantao Yu
zyu18@vols.utk.edu

To the Graduate Council:

I am submitting herewith a dissertation written by Zhantao Yu entitled "EFFECTS OF HEAT STRESS ON INFLAMMATION, INTESTINAL INTEGRITY, MICROBIOTA, AND PRODUCTIVITY IN DAIRY CALVES." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Agustin Rius, Major Professor

We have read this dissertation and recommend its acceptance:

Agustin Rius,Jun Lin, Guoxun Chen, Jennie Ivey,Gina Pighetti

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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ABSTRACT

Two studies were conducted to evaluate the effects of heat stress on growth performance, inflammation, intestinal integrity and microbiota changes, furthermore dexamethasone was tested to alleviate heat stress in dairy calves. In study one, we hypothesized that cyclical heat stress for 7 days alters pro- and anti-inflammatory signals, gut microbiota community composition, and intestinal barrier in dairy calves. Heat stress had a section-specific impact on intestinal integrity. Jejunal mRNA expression of *TJP1* and expression of heat shock transcription factor-1 decreased in heat stress calves. Heat stress decreased interleukin-1 alpha and tended to decrease interleukin-17A. Conversely, heat stress increased jejunal concentration of interleukin-36 receptor antagonist. Heat stress reduced concentrations of interleukin-6 in plasma. Alteration of the large intestine bacterial communities was characterized by increased butyrate-producing bacteria genus *Butyrivibrio_3* in heat stress calves. In study two, we hypothesized that a modulation of the immune response improves nutrient metabolism, intestinal morphology, barrier function, and gut microbial community composition in Holstein bull calves exposed to cyclical heat stress for 5 days. Treatments (saline vs dexamethasone), environments (thermoneutral vs heat stress), and time (study day or hour) were included in the statistical analysis. Feed intake did not change but a treatment by environment interaction showed that dexamethasone increased average daily gain and feed efficiency in heat stress calves. A treatment by environment by time interaction on study day 1 showed that the post prandial plasma glucose concentration declined in thermoneutral but increased in heat stress calves treated with dexamethasone. A treatment by environment by study day interaction showed that white blood cells and neutrophil concentrations increased more in heat stress than in thermoneutral calves treated with dexamethasone on study day 1. A treatment by environment by

study day interaction showed that blood lymphocyte concentration decreased in heat stress calves treated with dexamethasone on study day 1. Treatment by environment interaction showed that jejunal crypt depth was reduced in heat stress calves treated with dexamethasone only. A treatment by environment interaction showed that jejunal IL-6 concentration was increased in thermoneutral dexamethasone but reduced in heat stress dexamethasone calves. Collectively, heat stress triggered inflammation, intestinal barrier dysfunction, and jejunal microbial community imbalance and dexamethasone improved productivity, intestinal morphology and reduced the pro-inflammatory tone in dairy calves.

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CHAPTER 1 LITERATURE REVIEW

INTRODUCTION

Dairy calves are susceptible to heat stress during summer (Roland et al., 2016). Numerous publications have indicated the negative effects of heat stress on performance, behavioral response, immune response, and gastrointestinal tract (GIT) function in dairy cows (Wheelock et al., 2010, Fontoura et al., 2022). However, heat stress in early life of dairy cattle (e.g., fetus, preweaning) can have long-lasting effects on their productive life (Dahl et al., 2016). Therefore, it is vital to study the direct effect of heat stress on preweaning dairy calves.

Compared with thermoneutral, heat stress can diminish the activity and proliferation of immune cells, disrupt cytokine production, increase oxidative stress and inflammation, all of which can impair the animal's ability to combat infections in lactating dairy cows and goats (Wheelock et al., 2010, Sejian et al., 2018). Heat stress affects the immune response by infiltrating immune cells into intestine along with changes in leukocytes counts, Th1:Th2 ratio, and plasma cytokine concentrations compared with thermoneutral cows (Koch et al., 2019, Chauhan et al., 2021). While heat stress increased neutrophil counts and the neutrophils to lymphocytes ratio in cattle (Stull and McMartin, 1992, Naik et al., 2013), a decreased neutrophil count was also reported in heat-stressed dairy cattle (Elvinger et al., 1991). Additionally, activation of immune pathway is an energetically costly event, and could be one of the reasons for the compromised productivity in animals exposed to heat stress (Greer, 2008). Hence, there is value in studying hematology profiles and cytokine signals in heat-stressed animals.

The effects of thermal stress on intestinal barrier function of calves require additional investigations. The integrity of the intestinal epithelial barrier is crucial in preventing the invasion of intestinal microbiota and luminal contents into the epithelium. This barrier consists of tight junctions, adherence junctions, and desmosomes, to restrict the infiltration of pathogens and bacteria. Heat stress can directly impair the integrity of the intestinal barrier, as evidenced by

studies indicating a two-fold increase in ZO-1 mRNA abundance in heat-stressed cows compared to control cows (Koch et al., 2019). Furthermore, heat stress can induce gut permeability, enabling the entry of pathogens and endotoxins into the bloodstream, leading to an inflammatory response (Sejian et al., 2018).

The gastrointestinal tract (GIT) microbiome plays a critical role in nutrient fermentation, immune response, and mucus production (Wang et al., 2018, Zhao et al., 2019, Zhong et al., 2019). Furthermore, compared with pigs in thermoneutral conditions, heat stress reduced the diversity of the gut microbiota, rendering them more susceptible to pathogens and thereby compromising their immune functions (Liu et al., 2022). Therefore, there is a growing need to study the effects of heat stress on gut microbiota in dairy calves.

Dexamethasone is a steroid drug with anti-inflammatory and immunosuppressant properties that has been utilized for over five decades to treat various conditions, such as arthritis, asthma, and allergic reactions (Rhen and Cidlowski, 2005). Previous studies conducted on rats have indicated that dexamethasone increases the expression of heat shock proteins, suggesting its potential use in mitigating the negative effects of heat stress (Sun et al., 2000). It is possible that dexamethasone improves immune function, reduce inflammation and, subsequently, improve in acclimation, intestinal morphology, function, and microbial community composition. Our overall hypothesis was that heat stress-associated alterations of intestinal barrier function would be accompanied by a downregulation in the expression of tight junction proteins, an increase in pro- and anti-inflammatory signals, and intestinal dysbiosis. The immunomodulatory effect of dexamethasone would reduce inflammation and, subsequently, improve energy metabolism, and intestinal morphology, function, and microbial community composition.

HEAT STRESS IN LIVESTOCK

The economic cost of heat stress

Heat stress has emerged as a significant challenge due to the escalating global temperatures (IPCC, 2018) amidst the need for intensive agriculture (Renaudeau et al., 2012). Heat waves in 2006 (with temperatures above 38°C) killed 25,000 dairy cows in California (AFP, 2006). This equates to an overall loss of $37.5 million to $62.5 million, or $1,500 to $2,500 per cattle. The same news article also noted a daily drop of 1.1 million liters (400,000 gallons) of milk production in central California. Dairy cow deaths and performance losses totaling $28 million were attributed to a heat wave in Iowa in July 1995. The effects of summer heat stress on dairy and feedlot cows have been a continuing problem, since decades. In the United States, livestock producers suffer annual losses of approximately two billion dollars as a cause of heat stress. Within the dairy industry alone, losses amount to $900 million from reduced dry matter intake (DMI), milk production, body weight gain, increased mortality rates, and reproductive culling (St-Pierre et al., 2003).

Thermoneutral zone

Dairy cows are considered homeothermic animals, meaning they can regulate their body temperature within a specific range despite of fluctuations in the external environment. In a state of thermal comfort, a dynamic equilibrium exists between heat generation, heat gain, and heat dissipation. Heat generation primarily arises from basal metabolic rate, physical activity, food metabolic increment, milk synthesis, solar radiation absorption, while heat loss occurs through conduction, convection, evaporation, and radiation. However, in extreme conditions, peripheral sensors transmit a message to the thermostatic control unit, which comprises of the heat loss center (anterior hypothalamus) and the heat production center (posterior hypothalamus). The thermostatic control unit aims to maintain the core body temperature (central body temperature)

close to a pre-set point. The control of effectors responsible for heat loss and heat production is determined by the disparity between the actual core temperature and the desired core temperature (Hafez, 1968).

The thermoneutral zone refers to the range of temperatures within which no additional effort is needed to heat or cool the body. For lactating dairy cows, the thermoneutral zone is typically between −0.5 to 20°C (Johnson, 1987). However, studies have reported a higher upper critical air temperature, around 25 to 26°C, where dairy cows can maintain their core body temperature (Berman et al., 1985). The upper critical temperature is lower than the body temperature, which creates a thermal gradient that allows mammals to dissipate heat through conduction, convection, and radiation. Another way to achieve heat dissipation is through evaporation, which includes sweating and panting. The relative humidity is the controlling factor for evaporation heat loss, as it requires a vapor pressure gradient. When the environmental temperature is hot and humid, all heat dissipation pathways are compromised, and heat stress symptoms begin to show up.

Heat stress incidence condition

Heat stress occurs when animals are unable to dissipate excessive heat through mechanisms such as convection, conduction, radiation, and evaporation. Dairy cattle are particularly vulnerable to hot and humid environments due to their lower surface-volume ratio, which reduces their heat dissipation efficiency and the large amount of metabolic heat they generate. In comfortable thermal conditions, dairy cattle are able to maintain a balance between heat generation and heat dissipation, resulting in a relatively stable body temperature. However, in hot and humid conditions, increased heat load from solar radiation, coupled with compromised

heat dissipation efficiency, leads to higher body temperature and respiration rates, ultimately impacting animal welfare and productivity.

Heat stress indicators

Temperature-humidity index (THI)

THI is a commonly used indicator to quantify the heat stress experienced by animals. Most studies calculate the THI using temperature and relative humidity data, as these are readily available. However, thermal stress is not only caused by temperature but also by solar radiation, wind speed, and precipitation, especially in cattle raised outdoors (Mader et al., 2006). Therefore, these factors have been included in calculating various heat stress indices, which differ in their weighting of temperature and humidity (Table 1-1). It is important to note that the accuracy of predicting heat stress may vary based on the index used, as those with higher humidity weights are more suitable for humid climates. In contrast, those with higher temperature weights are better for semi-arid climates.

The threshold values for alerting heat stress vary across different studies. Some researchers categorize THI into normal (THI<=74), alert (75<=THI<=78), danger (79<=THI<=83), and emergency (THI>= 84) (LeRoy Hahn et al., 2009). Another categorization includes the comfort zone (THI<=71), mild heat stress (72<=THI<=79), moderate stress (80<=THI<=89), and severe stress (THI>=90) (Armstrong, 1994). Yet another study found that lactating cows exhibit behavioral changes when THI exceeds 68 (Cook et al., 2007). It is worth noting that the first two categorizations were based on studies conducted between 1950 to 1960 when the average milk yield was around 15.5 kg/d (range 2.7-31.8 kg/d). However, in the present-day United States, the milk yield ranges from 22.8 to 52.5 kg/d, and cows are more susceptible to heat stress due to the increased milk yield and accompanying heat generation.

Animal related indicators

Each cow may have different sensitivity levels to heat stress based on factors such as housing, parity, and milk yield. Therefore, animal-related indicators are also used to measure thermal stress. The most commonly used animal-based indicator is internal body temperature, which accounts for 28% of the 129 heat stress studies conducted on dairy cows. Rectal temperature (RT) is the most commonly used internal body temperature indicator, followed by vaginal temperature. The normal temperature range for dairy cows is reported to be 38.6 ± 0.5℃ (Wenz et al., 2011), and RT above 39.5℃ has been observed in dairy cattle exposed to high ambient temperatures. The second most commonly used group of indicators is physiological indicators, including respiration rate (RR), heart rate, and sweat rate (Galan et al., 2018). Any RR greater than 60 breaths per minute is considered as an indicator of heat stress. Feeding behavior is also used as an indicator of heat stress, with decreased dry matter intake (DMI) and feeding time being widely used. Other indicators, such as standing time, lying time, drinking frequency, and volume are also used to measure heat stress in dairy cows.

Thermoregulation in dairy cattle

Increased RT and RR

Body temperature increases as a response to heat stress (Armstrong, 1994). The increase in body temperature will expand the temperature gradient between skin surface and ambient temperature which will enhance heat loss from the body. Respiration is a main way to dissipate the heat from the body, in line with that higher RR in Jersey cows represents more efficient heat dissipation compared to Holstein cows. Increase in respiratory rate is one of the most sensitive phenotypic indicators of heat stress in dairy cows. Any RR >60 breaths/min is indicative of heat

stress (Berman et al., 1985). When RR is greater than 120 breaths per minute, the cow is under severe stress (Hahn, 1999).

Increased sweating rate

Sweating is one of the most effective means for evaporative cooling mechanisms ranges from a minimum of 0.1 kg m^{-2} h^{-1} to a maximum 0.6 kg m^{-2} h^{-1} (Spiers, 2012). When dairy cows were exposed to hot and dry condition (THI=79.6), a sweating rate of 668 g m^{-2} h^{-1} was reported (Gebremedhin et al., 2010). A similar rate was reported as 660 g m^{-2} h^{-1} when THI and wind velocity were slightly different (Gebremedhin et al., 2008).

Behavior

Upon receiving signals from the central nervous system, dairy cattle regulate their body temperature via behavioral and physiological responses (see Figure 1-1). Behavioral responses include increased standing time, shade seeking, drinking frequency. The standing position increases the efficiency of heat dissipation through convection, and heat stress-induced increased standing time has been reported in several studies of dairy cows (Zahner et al., 2004, Allen et al., 2015). However, standing for prolonged periods can limit the blood flow to the udder, contributing to decreased milk yield in dairy cows, along with increased maintenance requirements (Rulquin and Caudal, 1992). Increased water intake has been reported before in dairy cows when exposed to heat stress (Blackshaw and Blackshaw, 1994). Water makes up 75 - 81% of the body weight of dairy cows, and 87% of milk, resulting in a high-water turnover in dairy cattle. Increased water intake has been observed to cope with water loss due to increased evaporation. Meanwhile, water excreted through urine or feces helps with heat dissipation in the main body due to the high specific heat of water (Kadzere et al., 2002). Cows seek shade to avoid being exposed to the direct incoming solar rays and to mitigate heat load increases caused

by radiation. Seeking shake has been reported in heat stress cattle especially raised on pasture (Blackshaw and Blackshaw, 1994). This behavior was mostly prominent during mid-afternoon and Kendall et al. (2006) reported cows to have a decrease in vaginal temperature and an increase in milk yield when they had access to shade.

Cardiovascular changes

Heat stress has been reported to increase heart rate, increase heart output, and redirect the blood flow. During heat stress, elevated body temperature causes the circulatory system to attempt harder to cool down and sustain important physical processes. The greater load on the circulatory system causes an increase in heart rate (West, 2003). This is an adaptive response designed to enhance blood flow to the skin and aid in heat dissipation. Along with that, a increased cardiac output have been reported in heat stress animals (Hales and Dampney, 1975). The redistribution of cardiac output from the splanchnic tissue to peripheral tissues and respiratory tract will facilitate the heat loss (Lambert et al., 2002).

HEAT STRESS IN DAIRY CALVES

Heat stress indicators

In lactating dairy cows, the temperature-humidity index (THI) is often used as an indicator of heat stress. A THI range of 68-72 is considered comfortable for dairy cows (Polsky and von Keyserlingk, 2017). However, researchers are yet to establish a clear THI threshold for dairy calves. Recent studies have utilized reference THI values to evaluate thermic stress in preweaning calves, but the cutoff THI values varied in each study (Pena et al., 2016, Manriquez et al., 2018). As each calf responds to heat load individually and differently, animal-related parameters, including physiological, behavioral, and performance indicators, have been utilized.

Among these indicators, RR and body temperature have been found to be the two dominant parameters (Galan et al., 2018).

Thermoregulation in dairy calves

Thermoregulation is critical for maintaining a constant core body temperature in dairy calves and involves both behavioral and physiological changes. Behavioral changes in calves include postural adjustments, changes in water and food intake, alterations in diurnal and nocturnal activity patterns, locomotor activities, and seeking shade. Studies have shown that calves exposed to heat stress tend to stand more and lie down less, while their water intake and consumption increase (Tripon et al., 2014, Kim et al., 2021). Furthermore, calves dissipate heat by increasing their respiration rate, which may result in potential respiratory alkalosis (Ríus et al., 2022). Shade seeking is also an efficient method to prevent heat load in animals from solar radiation, although there are limited studies about providing shade for calves (Mitlohner et al., 2001, Schutz et al., 2010, Schutz et al., 2011).

Besides behavioral changes, dairy calves can also achieve thermoregulation through physiological adjustments. The nervous system in calves contains temperature-sensitive neurons that can trigger physiological and behavioral responses through the hypothalamus. Dairy calves, for instance, can increase peripheral blood flow to enhance heat dissipation and regulate their body temperature by decreasing their dry matter intake when exposed to heat stress (Lough et al., 1990, Fan et al., 2019).

HEAT STRESS ON IMMUNE SYSTEM

Immune system of cattle

Dairy cattle possess a robust immune system that relies on a collaboration between the innate and adaptive immune responses to maintain homeostasis. The innate immune components primarily consisting of physical barriers such as the skin and mucosal membranes, phagocytes,

and soluble molecules like complement. The skin and mucosal membranes form the first line of defense against invading microorganisms. If pathogens manage to breach these physical barriers, they are detected by pathogen pattern recognition (PPR) and taken up by phagocytes. Toll-like receptors (TLRs) serve as the main PPRs, with various types of TLRs having been confirmed in dairy cattle (Novák, 2014). The complement system, which circulates in an inactive state, is another essential part of innate immune defense that can damage the membranes of pathogens. In most cases, dairy cattle can eliminate microorganisms efficiently without activating the adaptive immune system. However, if the invading pathogens persist, the adaptive immune response is activated. Antigen-presenting cells (APCs), including macrophages, B cells, and dendritic cells, are capable of internalizing external antigens through phagocytosis or endocytosis and presenting them on the major histocompatibility complex class II (MHC). The T helper cells are activated when their T-cell receptors (TCRs) bind to the antigen expressed by MHC class II, resulting in the secretion of cytokines that can activate cytotoxic T lymphocytes (CTLs) and B cells. Subsequently, B cells differentiate into plasma cells, which secrete antibodies to facilitate the clearance of antigens.

Heat stress on innate immunity

Heat stress on immune cells

Leukocytes including granulocytes (neutrophils, eosinophils, and basophils), monocytes, and lymphocytes (T cells and B cells) are essential to both innate and adaptive immune system. Heat stress has been reported to affect leukocyte population.

Neutrophils are a type of white blood cell that plays a crucial role in the innate immune response, which is the first line of defense against infections. The primary function of neutrophils is to identify, engulf, and destroy invading pathogens such as bacteria, viruses, and fungi, a

process known as phagocytosis (Nauseef and Borregaard, 2014). Neutrophils have several mechanisms to kill pathogens, including the production of reactive oxygen species (ROS) and release of antimicrobial peptides. ROS are toxic to bacteria and help to kill them by oxidizing cellular components (Nauseef and Borregaard, 2014). Neutrophils also release neutrophil extracellular traps (NETs), which are composed of DNA, histones, and antimicrobial proteins that trap and kill pathogens (Brinkmann et al., 2004). In addition to their role in fighting infections, neutrophils also play a role in inflammation. When tissues become inflamed due to injury or infection, neutrophils are among the first cells to migrate to the affected area. They release inflammatory mediators, such as cytokines and chemokines, which help to attract other immune cells to the site of infection or injury, and promote tissue repair and healing (Kolaczkowska and Kubes, 2013). The effects of heat stress on neutrophil have been inconsistent. Heat stress increased the neutrophil counts and neutrophils to lymphocytes ratio (Stull and McMartin, 1992, Naik et al., 2013) and decreased the neutrophil counts in dairy cattle (Elvinger et al., 1991).

Lymphocytes are a type of white blood cell that plays a crucial role in the adaptive immune response, and includes B cells and T cells. B cells are responsible for producing antibodies, which are proteins that specifically recognize and bind to pathogens. Once bound, antibodies can neutralize the pathogen or mark it for destruction by other immune cells. B cells are also involved in the process of memory, which allows the immune system to mount a more rapid and effective response upon subsequent exposure to the same pathogen (Tarlinton and Good-Jacobson, 2013). T cells are involved in a range of immune responses, including the destruction of infected cells, the activation of other immune cells, and the regulation of the immune response. There are several different types of T cells, including helper T cells, cytotoxic

T cells, and regulatory T cells. Helper T cells are involved in the activation of other immune cells, while cytotoxic T cells are responsible for the destruction of infected cells. Regulatory T cells help to regulate the immune response and prevent it from becoming too strong and causing damage to tissues (Waldman et al., 2020). It has been reported that heat stress negatively affected lymphocytes proliferation (Kamanga-Sollo et al., 2011). However heat stress did not affect lymphocytes counts in dairy cattle (Elvinger et al., 1991).

Eosinophils are a type of white blood cell that plays a critical role in the immune response to parasitic infections and allergies. Their main function is to identify, neutralize, and eliminate invading parasites and other foreign substances, including allergens. Eosinophils accomplish this through several mechanisms, including the release of granules containing toxic proteins that can kill parasites and the activation of other immune cells. Eosinophils are also involved in the inflammatory response, and their activation can lead to the recruitment of other immune cells to the site of infection or inflammation. In addition to their role in parasitic infections and allergies, eosinophils are also involved in tissue repair and remodeling. They release growth factors and other proteins that can stimulate the growth and repair of damaged tissues (Waldman et al., 2020). One study on heat-stressed dairy cows found a decrease in the percentage of eosinophils in the blood, suggesting that heat stress may reduce eosinophil activity (Bernabucci et al., 2002). Another study on heat-stressed pigs found a reduction in eosinophil numbers (Ju et al., 2014). Overall, more research is needed to fully understand the effects of heat stress on eosinophils in livestock and the implications for animal health.

Monocytes are a type of white blood cell that plays a critical role in the immune response. Their main function is to identify, engulf, and eliminate foreign particles, including pathogens, dead cells, and debris, through a process called phagocytosis. In addition to

phagocytosis, monocytes are also involved in the activation of other immune cells, such as T cells and B cells, and the production of cytokines, which are signaling molecules that help to coordinate the immune response. Monocytes can differentiate into other immune cells, such as macrophages and dendritic cells, which have more specialized functions in the immune system (Ziegler-Heitbrock, 2014). Furthermore, monocytes play a crucial role in tissue repair and remodeling. They release growth factors and other proteins that can stimulate the growth and repair of damaged tissues (Ziegler-Heitbrock, 2014).

Heat stress on cytokine expression

Cytokines are signaling molecules that play a critical role in regulating the immune response and maintaining homeostasis in body. They are involved in a wide range of biological processes, including inflammation, hematopoiesis, cell proliferation and differentiation. Cytokines are produced by a variety of cell types, including immune cells, endothelial cells, and epithelial cells.

Heat stress has been shown to disrupt the balance of cytokines in livestock, leading to an altered immune response and increased susceptibility to disease. One study found that heat stress caused an increase in pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-alpha) in cattle (Collier et al., 2008). These cytokines are involved in initiating and sustaining inflammation in response to tissue damage or infection. However, overproduction of pro-inflammatory cytokines can lead to tissue damage and chronic inflammation, which can negatively impact animal health. In addition to affecting pro-inflammatory cytokines, heat stress can also impact anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-beta). These cytokines are involved in regulating the immune response and reducing inflammation. Heat stress has been

shown to cause a decrease in the production of these cytokines in pigs (Pearce et al., 2013a), which can lead to a reduced ability to modulate the inflammatory response. In conclusion, heat stress can disrupt the balance of cytokines in livestock, leading to an altered immune response and increased susceptibility to disease.

Heat stress on adaptive immunity

Heat stress on cell-mediated immune

Adaptive immunity consists of cell-mediated immune responses and humoral immune responses. Heat stress decreased the proliferation of peripheral blood mononuclear cells (PBMC) under in vitro condition (Kamwanja et al., 1994). Similarly, heat stress has been reported to suppressive lymphocyte proliferation in vitro in cows (Lacetera et al., 2006).

Heat stress on humoral immune

A total of five different immunoglobins (IgM, IgG, IgA, IgD, and IgE) were identified in bovine (Stanfield et al., 2018), in which IgM has two (IgM1 and IgM2), and IgG has three subclasses (IgG1, IgG2, and IgG3). The IgG is vital in cattle because it is the most abundant immunoglobin in colostrum and function for neutralizing virus, and bacterial agglutination and opsonization. It has been observed that heat-stressed cows had a lower colostrum IgGs concentration (around 22% less), while increased IgGs concentration was founded in heat stress cows (Nardone et al., 1997), suggesting an impaired transformation from maternal blood to colostrum. Further, calves exposed to heat stress at late gestation had a lower serum IgG concentration (Tao et al., 2012). The IgA is essential for mucosal antiviral function; however, bovine has a low quantity of IgA in serum. In addition, the primiparous cows exposed to heat stress had a lower (around half of their counterpart) colostrum IgA concentration compared to thermoneutral cows (Nardone et al., 1997).

Heat stress on mucosal immune functions

Mucosal immune barrier includes four major components: commensal microbiota, mucous layer, epithelial cells, and immune cells of the mucosa-associated lymphoid tissues.

Heat stress on mucous layer

The mucosal layer consists of mucous, antimicrobial peptides (AMPs), and IgA and is known as a "kill zone" to prevent microbial invasion of the epithelium. Mucous and mucins are mainly secreted by goblet cells, with epithelial cells producing fewer mucins. Meanwhile, AMPs are produced by enterocytes and ciliated epithelial cells (CEC). Plasma cells in lamina propria (LP) contribute to IgA secretion, followed by transportation of IgA to the surface of epithelial cells. The inner mucous layer along with the AMPs and IgA form a "killing zone" that few pathogens or commensals have evolved strategies to penetrate. In broilers, heat stress has decreased total wall thickness and mucous layer height (Mazzoni et al., 2022). Further, mucosa damage was observed in heat stress rats, along with reduced goblet cells which may be the reason for thinner mucous layer (Liu et al., 2012).

Heat stress on epithelium structure

In the gastrointestinal mucosa, a single layer of epithelial cells adheres to one another, through an intercellular junctional complex known as TJs. The TJ is composed of proteins, including occludin, claudins, zonula occludens, and junctional adhesion molecules. The TJ proteins function as a gate-and-fence, selectively facilitating paracellular transport of nutrients, ions, and water, but preventing diffusion of microorganisms and microbial-derived peptides. While the heat stress effects on the dysfunction of TJs remain unclear. At protein level, heat stress reduced the expression of occludin, ZO-1 in poultry (Song et al., 2014); similar results were observed in jejunum of heat stressed cow (Koch et al., 2019). However, increased occludin

expression was also founded in hyperthermia caco-2 cells (Dokladny et al., 2008). Surprisingly, mRNA expression often times was not matched with protein expression. For example, it has been reported that heat stress upregulated mRNA expression of ZO-1 (Koch et al., 2019). Additionally, the redistribution of occludin and ZO-1 has also been associated to heat stress (Mazzon et al., 2002). Research efforts are needed in this area to have better insight regarding the association of heat stress and epithelium structures.

IMMUNOPHYSIOLOGICAL RESPONSES IN THE INTESTINES

Heat stress on GIT

The GIT is the primary place where nutrients are digested and absorbed, and it plays a crucial role in maintaining immune health. It is well documented in different species that heat stress affects intestinal function and triggers immune responses (Lambert, 2009). Intestinal and liver blood flow was decreased because more blood was divided into periphery tissues to enhance the efficiency of heat dissipation when animals suffered from heat stress (Rowell, 1974, Kregel et al., 1988, Sakurada and Hales, 1998). Meanwhile, heat stress stimulated metabolism, decreased the splanchnic blood flow leading to cellular hypoxia, followed by intestinal barrier dysfunction (Hall et al., 2001). Failures in thermoregulation to keep steady core body temperature, lead to intestinal tissue hyperthermia which induces the opening of tight junction (Moseley et al., 1994). The hyperthermia associated enhanced intestinal permeability was also related to intestinal epithelium damage (Lambert et al., 2002).

Heat stress on intestinal barrier function

Intestinal epithelium barrier is formed with tight junction, adherence junction, and desmosome which play a crucial role in preventing intestinal microbiota and luminal contents into the epithelium, and cooperate with matter transport, like water and waste products. Tight junction plays role in size and charge selectivity through ‘pore’ and ‘leak’ pathways (Shen et al.,

2011). Tight junction formed by occludin, claudins, junctional adhesion molecule, and tricellukin define the permeability and control the paracellular space. Claudins are critical for tight junction permeability to define the charge selectivity (Turner, 2009). Zonula occludens serve as scaffold proteins interact with transmembrane proteins, and Zonula occludins connected with perijunctional actomyosin ring (Suzuki, 2013). E-cadherin (also known as cadherin-1) is a transmembrane protein that bind with catenin δ1 (also known as p120 catenin) and β-catenin. β-catenin interact with α-catenin to regulate perijunctional actomyosin ring assembly (Turner, 2009, Odenwald and Turner, 2017). There are several factors that can affect intestinal epithelium barrier function i.e., heat stress.

Tumor necrosis factor-α (TNF-α) was found to cause intestinal barrier dysfunction (Madara and Stafford, 1989), associated with inflammatory bowel disease (IBD) (Stallmach et al., 2004). Myosin light chain kinase (MLCK) has been indicated as the central intermediate of this pathway (Clayburgh et al., 2005, Clayburgh et al., 2006). Also, TNF-α caused diarrhea through disturbing the Na^+-H^+ exchange (Marchiando et al., 2010). In intestinal Caco-2 cells, TNF-α decreases the expression of ZO-1 (Ma et al., 2004).

Interleukin-13 (IL-13) increases claudin-2 expression, resulting in the permeability of pore pathway (Weber et al., 2015). Another study indicated that IL-13 cause barrier loss by increasing both claudin-2 expression and apoptosis (Heller et al., 2005). The IL-4 and IL-6 were found to induce the increasing of claudin-2 expression which triggers the increased permeability of intestinal barrier (Prasad et al., 2005, Weber et al., 2010). Heat stress can destroy the intestinal barrier directly, and heat-stressed cows had a 2-fold higher ZO-1 mRNA abundance than control (Koch et al., 2019). Given that plasma IL-6, TNF-α increased when dairy cattle were exposed to

heat stress (Chen et al., 2018), additional research is required to determine whether intestinal barrier function is always accompanied by inflammation caused by heat stress.

MICROBIOTA CHANGES IN GIT

Microbial ecosystem establishment in calves

Calves are born with sterile GIT, however, dynamic microbial ecosystem establishment undergoes immediately after birth by connecting with surrounding environment. A previous study indicated that even though different age animals shared the same dominant phyla (*Firmicutes*, *Bacteroidetes* and *Proteobacteria*), but difference was observed in abundance and numbers of genera, caused mainly by age and diet (Jami et al., 2013). More specifically, 1-day-old calves only share one taxon with more mature animals, the main groups detected at a very young age (less than 3 weeks old) were found as major populations in the human fecal bacterial community (i.e., Bacteroides-Prevotella, the Clostridium coccoides-Eubacterium rectal group, Faecalibacterium, and Atopobium). Bacteroides-Prevotella and the C. coccoides-E. rectal group comprised a major fraction of the microbiota (ca. 50%–70% of the total) throughout the first 12-week period after birth, whereas the numbers of Atopobium, Faecalibacterium, and some probiotic bacteria (such as those of the genera Lactobacillus and Bifidobacterium) decreased as the animal aged (Uyeno et al., 2015). Taking into account the diet, phylum Bacteroidetes, which was significantly less abundant in newborns, was composed mainly of the genus Bacteroides, whereas in the older animals it was prominent and composed almost exclusively of the genus Prevotella. This is primarily caused by diet differences, where newborn diet typically is of high calorific value, rich in protein, fat and sugar, whereas the older animal diet is composed mainly of plant fiber (Kehoe et al., 2007).

Function of microbiota

Microbiota is beneficial in nutrient digestion, immune system establishment, and healthy maintenance through inhibiting pathogen colonization, stimulating richness of healthy microbiota, and enhancing intestinal barrier function.

Microbiota and tight junctions

The TJs play a vital role in selectively facilitating paracellular transport of nutrients, ions, and water, preventing diffusion of harmful molecules and microbiome. The importance of feeding calves with colostrum immediately after birth has been empathized because high intercellular permeability only last for 24-36 hours which is helpful to absorb proteins (i.e., IgG) from colostrum. This permeability reduces dramatically after 36 hours of birth and continues to decrease over the first month of life (Araujo et al., 2015). The expression of occludin and claudin-5 increased in pathogen-free mice compared to germ-free mice showing the interaction between host and microbiota is critical for barrier function establishment (Braniste et al., 2014). In line with the results on mice, increased mRNA abundance of TJs coding genes were observed in small intestine of calves in the first week of life (Liang et al., 2016). More specifically, *Lactobacillus spp* and *Bifidobacterium spp* are the two dominant genes in early life which can upregulate the expression of TJs (Ewaschuk et al., 2008, Miyauchi et al., 2012, Sultana et al., 2013). Disrupted TJs expression triggered by heat stress has been reported in piglets (Pearce et al., 2013b), while it remains unclear whether this change is related to microbiota change. Hence it is worth digging into the effects of heat stress on TJs expression changes and microbiota changes.

Microbiota and mucosal immune response

Mucus serving as the physical barrier of immune response contributes to maintenance of gut integrity, and the presence of commensal microbiota enhances the production of mucus. It has been reported that germ-free rats had lower mucus production compared to conventional-microbiota rats (Szentkuti et al., 1990). Further, exposure epithelium of germ-free animals to bacteria source antigen (i.e., lipopolysaccharide) lead to normal mucus production, indicating microbiota facilitated mucus secretion (Enss et al., 1992). Antimicrobial peptides (AMPs) secreted by Paneth cells and enterocytes can prevent the penetration of bacterium, virus, fungi and protozoa. Similarly, the presence of commensal microbiota plays a role in stimulating the production of AMPs (Kato et al., 2014). On the opposite, studies in germ-free animals showed that the production of AMPs did not rely on the presentence of bacteria (Putsep et al., 2000). The IgA secreted by plasma cells is the dominant immunoglobulin in mucosal immunity, and it acts by neutralizing toxins and pathogens with a high-affinity and the commensal microbiota with a relative low-affinity (Macpherson et al., 2008). In germ-free animals, the count of IgA-secreting cells was significantly low, The reduced IgA secretion in neonatal animals was because the commensal microbiota colonization was missing (Benveniste et al., 1971). It has been reported that impaired IgA secretion leads to bacterial expansion followed by local and systemic inflammation (Cunningham-Rundles, 2001). Overall, intestinal commensal microbiota plays a vital role in mucosal immunity. Hence stressors (i.e., heat stress) causing shifts of commensal microbiota will ultimately impact immune system.

Effects of heat stress on microbial ecosystem in calves

It has been observed that heat stress enhanced the relative abundance of lactate producing bacteria (e.g., Streptococcus and unclassified *Enterobacteriaceae*), given that those bacteria can

generate lactate with soluble carbohydrate as substrate, thereafter, an increased lactate concentration was founded in lactating cows (Zhao et al., 2019). In goats, heat stress did not change the alpha diversity but had effects at the phylum level. However, with the increase of THI, dominant phylum switched from Firmicutes to Bacteroidetes, and the content of probiotics in the Lachnospiraceae_ND3007_group decreased, while the abundance of pathogenic bacteria increased (i.e., s Erysipelotrichaceae_UCG-004 and Treponema_2, increased) (Zhong et al., 2019). In contrast, heat stress increased alpha diversity in ileum of broilers, indicating increased microbiota species richness. Specifically Clostridium XIVb, Streptophyta, Faecalibacterium, Rothia, Alistipes, Azospirillum, and Oscillibacter were enriched at genera level in heat-stressed broilers (Wang et al., 2018). However, there were limited data about how thermal stress influence the microbial community of dairy calves, thus more studies are warranted in this area.

STRATEGIES TO INTERVENE HEAT STRESS

Alter the microclimate

Implementing shade during hot and humid weather is a more accessible, efficient, and economical way to provide comfortable conditions for animals. Even though different shade types have specific advantages and deficiencies, and shade orientations varies depending on the climate, it has been confirmed that a total of 30%-50% heat load can be reduced from a well-designed shade (Bond and Kelly, 1955). Decreased RT (38.9 vs 39.4℃), respiratory rate (54 vs 82 breaths/min) have been observed in shaded cows compared to no shade cows (Romanponce et al., 1977, Collier et al., 1981). Heat dissipation can be achieved through conduction, convection, evaporation, and radiation. Increased airspeed and decreased CO_2 concentration has been reported inside hutches when rear side was evaluated, making calves to have a reduced RR (44 vs 58 breaths/min) (Moore et al., 2012). Another way to increase ventilation inside hutches is to install fans, which can improve the microclimate of hutches, but the limitation is that calves are

needed to be housed indoors (Hill et al., 2011). The orientation of hutches affected the heat load on calves through solar radiation, south-facing hutches in summer season has been reported to have an elevated RR (103 bpm vs 90 bpm) (Bakony et al., 2021).

Breeding species for heat-tolerance

The thermoregulation ability varies among different breeds. For instance, cutting across different breeds, zebu cattle have larger sweat glands, and therefore can have better thermotolerance due to enhanced heat loss. This characteristic of zebu cattle made them robust to be utilized in crossbreeding systems. Although crossbreeding is an efficient way to incorporate thermotolerance, energy metabolism is compromised followed by a reduction in milk yield (Finch, 1986). With the development of new breeding techniques, more precise breeding could be accomplished without compromise milk yield through gene selection. For instance, slick hair gene which corresponds to a short, sleek, and glossy hair coat has been reported as a potential gene used to improve thermoregulatory ability (Dikmen et al., 2008). Coat color related to heat load from solar radiation, affects the susceptibility of dairy cattle to thermal stress. Cows with white coat have been observed to have lower core body temperature and greater milk yield compared to black coated ones in Florida (Hansen, 1990). However, MC1-R gene has been observed to be the dominant expression of black pigment in domestic species (Klungland and Vage, 2003). Therefore, further investigation is warranted to raise heat tolerance breeds while achieving high milk yield demand.

Nutritional management

It has been reviewed that heat stress reduced DMI, compromising the nutrient requirements in lactating dairy cows (West, 1999, West, 2003). Increasing energy density by reducing forage portion and increasing concentrate content has been accepted worldwide to

alleviate heat stress in lactating dairy cows. In dairy calves, Ríus et al., (2022) reported that there were no difference in DMI between heat stressed calves and their thermoneutral counterparts when exposed to moderate heat stress. However, calves in hutches where the environment was hot and humid showed a decreased starter intake (Hill et al., 2011).

The microbial ecosystem establishment starts after calves are born, accompanied by a decreased fraction of several probiotic bacteria (i.e., *Lactobacillus* and *Bifidobacterium*). However, diet changes also affect the gut microbiota, all together enhancing the susceptibility of young calves to pathogen colonization.

DEXAMETHASONE

Feature of Dexamethasone

Endogenous glucocorticoids are secreted by adrenal gland, regulated by hypothalamic-pituitary-adrenal axis (HPA) and coordinate several physiological processes. Furthermore, neutral glucocorticoids can bound to corticosteroid-binding globulins which can be converted to inactive form of cortisone by 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2). However, synthetic glucocorticoids (i.e., prednisolone, dexamethasone and budesonide) are not susceptible to 11β-HSD2 or corticosteroid-binding globulins resulting a more stable level (Weinstein, 2012). Dexamethasone is widely used as a potent immunosuppressive drug alternating gene expression and the immune response (Cain and Cidlowski, 2017). Upon dexamethasone penetration through cell membrane, dexamethasone binds to glucocorticoid receptor (GR) in cytoplasm, forming a complex. This complex has the ability to enter into the nucleus, where it can affects genomic transcription via binding to response elements and by interacting with other transcription factors (Cain and Cidlowski, 2017).

Dexamethasone regulates inflammation

Dexamethasone regulates inflammatory responses through different mechanisms. Dendritic cells (DCs) are antigen-present cells (APs) stimulating T helper cells (Th) via Major Histocompatibility Complex (MHC) class I and MHC class II. It has been reported that dexamethasone decreased the number of DCs, inhibit DCs maturation via deregulating the expression of MHC class II, reduce the expression of pro-inflammatory cytokines, and upregulate anti-inflammatory cytokine expression (Shodell et al., 2003, Cao et al., 2013). Further, dexamethasone can attenuate the pattern recognition receptors (PRRs) signaling pathways (i.e., TLR signaling) through inhibiting activator protein 1 (AP-1) or nuclear factor-κB (NF-κB) transcriptional factors resulting in reduced pro-inflammatory cytokines (Gottlicher et al., 1998). One of the mechanisms of how mammals regulate the inflammatory response is by recruiting neutrophils and monocytes in blood vessels to the inflammation site. E-selectin and chemokines play a vital role in this feature; glucocorticoids have been reported to inhibit the transcription of *SELE* encoding E-selection (Atsuta et al., 1998), while, decreased production of chemokines were also reported.

Given that heat-stressed cattle showed sustained systemic inflammation (Kaufman et al., 2020 and 2021), we hypothesized that heat stress-associated alterations of intestinal barrier function will be accompanied by a downregulation in the expression of tight junction proteins, an increase in pro- and anti-inflammatory signals, and intestinal dysbiosis in dairy calves. We hypothesized that the administration of therapeutic dose of dexamethasone would cause an immediate reduction in the inflammatory tone and, subsequently, improvements in intestinal morphology, function, and microbial community composition in heat-stressed calves. The

reduction of inflammation would result in a partial restoration of productivity in dexamethasone-treated calves exposed to heat stress.

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APPENDIX

Table 1-1. Formulas used to calculate temperature-humidity index (THI)

Item	Formula	Reference
THI1	$THI1 = (1.8 \times T_{db}^{1} + 32) - [(0.55 - 0.0055 \times RH^{2}) \times (1.8 \times T_{db} - 26.8)]$	(NRC, 1971)
THI2	$THI2 = (0.55 \times T_{db} + 0.2 \times T_{dp}) \times 1.8 + 32 + 17.5$	(NRC, 1971)
THI3	$THI3 = (T_{db} + T_{wb}^{3}) \times 0.72 + 40.6$	(NRC, 1971)
THI4	$THI4 = T_{db} + 0.36 \times T_{dp} + 41.2$	(Bianca, 1962)
THI5	$THI5 = (0.35 \times T_{db} + 0.65 \times T_{wb}) \times 1.8 + 32$	(Bianca, 1962)
THI6	$THI6 = (0.15 \times T_{db} + 0.85 \times T_{wb}) \times 1.8 + 32$	(Bianca, 1962)
THI7	$THI7 = [0.4 \times (T_{db} + T_{wb})] \times 1.8 + 32 + 15$	(Thom, 1959)
THI8	$THI8 = (0.8 \times T_{db}) + [(RH/100) \times (T_{db} - 14.4)] + 46.4$	(Mader et al., 2006)

[1]T_{db} = dry bulb temperature [2]RH = relative humidity [3]T_{wb} = wet bulb temperature

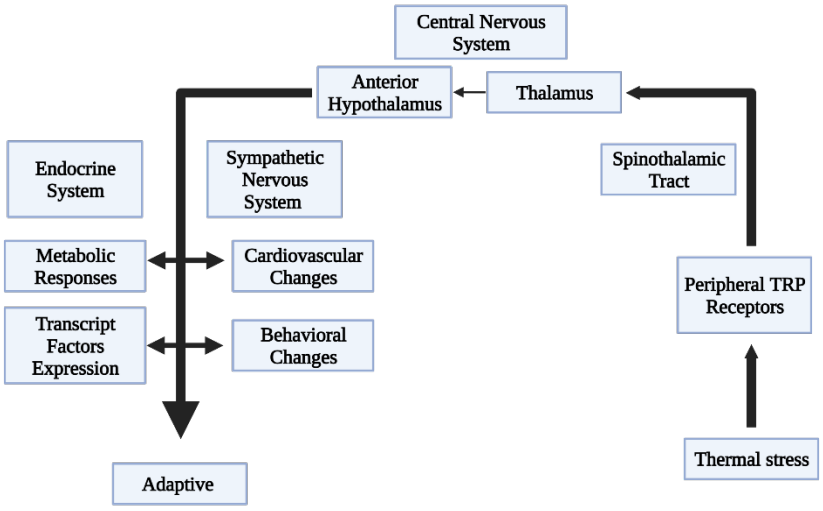

Figure 1-1 Metabolic responses to thermal stress. Abbreviation: TRP, transient receptor potential.

CHAPTER 2 HEAT STRESS-ASSOCIATED CHANGES IN THE INTESTINAL BARRIER, INFLAMMATORY SIGNALS, AND MICROBIOME COMMUNITIES IN DAIRY CALVES

A version of this chapter was submitted for publication in Journal of Dairy Science by: Z. Yu, J. M. Cantet, J. D. Kaufman, H. A. Paz, M.S. Orellano, I. R. Ipharraguerre, and A. G. Ríus.

Z.Y and J.M.C conducted the lab work. Z.Y analyzed the data and wrote the manuscript. J.D.K collected the samples and edited the manuscript. H.A.Z analyzed intestinal content samples and edited the manuscript. M.S.O and I.R.I edited the manuscript. A.G.R directed the project, acquired the funding, and edited and approved the manuscript as the corresponding author.

ABSTRACT

Recent livestock studies indicate that heat stress pathophysiology is associated with intestinal barrier dysfunction, local and systemic inflammation, and dysbiosis. Therefore, the objective of this study was to evaluate components of the intestinal barrier, pro- and anti-inflammatory signals, and microbiota community composition in Holstein bull calves exposed to heat stress. Animals (mean age = 12-week-old, mean body weight = 122 kg) penned individually in temperature-controlled rooms were assigned to 1) thermoneutral conditions (constant room temperature at 19.5 ℃) and restricted offer of feed (TNR, n = 8), or, 2) heat stress conditions (cycles of room temperatures ranging from 20 to 37.8 ℃) along with ad libitum offer of feed (HS, n = 8) for 7 days. Upon treatment completion, sections of the jejunum, ileum, and colon were collected and snap-frozen immediately to evaluate gene and protein expression, cytokine concentrations, and myeloperoxidase (MPO) activity. Digesta aliquots of the ileum, colon, and rectum were collected to assess bacterial communities. Plasma was harvested on days 2, 5, and 7 to determine cytokine concentrations. Overall, results showed a section-specific impact of HS on intestinal integrity. Jejunal mRNA expression of TJP1 was decreased by 70 % in HS calves. In agreement, jejunal expression of heat shock transcription factor-1 (HSF-1), a known tight junction protein expression regulator, decreased in HS calves. Jejunal analyses showed that HS

decreased concentrations of interleukin-1 alpha by 36.6 % and tended to decrease the concentration of interleukin-17A. Conversely, HS elicited a 3.5-fold increase in jejunal concentration of anti-inflammatory interleukin-36 receptor antagonist. Plasma analysis of pro-inflammatory cytokines showed that HS reduced concentrations of interleukin-6. Heat stress alteration of the large intestine bacterial communities was characterized by increased genus Butyrivibrio_3 and changes in bacteria metabolism of energy and amino acids. A strong positive correlation between the rectal temperature and pro-inflammatory Eggerthii spp. was detected in HS calves. In conclusion, this work indicates that HS impairs the jejunal barrier function but in a segment-specific fashion. The pro- and anti-inflammatory signal changes may be part of a broader response to restore intestinal homeostasis in jejunum. The changes in large intestine bacterial communities favoring butyrate-producing organisms may be part of a successful response to maintain the integrity of the colonic mucosa of HS calves. The alteration of intestinal homeostasis should be the target for heat stress therapies to restore biological functions, and, thus highlights the relevance of this work.

Keywords: heat stress, tight junction, inflammation, microbiome

INTRODUCTION

Livestock producers lose about two billion dollars to heat stress each year including $900 million losses in the dairy industry. Reduced feed efficiency is a major factor associated with economic loss as well as reduced dry matter intake (DMI), milk production and body weight gain, and increased deaths and reproductive culls (St-Pierre et al., 2003). Cattle are particularly prone to heat stress due to their limited capability for heat dissipation. To increase heat dissipation through the skin, heat-stressed cattle redistribute blood flow from splanchnic to peripheral tissues, decreasing oxygen and nutrients reaching the gastrointestinal tract. Subsequently, the reduced blood flow limits the supply of oxygen and nutrients, blunting blunts

intestinal mucosa development, increasing increases epithelial stress, and altering alters intestinal barrier function (Hall et al., 2001). The impairment of the intestinal barrier integrity leads to bacterial infiltration and penetration of antigens causing the activation of the innate immune system. Consequently, heat stress leads to local and systemic activation of inflammatory pathways shifting nutrient availability and utilization away from anabolic functions such as skeletal muscle synthesis in growing calves and milk synthesis in lactating cows (Hall et al., 2001, Koch et al., 2019, Fontoura et al., 2022, Ríus et al., 2022).

Direct and indirect evidence of impaired intestinal barrier function and homeostasis has been reported in cattle and other species (Kaufman et al., 2020, Cantet et al., 2021, Kaufman et al., 2021a). Studies in rodents (Hall et al., 2001, Lambert et al., 2002) and lactating cows (Koch et al., 2019) indicate that impairment of intestinal barrier function is associated with dysfunctional tight junction proteins between adjacent epithelial cells as well as shifted immune responses to the altered local environment. The balance of pro- and anti-inflammatory cytokines produced by epithelial and immune cells modulate the host response against lumen antigens by altering tight junction protein expression and intestinal barrier function (Dokladny et al., 2008). Altered expression of tight junction proteins and inflammatory signals may be part of the deleterious consequences of heat stress in cattle. Therefore, the study of local mechanisms associated with altered homeostasis in cattle should enhance our understanding of heat stress pathophysiology.

Intestinal microorganisms have a symbiotic relationship with the host and the disruption of host-microbial homeostasis, i.e., dysbiosis, has been linked to intestinal barrier dysfunction. Hooper et al., (2001) showed that gut colonization with commensal Bacteroides thetaiotaomicron elicited the upregulation of seven genes linked to mucosal barrier function and the upregulation

of several genes involved in the cytoskeleton and extracellular matrix, suggesting the stimulation of the mucosal barrier integrity. In chickens, heat stress affected the composition of intestinal microbiota and reduced viable counts of beneficial Lactobacillus and Bifidobacterium, resulting in dysbiosis (Al-Fataftah and Abdelqader, 2014, Song et al., 2014, Kers et al., 2018). Studies conducted on sheep and heifers showed that heat stress negatively affected rumen microbiota composition (Tajima et al., 2007, Duffy et al., 2018). Although heat stress had little effect on the alpha diversity in rumen samples, alterations at the phylum and genus levels were observed in the rumen of goats (Zhong et al., 2019). Our group showed that feeding a postbiotic from Aspergillus oryzae to heat-stressed lactating cows and calves reduced the pro-inflammatory state and improved intestinal barrier function and metabolism of nutrients, suggesting such beneficial effects derived from the modulation of gut microbiota by the Aspergillus oryzae postbiotic (Kaufman et al., 2021b, Ríus et al., 2022). Heat stress alterations of intestinal microbial communities in cattle are not well described; however, this information may prove necessary to develop prophylactic interventions to maintain gut health in heat-stressed cattle.

A comprehensive study was performed to understand the effects of heat stress on intestinal homeostasis. In this regard, this work aimed to evaluate the components of the intestinal barrier and mediators of the inflammatory response by measuring gene and protein expression and the composition of intestinal bacterial communities in dairy calves. We hypothesized that heat stress-associated alterations of intestinal barrier function will be accompanied by changes in the expression of tight junction proteins, pro- and anti-inflammatory signals, and alterations of bacterial communities. A better understanding of the events altering intestinal homeostasis would illuminate ways for developing strategies to combat the negative consequences of heat stress.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of the University of Tennessee (IACUC, protocol #2665-0219) following animal ethics approval and regulations.

Animal management and experimental design

A total of 16 Holstein bull calves (body weight = 122.2 ± 8.8 kg) were divided into two cohorts of 8 calves. Each calf was housed in an individual pen in a temperature-controlled room in the Johnson Research and Teaching Unit building (East Tennessee Research and Education Center, Knoxville, TN) for a total of 10 days. Animals were allowed to acclimate for 3 days and then randomly assigned to one of two treatments for 7 days: 1) thermoneutral conditions (constant room temperature at 19.5 ℃) and ~8 % restricted offer of starter (TNR, n = 8), and 2) heat stress conditions (10 hours of room temperature at 37.8 ℃ followed by 14 hours of room temperature at 20 ℃) and fed ad libitum amounts of starter (HS, n = 8) for 7 days. A commercial milk replacer was administered once daily, and calf starter was provided four times daily. On a dry matter basis, the chemical composition of the milk replacer contained 26% crude protein, 20% crude fat, 0.15% crude fiber, minerals, and vitamins. The calf starter contained 18% crude protein, 2% crude fat, 21% acid detergent fiber, minerals, and vitamins. The restriction of starter intake in the TNR treatment was designed to account for the effect of the expected dissimilar nutrient consumption between heat-stressed and thermoneutral animals (Koch et al., 2019; Fontoura et al., 2022). Additional details regarding study design, housing, management, and performance results were described previously (Ríus et al., 2022).

On days 2, 5, and 7 of the experiment, blood samples were collected from the jugular vein using sodium heparin tubes (BD and Co., Franklin Lakes, NJ). Blood samples were centrifuged at 1,200 × g for 12 min at 4 ℃ and plasma was harvested and stored at -80 ℃ until

further analysis. At the end of the treatment period, calves were euthanized by administration of pentobarbital, and tissue samples were collected and processed within 15 min of auscultated cardiac arrest. A 40-cm piece of whole jejunum was collected approximately 10 m distal from the descending duodenum. A piece of the whole ileum was collected approximately 1 m proximal from the ileum-cecum anastomosis. A piece of the whole colon was collected approximately 0.5 m proximal from the transverse colon. Samples of digesta content from the ileum, colon, and rectum segments were collected, snap-frozen, and stored at -80 °C until bacteria community analyses were conducted. Total digesta samples were collected based on prior heat stress work reporting intestinal injury and microbiome changes in pigs (Pearce et al., 2013b, Xiong et al., 2020) and poultry (He et al., 2019). Tissue samples of jejunum, ileum, and colon were collected, rinsed with 1× phosphate buffer saline, snap-frozen, and stored at -80 °C. Tissue samples of jejunum and ileum were chosen due to the importance of these segments in nutrient absorption. Additionally, jejunum and ileum have shown high sensitivity to hypoxia and heat stress in rodents, poultry, and swine research models. Colon samples were collected due to the importance of this segment in water absorption and the alteration of the mucosal barrier in heat-stressed mice (Hall et al., 2001, Lambert et al., 2002) and pigs (Pearce et al., 2013b, Pearce et al., 2014).

Isolation of RNA and performance of quantitative PCR

Isolation of jejunum, ileum, and colon mRNA was conducted according to the protocol provided by Direct-zol RNA MiniPrep kit (Zymo Research, Irvine; catalog No. R2050). The mRNA quality was evaluated using Nanodrop (Thermo Scientific, Waltham, MA) and agarose gel electrophoresis. Eleven target genes were evaluated using reverse transcription (Table A1; SuperScript First-Strand Synthesis System; Eppendorf, Louis, MO). The qPCR procedure

included 40 cycles, each cycle consisted of 4 periods (95 °C for 15 min, 95 °C for 10 sec, 55 °C for 30 sec, and 72 °C for 30 sec). Genes that had one melting curve and amplification efficiency between 90 %-110 % were used in this study (Hellemans and Barbara, 2010). Hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein S9 (RPS9), ribosomal protein L0 (RPL0), beta-actin (ACTB), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were tested as reference genes. Student's T-test analysis on delta CT results of references genes (Schmittgen and Livak, 2008) determined that RPS9, RPL0, and ACTB were deemed reference genes. The 2-delta CT method was used to analyze the results of target genes (Schmittgen and Livak, 2008).

Tissue protein analysis

The abundance of beta-actin, heat shock transcription factor-1 (HSF-1), and occludin in jejunum were determined using WES system technology following manufacturer recommendations (ProteinSimple, San Jose, CA, USA). Briefly, sample lysates described above were diluted with sample buffer, then mixed with fluorescent master mix and denatured under 95 ℃ for 5 min. Next, the following was added: 3 µl lysate, 10 µl antibody diluent, 10 µl primary antibody, 10 µl secondary antibody, and 15 µl luminol-peroxide to each well, and the plate was centrifuged at 2,500 rpm for 5 min. The electrophoresis run (30 min) at 475 V followed by blocking (5 min), primary and secondary antibody (30 min,) and luminol/peroxide chemiluminescence detection (15 min). Exposure signals, baseline, peaks, and quality control analysis were monitored to ensure adequate data quality. The area under the curve was calculated using commercial software (Compass for SW Version 3.1.7; Estoppey et al., 2021). Target protein results were normalized to beta-actin results presented as the ratio of each target protein to beta-actin * 1,000.

Myeloperoxidase analysis

The neutrophil infiltration marker, myeloperoxidase (MPO) activity, was determined to investigate its relationship with inflammatory pathways in the gastrointestinal tract. The MPO activity was measured based on the protocol established by (Suzuki et al., 1983) as modified by (Nieto-Veloza et al., 2019). About 20 ± 5 mg of tissue (jejunum, ileum, and colon) was mixed with 500 µl of hexadecyltrimethylammonium buffer (TCI, Montgomeryville, PA), in a 2 ml tube with 0.2 mg beads and homogenized with a tissue homogenizer for 4 min at 30 Hz and centrifuged for 6 min at 13,400 × g and 4 °C (Qiagen, Germantown, MD). The supernatant was collected and transferred to a 1.5 ml tube. The supernatant was mixed with 7 µl of homogenate and 200 µl of the o-dianisidine dihydrochloride solution (TCI, Montgomeryville, PA) then transferred to a 96-well clear plate and read every 60 sec for 10 min at 450 nm using a spectrophotometer (Biotek, Santa Clara, CA). Units of MPO in each sample were determined as the change in absorbance following the formula:

$$\text{MPO activity} = \frac{[\sum_{i=2}^{10} \Delta A(t_i - t_{i-1})/(w \times 1.13 \times 10^{-2})]}{9}$$

where ΔA(t2-t1) stands for the absorbance margins between two adjacent readings; w stands for tissue weight. Protein concentration was quantified using a commercial kit and spectrophotometry (Pierce 660 nm Protein Assay ThermoFisher, Waltham, MA; catalog No. 22662). Protein concentrations were used to normalize the results of MPO activity and reported.

Cytokine analysis

To assess the activation of inflammatory pathways in jejunum, concentrations of interferon-gamma (IFNg), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-17 A (IL-17A), interleukin-36 receptor antagonist (IL-

36RA), interferon gamma-induced protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 β (MIP-1β), tumor necrosis factor-alpha (TNFα), and vascular endothelial growth factor A (VEGF-A) were measured using a commercial kit (Milliplex, Burlington, catalog No. MA BCYT1-33K). Briefly, around 200 mg tissue samples were homogenized in a mixture of 2 ml lysis buffer and protease inhibitor buffer (the ratio of tissue weight to lysis buffer volume remained consistent for all the calves). Lysis buffer contained triton x-100 (0.2 %), glycerol (10 %), nonidet P-40 (NP-40; 1 %), 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (Hepes; 50 mM), ethylenediamine tetraacetic acid (EDTA;10 mM). The protease inhibitor buffer contained MSF, sodium fluoride, sodium vanadate, aprotinin, pepstatin, and leupeptin (Kuang et al., 2019). The mixture was centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant was collected and kept at −80 °C. Total protein concentrations were determined using Pierce 660 kit (Pierce 660 nm Protein Assay ThermoFisher, Waltham, MA; catalog No. 22662). Subsequently, cytokine concentrations were determined using xPONENT® software (Luminex® 200™, Luminex, Walpole, MA) following vendor's protocol.

Plasma interleukin-6 (IL-6) and IL-1β concentrations were detected using enzyme-linked immunoassay reagent kits (Invitrogen, Waltham, MA; catalog No. ESS0027 and ESS0029). Briefly, plasma was added to each well in a 96-well microplate (Corning, Tewksbury, MA, catalog No. 9018) incubated for 1 hour at room temperature, detection antibody was added, and incubated for another 1 hour at room temperature. Streptavidin-HRP reagent was added followed by a 30 min incubation at room temperature. Substrate solution was added and incubated in dark conditions for 20 min at room temperature followed by stop solution. Readings were conducted at 450 nm using a spectrophotometer (Biotek, Santa Clara, CA) following manufacturer recommendations.

DNA isolation and 16S rRNA amplicon library preparation and sequencing

Digesta contents were processed to determine bacterial communities. Previous research has shown that heat stress affects the microbiome community, in the ileum, colon, and feces causing gut dysbiosis in pigs and broilers (Zhu et al., 2019, Liu et al., 2020, Xia et al., 2022), hence we targeted the sequencing work in ileum, colon, and rectum contents. Genomic DNA extraction and quality control were performed as described by (De la Guardia-Hidrogo and Paz, 2021) and amplicon libraries of the V4 region from the 16S rRNA gene were prepared as described by (Paz et al., 2018). Following amplification, PCR products were analyzed on a 2% gel to verify the correct product size. Then, amplicons from each sample were normalized (1 to 2 ng/μL) using the SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA, USA). Normalized samples were pooled by plate and subsequently purified using the MinElute PCR Purification Kit (Qiagen, Valencia, CA, USA). The resulting libraries were quality controlled using the Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and quantified using the DeNovix QFX Fluorometer (Wilmington, DE, USA). Libraries were paired-end sequenced (2 × 250) using the Illumina Miseq System (Illumina, San Diego, CA, USA) according to the manufacturer protocol. Bioinformatics analyses were performed using QIIME 2 v2019.7 (Bolyen et al., 2019). Denoising was done with an initial quality filtering (Bokulich et al., 2013) using q2-quality-filter followed by the Deblur algorithm (Amir et al., 2017). Amplicon sequence variants (ASV) were aligned with MAFTT (Katoh et al., 2002) and then a phylogenic tree was generated with FastTree (Price et al., 2010). Samples were rarefied to an even depth (5168 quality-filtered reads) and rarefaction curves and Good's coverage index (Good, 1953) were used to evaluate sequencing depth. Alpha and beta diversity metrics were computed using q2-diversity. The principal coordinate analysis (PCoA) based on the weighted Unifrac distances

(Lozupone et al., 2011) was used to visualize clustering among samples. Representative sequences were assigned taxonomy using Naives Bayes classifier trained on the Greengenes 13 8 99% operational taxonomic units reference (McDonald et al., 2012). Heatmaps were generated in R v4.0.3 (RCoreTeam, 2020) using the heatmap.2 function from the gplots package (Warnes et al., 2020). Predictions of functional profiles were done using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) software via q2-picrust (Douglas et al., 2019). A phylogenetic tree was generated by The Molecular Evolutionary Genetics Analysis (MEGA) software.

Statistical analyses

Tissue and plasma results were analyzed to determine the normal distribution of residuals and homoscedasticity using Shapiro–Wilk's, and Levine's tests in SAS v. 9.4 (SAS Inst. Inc., Cary, NC). Results that did not meet the above criteria were log-transformed prior to conducting statistical analysis. Results were analyzed using mixed-effect models (SAS, version 9.4, SAS Institute Inc., Cary, NC) that included the overall mean, the fixed effect of treatment and day (i.e., plasma cytokines), the random effect of calf, and the random error. Least square means and SEM were reported, and effects were declared significant at $P \leq 0.05$, and trends were discussed for $P < 0.10$. Statistical analyses of microbiota results were performed in R v4.0.3 (RCoreTeam, 2020). Differences in alpha diversity metrics were evaluated using the Kruskall-Wallis test, while differences in beta diversity were evaluated using the permutational multivariate analysis of variance (Permanova, permutations = 999). Pairwise comparisons for functional profiles were done using the statistical analysis of taxonomic and functional profiles (STAMP) software v2.1.3 (Parks et al., 2014). The P-values were corrected for multiple testing using the false discovery

rate (FDR) method (Benjamini and Hochberg, 1995) and statistical significance was declared at adjusted $P \leq 0.05$.

RESULTS

As expected, treatments increased rectal temperature in HS calves and reduced starter intake in TNR calves (discussed in Ríus et al., 2022).

Epithelial barrier, cell stress response, and inflammation

To assess local changes in components of the intestinal barrier and inflammation, gene and protein expression were determined in the intestine of calves (Table 2-1). The HS treatment elicited a 2.4-fold decrease ($P < 0.05$) in mRNA abundance encoding the tight junction protein zonula occludens-1 (i.e., TJP1) in the jejunum but not in the ileum and colon (Table 2-1). Zonula occludens-1 is an essential structural protein as well as a gene expression regulator and a component of signal transduction pathways. In the jejunum, ileum, and colon, the mRNA abundance of other major tight junction proteins with functional and regulatory roles was not affected. Protein abundance analysis in jejunum showed that treatments did not change the expression of tight junction protein occludin ($P > 0.05$, Figure 2- 1).

Concerning markers of cellular stress, HS tended ($P = 0.098$) to increase the mRNA abundance of haptoglobin (HP) in jejunum but not in ileum and colon (Tabel 2-2). The expression of transcription factor and regulator of the stress response HSF-1 in the jejunum was reduced by HS ($P < 0.05$, Figure 2-1). Gene expression analyses of key inflammatory markers in the jejunum, ileum, and colon were not affected by treatments (Tabel 2-2).

Pro- and anti-inflammatory cytokine concentrations and MPO activity

The jejunal analysis of major pro-inflammatory cytokines showed that HS calves had a 36 % reduction in IL-1α concentration, compared with their TNR counterparts ($P < 0.05$, Tabel 2-3). In line with this result, HS tended to decline jejunal IL-17A concentrations ($P = 0.0734$).

Furthermore, HS resulted in a 1.5-fold decline in plasma IL-6 concentration ($P < 0.05$, Tabel 2-3). The analysis of anti-inflammatory cytokines showed that jejunal IL-36RA concentration increased 3.8-fold in HS animals ($P < 0.05$). The activity of neutrophil infiltration marker MPO was assessed in intestinal samples as a possible mediator in the pathogenesis of heat stress, but the MPO activity was not altered (Tabel 2-4, $P > 0.1$).

Microbiome analyses

To investigate changes in the intestinal microbiota and the possible role in the pathogenesis of heat stress, analyses of bacterial communities in the small and large intestines were performed. Statistics assessing alpha diversity indicated that community richness and evenness were less in the ileum than colon and rectum digesta whereas dominance was more in the ileum than colon and rectum digesta (Tabel 2-5). Taxonomic profiling indicated a diverse microbial community in the intestine of TNR and HS calves (Figures 2, 3, and 4). In the small intestine, Firmicutes was the predominant phylum with a small presence of Actinobacteria. In the large intestine, however, Firmicutes and Bacteroidetes shared similar proportions and constitute the main phyla (Figures 3 and 4). The results of the alpha diversity indexes showed that the homogeneity of samples in each group was high, and Shannon diversity was affected by the intestinal segment but was not affected by treatment (Tables 5 and 6). The permanova analysis showed differences in the bacterial communities between the ileum and large intestine ($P = 0.01$; Figure 2-A3). Ileum bacterial communities were not affected by treatment ($P = 0.79$). The treatments affected bacterial communities in the large intestine (colon and rectum, $P = 0.05$) and the LEfSe analysis identified twenty-two discriminative species between groups. Compared with TNR calves, the species enriched with an LDA score >2 were numerically lower in HS. Differentially abundant species in the colon and rectum microbiota of TNR calves were

dominated by the genus Butyrivibrio_1 while that in HS calves was dominated by the genus Butyrivibrio_3 (Figure 2-6). The phylogenetic tree identified that Butyrivibrio fibrisolvens is the closest reference strain with Butyrivibrio_3 (Figure 2-7). The functional profile of ileum and colon microbiome was assessed, and results suggested that functional genes associated with energy and amino acids metabolism and DNA synthesis were affected by treatment (Figures 8 and 9). Spearman correlation coefficients were calculated to reveal relationships between all bacteria abundance in the large intestine with rectal temperature and only significant correlations were presented (mean rectal temperature TNR = 38.8 ± 0.068; HS = 39.4 ± 0.331). Our data indicated that rectal temperature was significantly correlated with Eubacterium cylindroides in both groups (TNR: r = -0.61, P = 0.01; HS: r = -0.57, P = 0.03; Figure 2-10), and Eggerthii was correlated with rectal temperature in HS calves (HS: r = 0.64, P = 0.01; Figure 2-10)

DISCUSSION

Heat stress disrupts homeostasis triggering a response characterized by changes in physiology and metabolism that impair animal productivity. The current view that heat-stressed animals display intestinal and systemic inflammation has been found in rodents, swine, poultry, and bovine models (Lambert et al., 2002, Pearce et al., 2014, Koch et al., 2019, Cantet et al., 2021), but the alteration of homeostasis has been described poorly in heat-stressed dairy cattle. We suggest that this shortcoming needs to be addressed to understand and alleviate health and production concerns. By design, our heat stress protocol resulted in marked hyperthermia (Ríus et al., 2022). The TNR group accounted for the heat stress-depressed feed intake in the calves receiving an anti-inflammatory treatment (discussed in Ríus et al., 2022) because this finding was observed previously in ruminants and nonruminants (Koch et al., 2019; Fontoura et al., 2022). It is worth noting that the TNR calves resulted in a 6% drop in feed intake compared with the HS counterparts. To this point, reduced metabolic efficiency and elevated intestinal

permeability observed in HS but not in TNR calves suggested that the small difference in feed intake had negligible effects on nutrient metabolism (discussed in Ríus et al., 2022). Therefore, the results presented in the present study should be regarded as heat stress-mediated changes in pro- and anti-inflammatory pathways, intestinal integrity, and microbial community.

Our analysis of jejunum tissue showed that HS reduced TJP1 abundance. In comparison, jejunum TJP1 abundance increased in lactating dairy cows and growing pigs exposed to constant heat stress for 4 and 7 days (Pearce et al., 2013a, Koch et al., 2019). Meanwhile, we found no changes in ileum and colon mRNA abundance of tight junction proteins and these results are similar to those found in the ileum of pigs exposed to heat stress for 6 hours (Pearce et al., 2014). The segment-specific changes in the intestine may be associated with variations in mucus thickness. Compared with the duodenum, ileum, and large intestine, the jejunum has the thinnest total mucus and the mucus in the ileum and colon is 2-4 times thicker than that in jejunum and duodenum (Atuma et al., 2001). Gut mucus functions as a physical barrier that protects the intestinal mucosa from mechanical stress and the infiltration of pathogens (Johansson et al., 2013). A thin mucus layer in the jejunum of calves might be associated with increased susceptibility to stress. Furthermore, studies have reported that heat stress reduced the expression of jejunum MUC2 in broilers and mice (Goel et al., 2023, Lu et al., 2023). In addition, protein junction ZO-1 is the product of TJP1 expression and a regulator of paracellular permeability between adjacent enterocytes. Although ZO-1 was not measured in this study, jejunal downregulation of TJP1 suggests a disruption in tight junction and, subsequently, augmented paracellular permeability. Alternatively, increased intestinal permeability was associated with cellular redistribution of occludin and ZO-1 from membrane to cytosolic location in stressed rats (Mazzon et al., 2002) and might offer a possible explanation for our results. Together, the

alterations in tight junction appear to be limited to TJP1, segment- and species-specific, and dependent on heat stress insult characteristics. Tight junction changes associated with HS were paralleled with an increase in intestinal permeability (Ríus et al., 2022) and, thus, supports the notion of tight junction protein dysregulation and impairment of barrier function in HS calves.

In our study, we observed a reduction in the expression of the transcriptional regulator of the heat shock protein pathway HSF-1 in the jejunum of HS animals. In comparison, work conducted in pigs showed that heat stress did not affect jejunum HSF1 mRNA abundance (Pearce et al., 2014). Specific to thermal biology, heat shock factors are rapidly upregulated within minutes to a few hours after initial exposure to thermal insults (Takii et al., 2010). This response induces a transcriptional response of heat shock proteins (HSP) to cope with the deleterious effects of stress on the synthesis, folding, and translocation of proteins as well as to prevent the aggregation of misfolded proteins (Hartl, 1996). A key feature of HSF-1 activation is the transient nature of the transcriptional response, a consequence of both the intensity and the duration of exposure to the stress. Upon recovery from thermal stress, HSF-1 rapidly associates with HSP70 to decrease HSF-1 expression and transcriptional activity (Abravaya et al., 1992, Satyal et al., 1998). Together, in our study, the HSF-1 results appear to be linked to the downregulation of tight junctions and impairment of mucosal barrier function.

In our study, we observed heat stress-associated changes in tight junction proteins and inflammatory signal expression in jejunum mucosa, in line with the results in heat-stressed cows (Koch et al.,2019). Subsequently, we exclusively measured cytokine concentrations in jejunum samples. The pro-inflammatory cytokines IL-1α and IL-17A were reduced in the jejunum of HS animals, which suggests a modulation in inflammatory tone, transitioning from a pro-

inflammatory to an anti-inflammatory state, to enable intestinal healing and restoration of homeostasis.

In support of this view, mice deficient in IL-1α were protected from enteritis, and induced inflammation inflicted by the administration of dextran sodium sulfate (Bersudsky et al., 2014, Malik et al., 2016). Conversely, the activation of IL-1α signaling can lead to self-perpetuating inflammation (i.e., dysregulation of inflammation) unless a potent anti-inflammatory signal like IL-36RA suppresses the IL-1α signaling. Alternatively, heat-induced hypothermia has been observed in several species, including guinea pigs (Romanovsky et at., 1996), mice (Leon et al., 2006), and rats (Bouchama et al., 1991), and is important in homeostasis recovery after a heat stress insult. Chang (1993) reported that the ability to cool hyperthermic humans from 40 to 38°C was dependent on serum IL-1β levels, demonstrating a role for an endogenous cytokine in cooling. Therefore, a decline in jejunal IL-1α may be a needed signal to promote homeostasis of heat-stressed cattle.

In our study, the increase in jejunal IL-36RA concentration suggests the downregulation of pro-inflammatory pathways, and this is required for tissue regeneration and to restore homeostasis. This notion is supported by data in vivo and in vitro indicating that IL-36RA binds to the IL-36 receptor with higher affinity than IL-36 agonists resulting in the downregulation of inflammation (Gunther and Sundberg, 2014). In support of this view, IL-36RA gene knockout resulted in hypersensitivity to dextran sodium sulfate-induced enteritis (Yang et al., 2022). Interleukin 36 is a group of cytokines with pro-inflammatory effects. Intriguingly, IL-36 receptor ligands are overexpressed in animal colitis models and may play both pathogenic and protective roles, depending on the context. The decline in plasma IL-6 observed in HS calves may be part of a coordinated anti-inflammatory response to restore homeostasis. Together, it is possible that

an increase in jejunal IL-36RA concentration regulated the maintenance and restoration of the intestinal structure in heat-stressed calves.

Observed MPO results suggest that a neutrophil-derived response may not be related to the changes observed in the jejunum on day 7 of the study. In comparison, pigs exposed to heat stress for 24 hours displayed enhanced MPO activity in the ileum (Pearce et al., 2013b). Neutrophils elicit an early response and produce and release MPO to combat the translocation of antigens from the intestinal lumen to the gut mucosa and submucosa. Thus, the lack of changes in MPO activity in the current study suggests the negligible involvement of neutrophils in preventing the translocation of antigens and activating inflammation at the time of sample collection in our study.

Gut microorganisms influence the host's homeostasis and exert strong influences on physiology, regulating metabolism and immune function, as well as complex animal behaviors (Lynch and Hsiao, 2019). In our study, the general characterization of the core microbial communities shows similar intestinal microbial community composition relative to those found in the intestine of pigs (Xiong et al., 2020). Our analysis showed that treatment explained 44% of the variation in microbial communities in the large intestine. The microbiome analysis showed that Firmicutes were the dominant phylum in all three intestinal segments. Bacteroidetes were predominating in the colon and rectum but not in the ileum, and these results are in line with previous reports (Jami et al., 2013). In our study, the genus Butyrivibrio was affected by treatment. Butyrivibrio fibrisolvens (cluster XIVa) is the main butyrate-producing bacteria (Louis and Flint, 2009) and is one of the most common in the rumen (Merchen, 2002). Previous work showed that butyrate stimulated the production of tight junction proteins and mucosal epithelium in the large intestine (Peng et al., 2009). Butyrate-producing bacteria may be

responsible for the lack of molecular changes in the colon samples analyzed in our study. Also, butyrate promoted the differentiation and abundance of regulatory T cells and modulated the inflammatory response in intestinal segments in mice models of enteritis (Josefowicz et al., 2012, Arpaia et al., 2013). Although microbial metabolites were not measured in our study, the enrichment of a specific population of butyrate-producing bacteria (i.e. B3) may be related to an increase in the anti-inflammatory signal suggested by the augmented IL-36RA concentration in the jejunum, thus, abundance change of butyrate-producing bacteria could aid in restoring gut homeostasis in heat-stressed calves.

A correlation heatmap analysis was conducted to determine relationships between the relative abundance of large intestine bacteria and rectal temperature in TNR and HS calves. A negative correlation between the rectal temperature and the abundance of Eubacterium cylindroides was found. There is a strong correlation between the abundance of Eubacterium spp. and short chain fatty acid (SCFA) concentrations (Mukherjee et al., 2020), which is beneficial for the treatment of inflammatory bowel disease, metabolic syndrome, and colorectal cancer (Smith et al., 2013, Morrison and Preston, 2016, Venegas et al., 2019). This negative correlation may reflect a change in SCFA that leads to an inflammatory response during HS when RT changes. In HS calves only, there was a strong positive correlation between the rectal temperature and Eggerthii spp. While B. eggerthii has not been studied extensively, it was shown to be abundant in people with type 2 diabetes (Medina-Vera et al., 2019). Bacteroides eggerthii was identified as colitis-promoting species in mice with undisturbed intestinal microflora and in mice with antibiotic-depleted intestinal microflora (Dziarski et al., 2016). These results suggest that HS may have created an environment favoring B. eggerthii colonization in the large intestine, but the nature of this relationship remains unknown. Collectively, our results indicate

that heat stress plays a role in altering butyrate-producing bacteria, which might contribute to the inflammatory response in the intestine in calves.

CONCLUSION

Heat stress-associated alterations of intestinal barrier function appear to be segment specific and accompanied by changes in the expression of tight junction proteins, pro- and anti-inflammatory signals, and alterations of bacterial communities. The low abundance of TJP1 and HSF-1 in the jejunum may be associated with the impairment of the intestinal barrier during heat stress. Additionally, changes in pro- and anti-inflammatory cytokines may be part of a broader response to restore intestinal homeostasis during heat stress. The changes in colonic bacterial communities in favor of butyrate-producing bacteria in the HS group may be a contributing factor to maintaining the integrity of the intestinal mucosa, particularly in the large intestine. The alteration of intestinal homeostasis should be the target for heat stress therapies to restore biological functions, and, thus highlights the relevance of this work.

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APPENDIX

Table 2-1. Expression of tight junction genes in calves exposed to heat stress (HS) or thermoneutral feed-restricted (TNR) conditions (n=8/treatment).

	Genes	[1]Treatments		SEM	*P*-value
		TNR	HS		
Jejunum	[2]CDH1	0.136	0.123	0.0210	0.673
	[3]CLDN1	0.005	0.005	0.0006	0.763
	[4]CLDN2	0.025	0.024	0.0025	0.777
	[5]CTNNA1	0.055	0.166	0.0634	0.238
	[6]MARVELD2	0.002	0.002	0.0003	0.936
	[7]MLCK	0.064	0.046	0.0150	0.395
	[8]OCLN	0.010	0.007	0.0038	0.593
	[9]TJP1	0.055	0.016	0.0111	0.028
Ileum	CDH1	0.078	0.069	0.0304	0.837
	CLDN1	0.003	0.004	0.0014	0.664
	CLDN2	0.013	0.013	0.0037	0.970
	CTNNA1	0.032	0.034	0.0098	0.892
	MARVELD2	0.001	0.001	0.0005	0.886
	MLCK	0.016	0.024	0.0063	0.371
	OCLN	0.001	0.001	0.0005	0.690
	TJP1	0.015	0.006	0.0046	0.175
Colon	CDH1	0.342	0.312	0.0758	0.776
	CLDN1	0.008	0.008	0.0018	0.804
	CLDN2	0.022	0.030	0.0057	0.221
	CTNNA1	0.235	0.242	0.0533	0.917
	MARVELD2	0.006	0.006	0.0019	0.949
	MLCK	0.120	0.275	0.0838	0.197
	OCLN	0.012	0.015	0.0047	0.617
	TJP1	0.032	0.032	0.0056	0.937

[1]The number shown in the table was computed using 2-ΔCT method (Schmittgen and Livak, 2008), genomic mean of reference genes was used to calculate ΔCT. [2]CDH1: E-cadherin. [3]CLDN1: claudin-1. [4]CLDN2: claudin-2. [5]CTNNA1: catenin alpha-1. [6]MARVELD2: tricellulin. [7]MLCK: myosin light-chain kinase. [8]OCLN: occluding. [9]TJP1: zonula occludens-1.

Table 2-2. Expression of inflammation-related genes in the intestinal mucosa of calves exposed to heat stress (HS) or thermoneutral feed-restricted (TNR) conditions (n=8/treatment).

	Genes	[1]Treatments		SEM	*P*-value
		TNR	HS		
Jejunum	[2]HP	0.005	0.017	0.0046	0.098
	[3]HSP70	0.162	0.127	0.0282	0.383
	[4]INOS	0.001	0.001	0.0003	0.637
	[5]LCN2	0.001	0.001	0.0008	0.918
	[6]TLR4	0.001	0.002	0.0003	0.137
	[7]TNF-α	0.006	0.005	0.0007	0.427
Ileum	HP	0.099	0.089	0.0263	0.791
	HSP70	0.043	0.025	0.0154	0.394
	INOS	0.006	0.003	0.0024	0.373
	LCN2	0.010	0.006	0.0021	0.139
	TLR4	0.002	0.002	0.0002	0.140
Colon	HP	0.006	0.004	0.0014	0.361
	HSP70	0.101	0.092	0.0224	0.774
	TLR4	0.008	0.008	0.0016	0.928
	TNF-α	0.001	0.003	0.0004	0.320

[1]Computed using 2-ΔCT method (Schmittgen and Livak, 2008), mean of reference genes was used to calculate ΔCT. [2]HP: haptoglobin. [3]HSP70: heat shock protein 70. [4]INOS: inducible nitric oxide synthase. [5]LCN2: lipocalin 2. [6]TLR4: toll-like receptor 4. [7]TNF-α: tumor necrosis factor-alpha.

Table 2-3. Cytokine concentrations (pg/mL) in jejunum and plasma of calves exposed to heat-stress (HS) or thermoneutral feed-restricted (TNR) conditions (n=8/treatment).

	Treatments		SEM	*P*-value
	TNR	HS		
Jejunum				
IFNg	7.61	6.73	3.375	0.855
IL-1α	49.9	36.5	3.516	0.015
IL-1β	504	601	49.95	0.182
IL-8	91.3	68.4	18.490	0.395
IL-10	17.3	17.9	2.853	0.882
IL-17A	0.361	0.304	0.0219	0.073
[1]IL-36RA	386	1483	125	0.046
IP-10	694	374	176.1	0.220
MCP-1	356	356	56.75	0.999
MIP-1β	429	300	57.94	0.138
TNFα	4.81	4.57	0.213	0.435
VEGF-A	70.8	70.5	7.620	0.973
Plasma				
[2]IL-1β	7.80	8.20	1.80	0.870
[3]IL-6	26.5	17.6	2.69	0.024

[1]*P*-value was reported for the logarithmic transformation of data (TNR=4.77, HS=6.53 ± 0.585).
[2]*P*-value was reported for the logarithmic transformation of data (TNR=7.8, HS=8.2 ± 1.80).
[3]*P*-value was reported for the logarithmic transformation of data (TNR=26.5, HS=17.6 ± 2.69).

Table 2-4. Myeloperoxidase activity (MPO, U/mg protein[1]) in intestinal mucosa of calves exposed to heat stress (HS) or thermoneutral feed-restricted (TNR) conditions (n=8/treatment).

	Treatments		SEM	*P*-value
	TNR	HS		
Jejunum	67.2	85.3	9.54	0.200
Ileum	107.0	90.6	15.29	0.460
Colon	52.5	34.1	10.54	0.235

[1]One unit of MPO activity was expressed as the amount of MPO needed to degrade 1 mmol of hydrogen peroxide/min/mL.

Table 2-5. Alpha diversity analysis of the bacterial communities in ileum, colon, and rectum digesta of dairy calves

	Site				
	Ileum	Colon	Rectum	SEM	*P*-value
Observed ASV[1]	95.4[b]	430.4[a]	440.1[a]	14.9	<0.01
Shannon	4.01[b]	6.88[a]	7.00[a]	0.13	<0.01
Evenness	0.61[b]	0.79[a]	0.80[a]	0.02	<0.01
Dominance	0.13[a]	0.02[b]	0.02[b]	0.02	<0.01

[ab]Values within the same row with different superscripts indicate significant differences ($P < 0.05$)

[1]ASV: Amplicon sequence variants.

Table 2-6. Alpha diversity analysis of the bacterial communities in dairy calves assigned to thermoneutral feed-restricted (TNR) or heat stress (HS), values shown in the table are the mean of three intestinal segments (ileum, colon, rectum)

	Treatments			
	TNR	HS	SEM	*P*-value
Observed ASV[1]	372.1	332.5	35.6	0.39
Shannon	6.37	5.99	0.32	0.82
Evenness	0.76	0.73	0.02	0.82
Dominance	0.04	0.06	0.01	0.60

[1]ASV: Amplicon sequence variants.

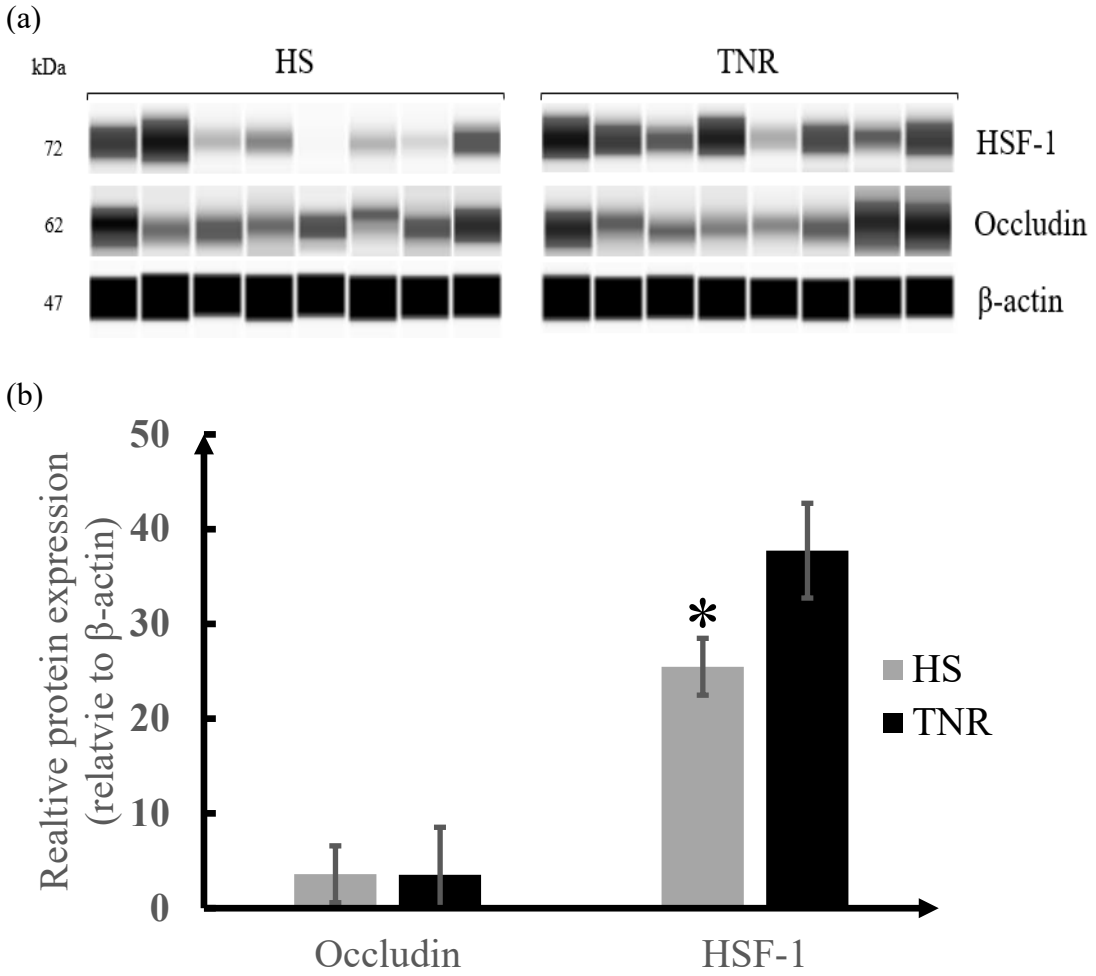


Figure 2-1. Expression of Occludin and HSF-1 in the jejunum of dairy calves (n=8/treatment). The upper panel (a) showed bands of HSF-1, Occludin, and β-actin. The lower panel (b) showed the relative expression of Occludin and HSF-1 (* denotes $P < 0.05$)

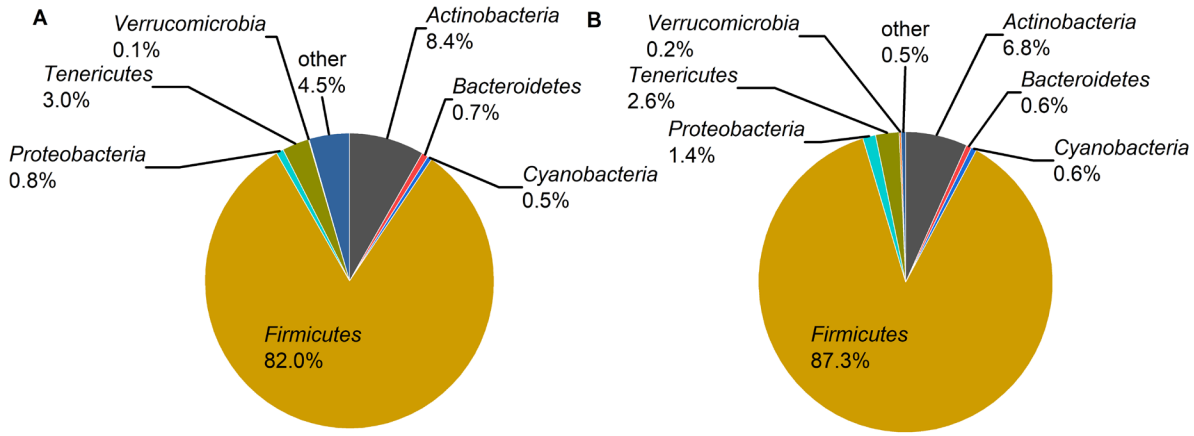


Figure 2-2. The taxonomic profiles of the ileum microbiota at phylum level in thermoneutral feed-restricted (TNR, panel A) and heat stress (HS, panel B) dairy calves. The taxonomic composition was compared between two groups based on relative abundance (reads of a taxon/total reads in a sample).

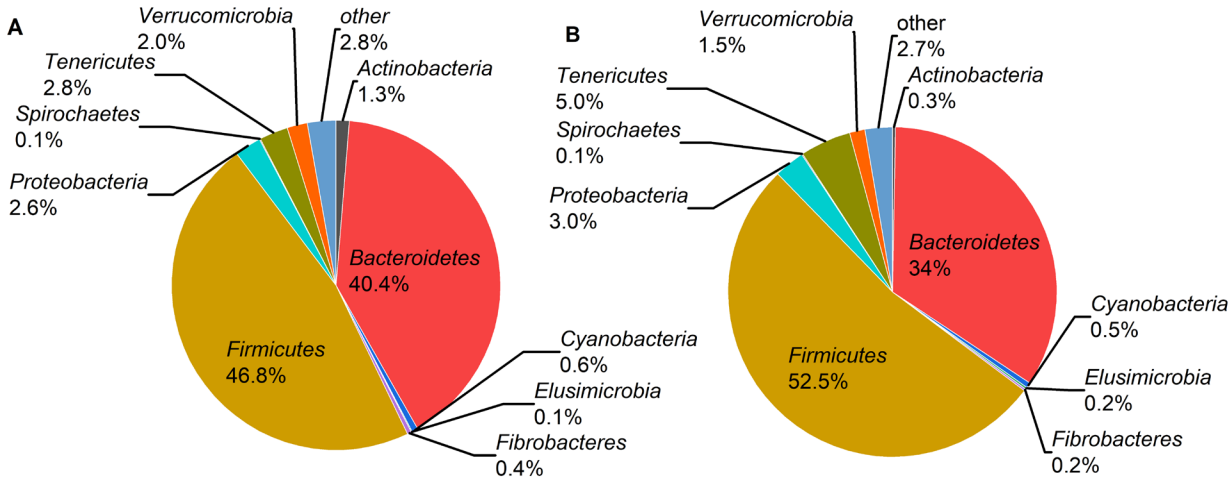


Figure 2-3. The taxonomic profiles of the colon microbiota at phylum level in thermoneutral feed-restricted (TNR, panel A) and heat stress (HS, panel B) dairy calves. The taxonomic composition was compared between two groups based on relative abundance (reads of a taxon/total reads in a sample).

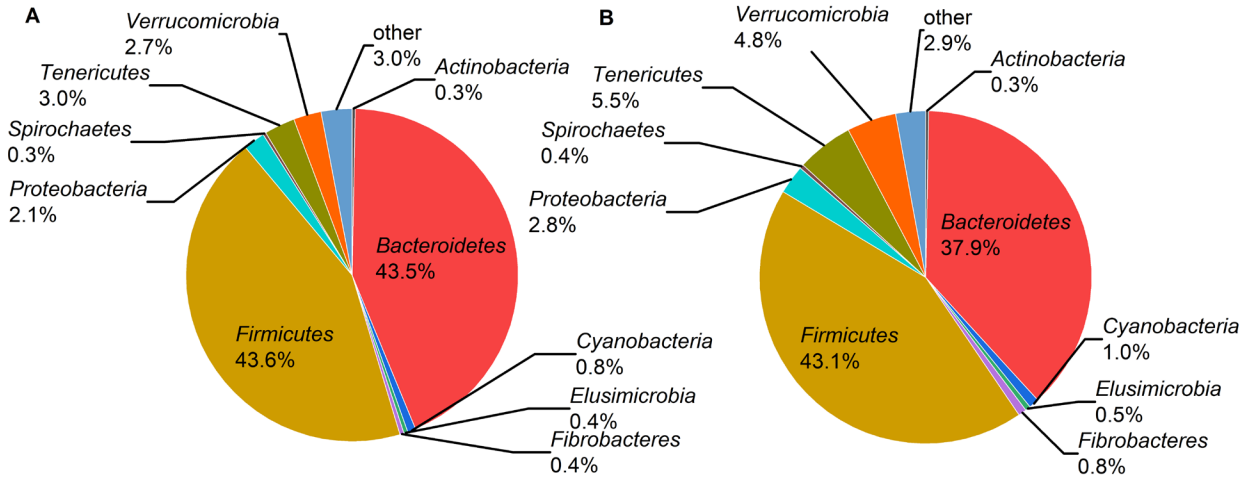


Figure 2-4. The taxonomic profiles of rectum microbiota at phylum level in thermoneutral feed-restricted (TNR, panel A) and heat stress (HS, panel B) dairy calves. The taxonomic composition was compared between two groups based on relative abundance (reads of a taxon/total reads in a sample).

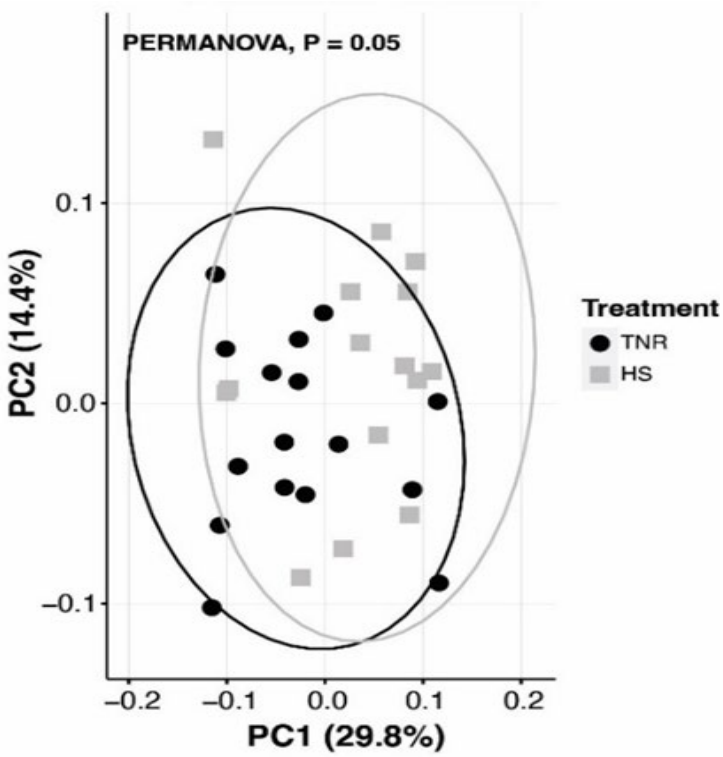


Figure 2-5. Beta diversity analysis of large intestine microbiome from thermoneutral feed-restricted (TNR) or heat stress (HS) dairy calves. Results were obtained using principal coordinate analysis (PCoA) based on the weighted Unifrac distances. Differences in diversity were evaluated using the permutational multivariate analysis of variance (PERMANOVA). Ellipses represent 95% confidence intervals.

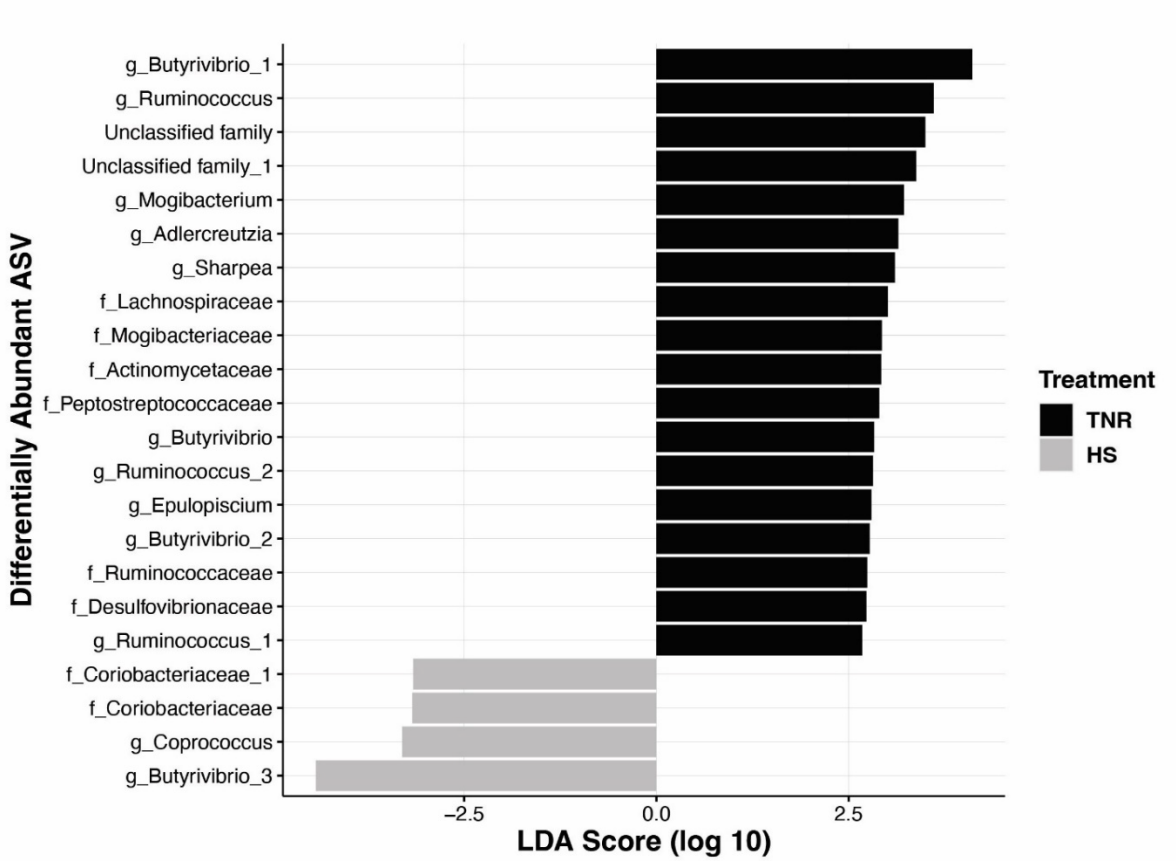


Figure 2-6. The LEfSe analysis identified the biomarker bacterial species in the large intestine of thermoneutral feed-restricted (TNR) and heat stress (HS) dairy calves. The linear discriminant analysis (LDA) scores represent the effect size of each abundant species. Species enriched in each group with an LDA score >2 are considered. Prefix f represents family, g represents genera; suffix 1,2,3 represent different strains.

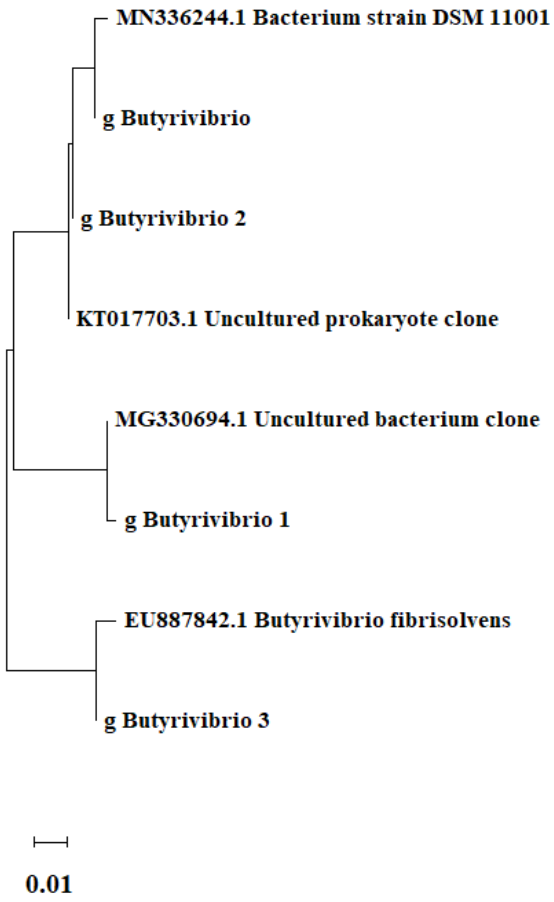


Figure 2-7.Maximum-likelihood tree showing specific *Butyrivibrio* detected by LEFSe's analysis of reference strains, obtained from Basic Local Alignment Search Tool (BLAST). GenBank accession numbers of the reference strains are shown together with the name of the strains. The scale bar represents the number of substitutions per sequence position.

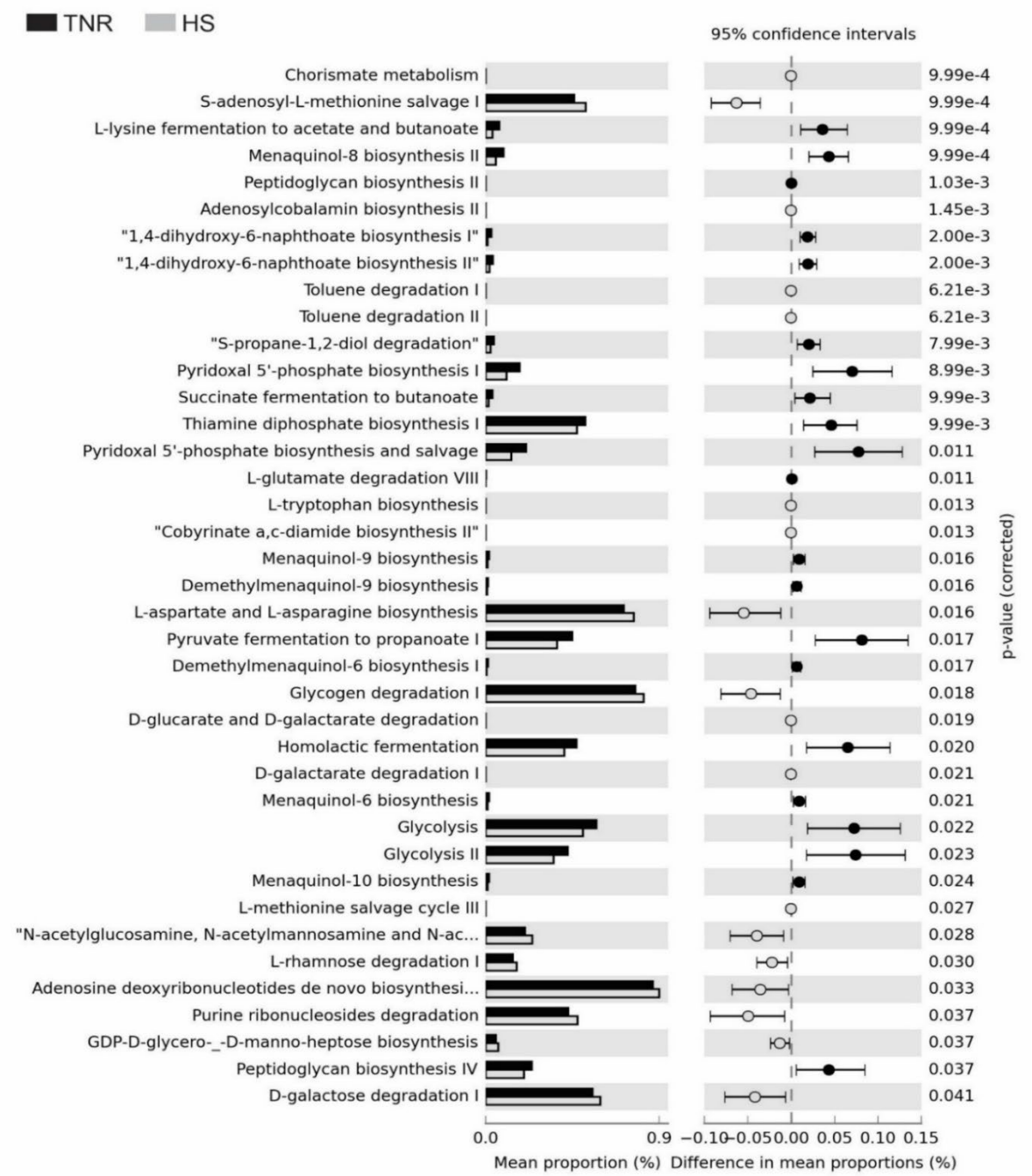


Figure 2-8. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) predictions of the functional profile of large intestine microbiome collected from calves exposed to thermoneutral feed restricted (TNR) and heat stress (HS). Data from PICRUSt were imported into STAMP software for statistical analysis and visualization. Bars represent predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology and associated proportions among samples. P- values < 0.05 are displayed.

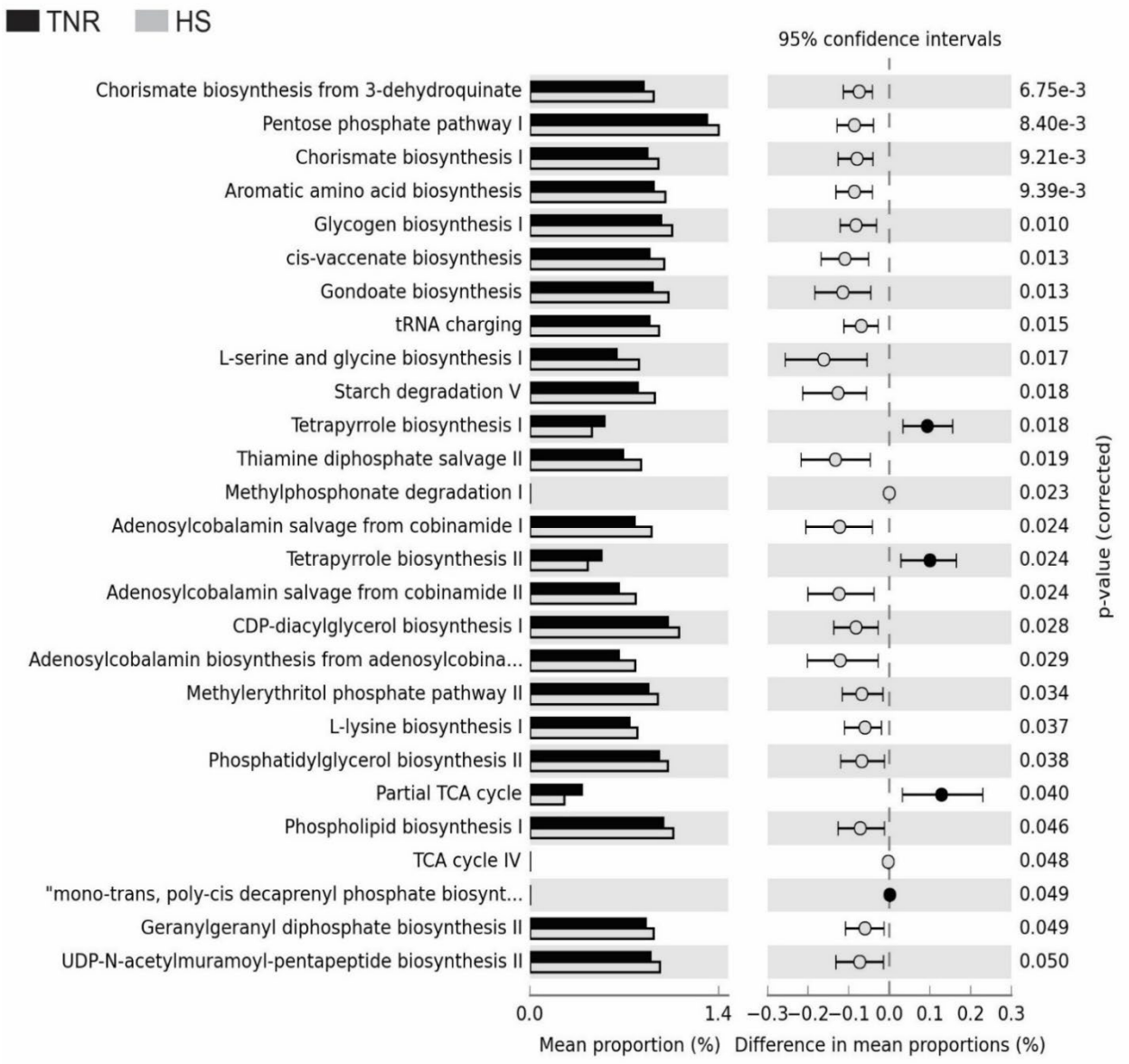


Figure 2-9. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) predictions of the functional profile of ileum microbes collected from thermoneutral feed-restricted (TNR) and heat stress (HS) calves. Data from PICRUSt were imported into STAMP software for statistical analysis and visualization. Bars represent predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology and associated proportions among samples. P-value < 0.05 was displayed.

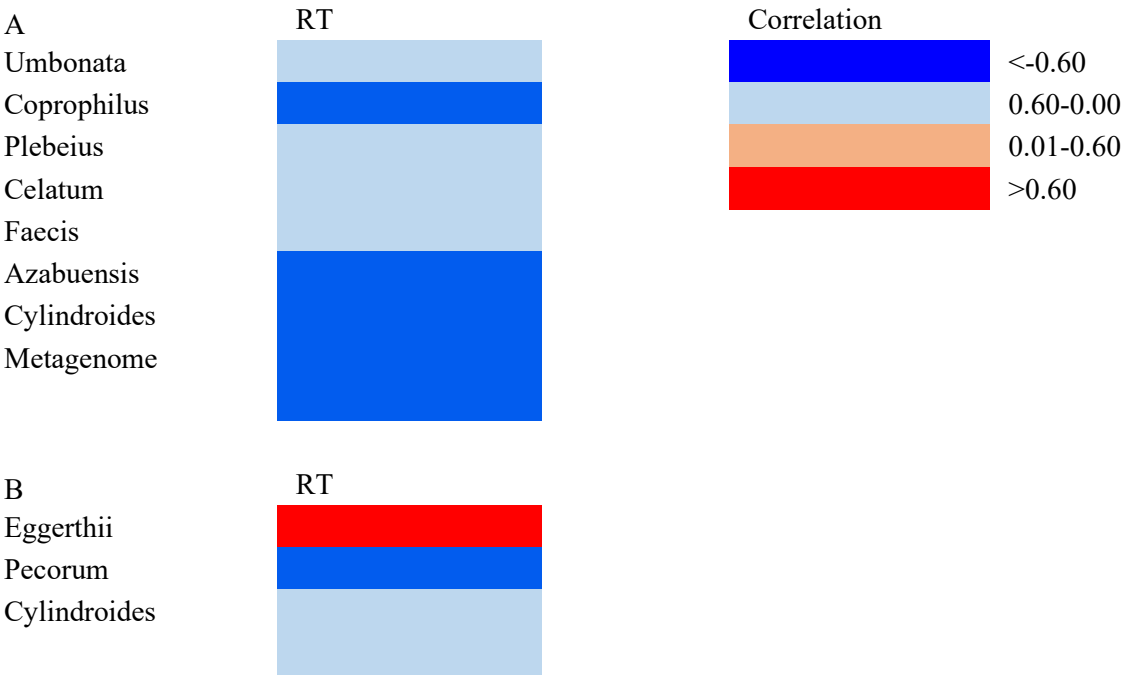


Figure 2-10. Heatmap of Spearman correlation between rectal temperature (RT) and specific bacteria species in the large intestine of calves exposed to thermoneutral (TNR, A) or heat stress (HS, B). All correlations listed in the heatmap are significant ($P < 0.05$).

SUPPLEMENTARY

Table 2-A1. Primer sequences used for mRNA expression analysis conducted in dairy calf tissues

Gene name	Gene symbol	Primer sequence (5'-3')	Origin
Claudin-1	*CLDN1*	Forward: TTCGACTCCTTGCTGAATCTG Reverse: GGCTATTAGTCCCAGCAGGATG	(Koch et al., 2019)
Claudin-2	*CLDN2*	Forward: CCAGGCCATGATGGTGACAT Reverse: GAAGAAGACTCCGCCCACAA	(Walker et al., 2015)
E-cadherin	*CDH1*	Forward: GATTGCAAGTTCCCGCCATC Reverse: ACATTGTCCCGGGTGTCATC	(Sakumoto et al., 2015)
Tricellulin	*MARVELD2*	Forward: CCGATCTGGGTTTCGGAGC Reverse: AGTGTCTGTCCCGATTCCTG	(Connor et al., 2017)
Lipocalin-2	*LCN2*	Forward: AAGGAAGACGGCAGCTACAA Reverse: ATATTGCCCAGGGTGAACTG	(Designed by our lab)
Haptoglobin	*HP*	Forward: TTGGTTCGCTATCAGTGCAA Reverse: ATGATCCTCTGCACCTGGTC	(Designed by our lab)
α-catenin	*CTNNA1*	Forward: TCTCAAGGAGGAGCTTGTGG Reverse: AGCAGGGATCATCTGCAAAC	(Designed by our lab)
TJP1	*TJP1*	Forward: GAAAGATGTTTATCGTCGCATCGT Reverse: ATTCCTTCTCATATTCAAAATGGGTTCTGA	(Koch et al., 2019)
Occludin	*OCLN*	Forward: CCTTTTGAAAGTCCACCTCCTTAT Reverse: TGTCATTGCTTGGTGTGTAGT	(Koch et al., 2019)
Myosin light chain kinase	*MLCK*	Forward: AGTACATCCACAAGCAGGGC Reverse: GGATGTAGCAGATGACCCCG	(Koch et al., 2019)
HSP70	*HSPA1A*	Forward: GGGGAGGACTTCGACAACAGG Reverse: CGGAACAGGTCGGAGCACAGC	(Block et al., 2008)
Beta-actin	*ACTB*	Forward: ATCACCATCGGCAATGAGC Reverse: TGATCCACATCTGCTGGAAG	(Kruger et al., 2005)
Ribosomal Protein S9	*RPS9*	Forward: CCTCGACCAAGAGCTGAAG Reverse: CCTCCAGACCTCACGTTTGTTC	(Ostrowska et al., 2014)
Ribosomal Protein L0	*RPL0*	Forward CAACCCTGAAGTGCTTGACAT Reverse AGGCAGATGGATCAGCCA	(Koch et al., 2019)
Hypoxanthine phosphoribosyltransferase 1	*HPRT1*	Forward: TACTGCTACTGTGTGCTTAGG Reverse: CTACTGAAACACTGGCGGGAC	(Koch et al., 2019)

Table 2-A2 Checking list for quality control and validation analysis of Real-Time PCR conducted in dairy calf tissues

Tissue	Genes	Efficiency (%)	Melt curve peaks	Replicates variation	Amplification graph
Jejunum	RPL0[1]	98.4	One	Acceptable	OK
	TJP1[2]	100.0	One	Acceptable	OK
	OCLN[3]	107.7	One	Acceptable	OK
	MLCK[4]	108.9	One	Acceptable	OK
	CTNNA1[5]	98.6	One	Acceptable	OK
	HP[6]	106.5	One	Acceptable	OK
	CDH1[7]	93.6	One	Acceptable	OK
	CLDN1[8]	108.4	One	Acceptable	OK
	CLDN2[9]	100.4	One	Acceptable	OK
	MARVELD2[10]	110.1	One	Acceptable	OK
	TLR4[11]	94.5	One	Acceptable	OK
	HSP70[12]	110.4	One	Acceptable	OK
	INOS[13]	90.4	One	Acceptable	OK
	LCN2[14]	91.8	One	Acceptable	OK
	RPS9[15]	93.6	One	Acceptable	OK
	ACTB[16]	93.7	One	Acceptable	OK
Ileum	RPL0	117.9	One	Acceptable	OK
	TJP1	100.0	One	Acceptable	OK
	OCLN	97.3	One	Acceptable	OK
	MLCK	96.6	One	Acceptable	OK
	CTNNA1	104.9	One	Acceptable	OK
	HP	101.5	One	Acceptable	OK
	CDH1	102.0	One	Acceptable	OK
	CLDN1	101.3	One	Acceptable	OK
	CLDN2	100.9	One	Acceptable	OK
	MARVELD2	120.9	One	Acceptable	OK
	TLR4	90.0	One	Acceptable	OK
	HSP70	92.4	One	Acceptable	OK

Table 2-A2 continued

	INOS	119.6	One	Acceptable	OK
	LCN2	95.6	One	Acceptable	OK
	RPS9	94.7	One	Acceptable	OK
	ACTB	106.7	One	Acceptable	OK
	RPL0	125.5	One	Acceptable	OK
	TJP1	102.1	One	Acceptable	OK
	OCLN	90.0	One	Acceptable	OK
	MLCK	108.1	One	Acceptable	OK
	CTNNA1	103.4	One	Acceptable	OK
	HP	99.8	One	Acceptable	OK
	CDH1	98.4	One	Acceptable	OK
Colon	CLDN1	110.8	One	Acceptable	OK
	CLDN2	104.1	One	Acceptable	OK
	MARVELD2	99.3	One	Acceptable	OK
	TLR4	100.2	One	Acceptable	OK
	HSP70	91.8	One	Acceptable	OK
	TNF-α	96.2	One	Acceptable	OK
	RPS9	125.1	One	Acceptable	OK
	ACTB[16]	115.1	One	Acceptable	OK

[1] RPL0: ribosomal protein L0. [2]TJP1: zonula occludens-1. [3]OCLN: occludin. [4]MLCK: myosin light-chain kinase. [5]CTNNA1: catenin alpha-1. [6]HP: zonulin. [7]CDH1: E-cadherin. [8]CLDN1: claudin-1. [9]CLDN2: claudin-2. [10]MARVELD2: tricellulin. [11]TLR4: toll-like receptor 4. [12]HSP70: heat shock protein 70. [13]INOS: inducible nitric oxide synthase. [14]LCN2: lipocalin2. [15]RPS9: ribosomal protein S9. [16]ACTB: beta-actin. [17]TNF-α: tumor necrosis factor-alpha.

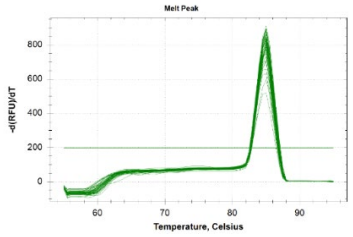
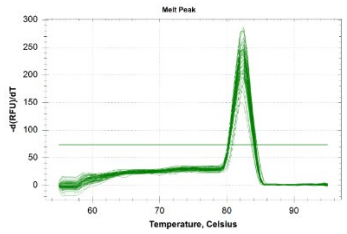
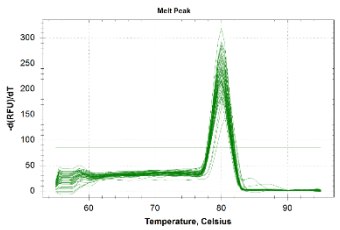
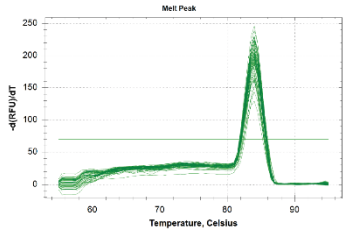
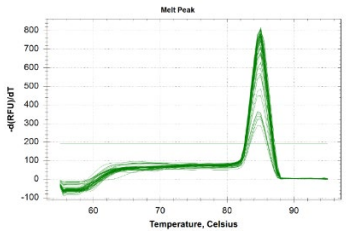
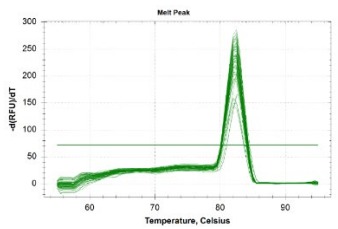
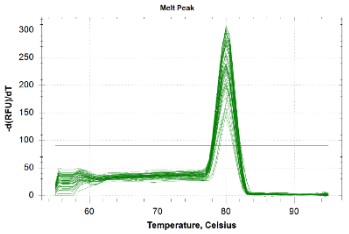
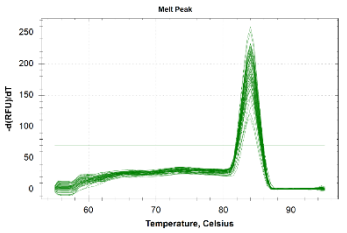
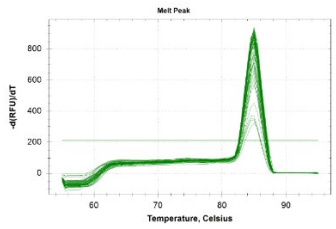
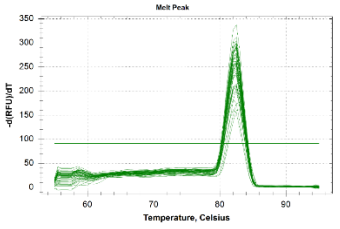
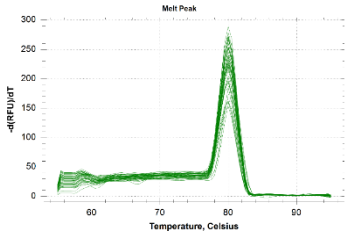
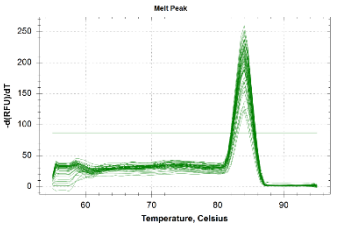
	RPL0[1]	CDH1[2]	TLR4[3]	CLDN2[4]
Jejunum				
Ileum				
Colon				

Figure 2-A1**.** Representation of melting curves of target genes listed in table A2 displaying quality control endpoints
[1] RPL0: ribosomal protein L0. [2] CDH1: E-cadherin. [3] TLR4: toll-like receptor 4. [4] CLDN2: claudin-2

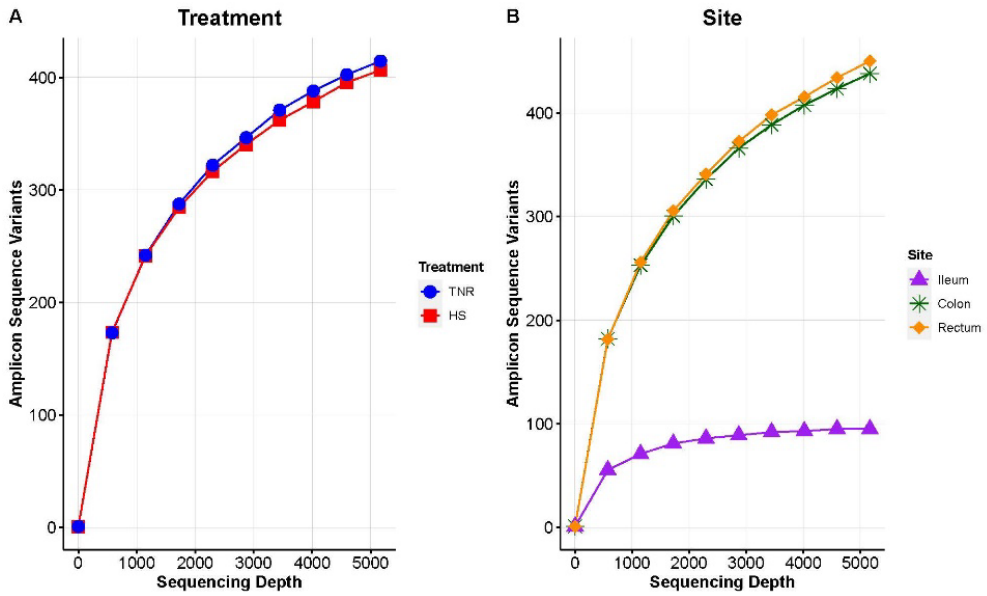


Figure 2-A2**.** Rarefaction curves displaying species richness at an even sampling depth. Curves among (A) treatments and (B) collection sites across the gastrointestinal tract. Samples were rarefied to 5168 quality-filtered reads. Each symbol represents the median value of 10 iterations (thermoneutral feed-restricted, TNR and heat stress, HS)

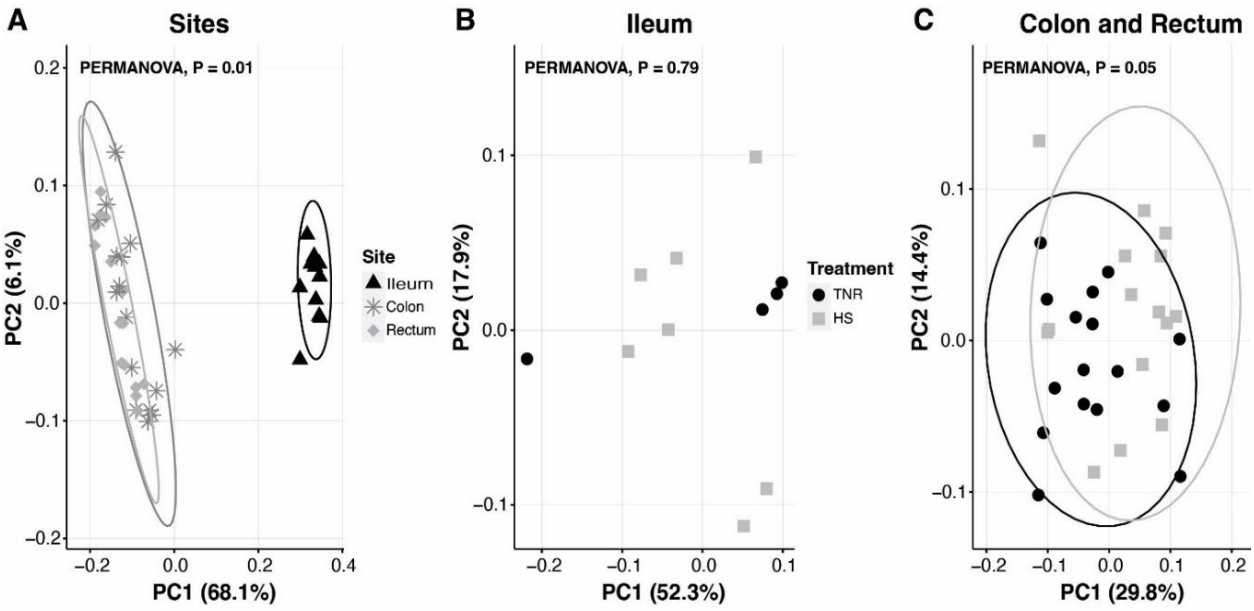


Figure 2-A3**.** Beta diversity analysis in digesta microbiome was conducted using principal coordinate analysis (PCoA) to visualize clustering among samples based on weighted Unifrac distances. Differences in diversity were evaluated using the permutational multivariate analysis of variance (PERMANOVA). Ellipses represent 95% confidence intervals. (A) PCoA plot using microbiome data from different intestinal segments. (B) PCoA plot of ileum microbiome from thermoneutral feed-restricted (TNR) or heat stress (HS) calves. (C) PCoA plot of large intestine microbiome from TNR and HS claves.

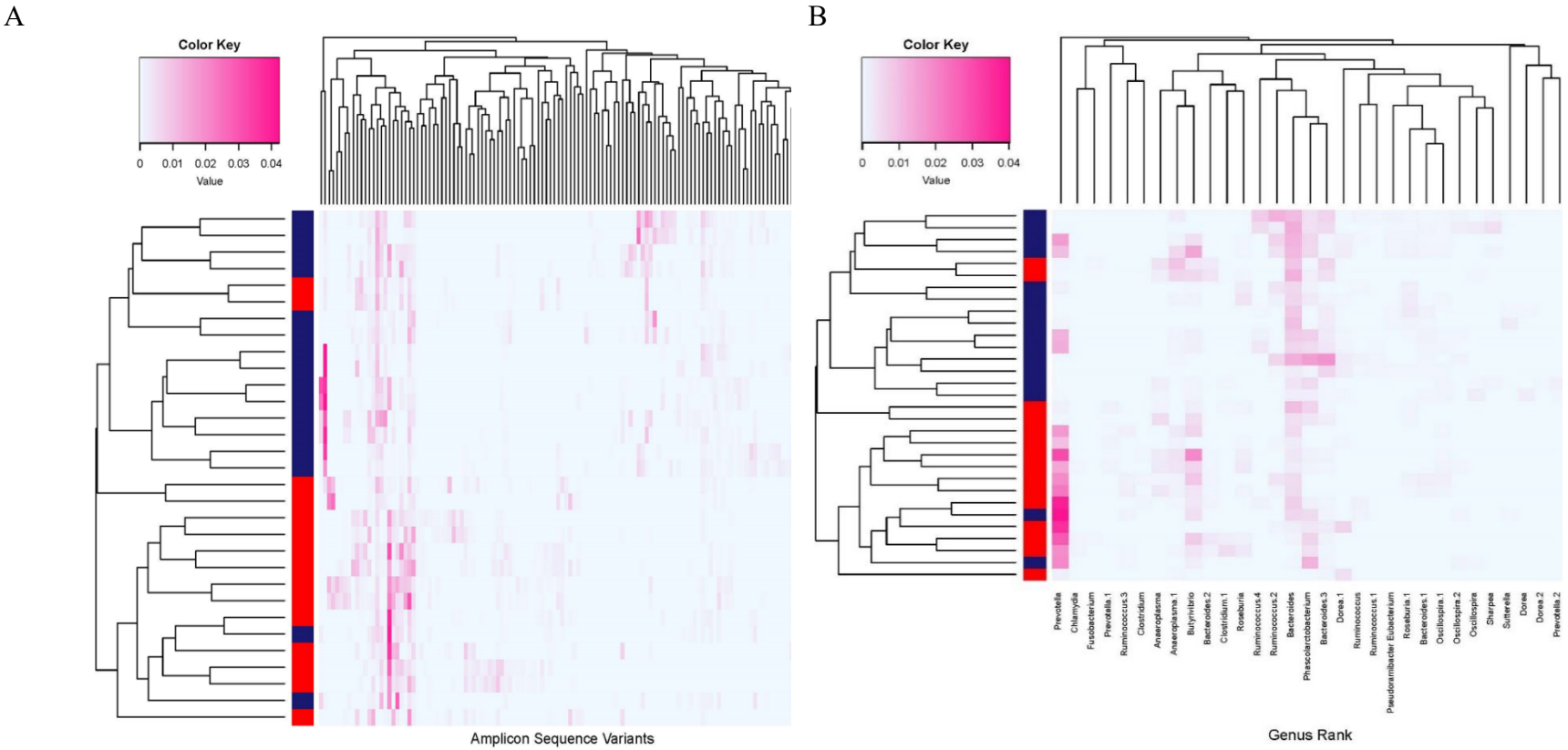


Figure 2-A4. Heatmap showing the distribution of large intestine abundant bacterial Amplicon Sequence Variant (ASV) in blue (thermoneutral feed-restricted, TNR) and red (heat stress, HS). The heatmap ranges from white (less occurrence) to pink (more occurrence), indicating if the different ASVs were more occurrent and abundant in the different samples. The names in the Y-axis on the right side of the figure correspond to the code given to each sample. (A) Heatmap of all differentially abundant bacterial ASV and (B) heatmap of differentially abundant ASV with a relative abundance > 0.5%. Clustering based on average linkage hierarchical clustering.

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CHAPTER 3 DEXAMETHASONE ALLEVIATED HEAT STRESS ASSOCIATED INFLAMMATION, INTESTINAL INJURY, AND IMPROVED PRODUCTIVITY IN DAIRY CALVES

This chapter is a version of a manuscript in preparation for journal submission by Z. Yu, J. M. Cantet, M. R. R. Nair, L. Cao, J. Lin, V. P. Dia, M. M. Fry, F. Cifuentes, A. B. Ferguson, and A. G. Ríus1

The following individuals conducted the work described in this study: study design and idea were from A.G.R. Animal study was organized and conducted by Z.Y, J.M.C, and M.R.R.N, sample analysis was performed by Z.Y, J.M.C and commercial labs. J.L funded sequencing and L.C assisted the lab work. V.P.D aided in cytokine measurement, M.M.F and A.B.F aided in hematology analysis, and F.C measured intestinal histology. Z.Y and A.G.R have conducted writing and editing of the manuscript.

ABSTRACT

Emerging evidence suggests that heat stress is associated with changes in immune response, nutrient metabolism, intestinal integrity and microbiome imbalance. We hypothesized that the administration of dexamethasone would reduce inflammation and subsequently improve nutrient metabolism, intestinal morphology, barrier function, and intestinal microbial community composition in dairy calves subjected to heat stress. Thirty-two Holstein bull calves (n=8/treatment) were housed in individual pens (68.5 ± 1.37 kg; Mean ± SEM) based on body weight, starter intake, and average daily gain (ADG) collected during two weeks pretreatment period. Calves were allowed to adapt to facilities for five days before treatments started and then were randomly assigned to one of four treatments for five days: 1) thermoneutral and administration of saline (~ 20°C ambient temperature, 24 h/d); 2) thermoneutral and administration of dexamethasone (~ 20°C ambient temperature, 24 h/d); 3) heat stress and administration of saline solution (~40°C ambient temperature, from 0800 to 1900 /d); 4) heat stress and administration of dexamethasone (~40°C ambient temperature, from 0800 to 1900 /d). Dexamethasone and saline solution were intramuscularly injected one hour prior to heat stress

on-site with a dosage of 0.05 mg/kg BW. Mixed models (SAS, version 9.4, SAS Institute Inc., Cary, NC) were used to analyze the results. Sequence reads were processed and data were analyzed using R (version 4.0.3). An envir by treat interaction showed that dexamethasone increased rectal temperature in heat stressed calves. Feed intake did not change but an envir by treat interaction showed that dexamethasone increased ADG by 66% and feed efficiency tended to increase by 72% in heat stress calves only. An envir by treat by time interaction on day 1 showed that the plasma glucose concentration declined in thermoneutral but increased in heat stress calves treated with dexamethasone. An envir by treat by study day interaction showed that dexamethasone increased the post prandial plasma glucose concentration on day 2. An envir by treat interaction showed that dexamethasone decreased the plasma concentration of NEFA by 36% in heat stressed calves. An envir by treat by study day interaction showed that white blood cells and neutrophil concentrations tended to increase more in heat stress than in thermoneutral calves treated with dexamethasone on study day 1. An envir by treat by day interaction showed that blood lymphocyte concentration decreased in heat stress calves treated with dexamethasone on study day 1. An envir by treat interaction showed that jejunal crypt depth was reduced in heat stress calves treated with dexamethasone. An envir by treat interaction showed that jejunal IL-6 concentration was increased in thermoneutral but reduced in heat stressed calves by dexamethasone. Regarding intestinal microbiome, an envir by treat interaction indicated elevated abundance of *Bacteroides* and *Corynebacterium* in heat stress calves treated with dexamethasone. Overall, the administration of dexamethasone altered the immune response, improved productivity, sustained intestinal morphology and changed gut microbiome in heat-stressed calves.

INTRODUCTION

Heat stress has emerged as a significant challenge due to the escalating global temperatures (IPCC, 2018) and the promotion of intensive agriculture (Renaudeau et al., 2012). Dairy calves are susceptible to heat stress, especially in rear of hutches during summer but receive less attention from scientific and management standpoints (Roland et al., 2016). Additionally, the effects of stress that calves experience in their early life (e.g., fetus, preweaning) can have long-lasting effects on their entire lifetime (Dahl et al., 2016). Therefore, it is vital to focus on the impact of heat stress on preweaning calves.

Disagreement exists over heat stress effects on metabolism of nutrients. Previous studies have shown a decrease or a rise in blood glucose concentrations (O'Brien et al., 2010, Pearce et al., 2013, Opgenorth et al., 2021, Ríus et al., 2022). Increased plasma NEFA concentrations have been observed in pigs (Pearce et al., 2013), goats (Al-Dawood, 2017), and calves (Opgenorth et al., 2021, Ríus et al., 2022) exposed to heat stress relative to thermoneutral conditions. Additionally, heat stress appears to increase skeletal muscle catabolism in chickens and dairy cattle in some studies (Yunianto et al., 1997, Wheelock et al., 2010) but not in other studies (Srikandakumar and Johnson, 2004, Huang et al., 2018). Additional research to determine heat stress effects in metabolism is necessary to better understand changes in physiology and productivity.

The increased incidence of immune-mediated disorder in heat-stressed animals can be attributed to immune dysfunction (Cantet et al., 2021). Short-lived basophil counts increased in heat-stressed chickens compared to their thermoneutral counterparts (Maxwell et al. 1992, Altan et al., 2000); however, their role in protective immunity and the specific insults that elicit basophil responses are not fully understood. Heat stress reduced eosinophil and neutrophil counts (Elvinger et al., 1991, Park et al., 2021), and MPO expression (Park et al., 2021) in cattle. The

neutrophil phagocytic activity of dairy cattle was observed to be lower during the summer season in comparison to the winter season (Tejaswi et al., 2020). The immune response, as assessed by lymphocyte proliferation, was found to be compromised under conditions of heat stress. For instance, while comparing the spring season to the summer season, it was shown that blood lymphocytes obtained from cows during the summer had reduced synthesis of DNA (Lacetera et al., 2005). In an in vitro investigation, peripheral blood mononuclear cells obtained from cows exposed to heat stress resulted in a notable alteration of their proliferative response (Lacetera et al., 2006). In comparison, heat stress increased the expression of genes involved in immune response and immunity-related signaling pathways in white blood cells of Holstein bull calves (Srikanth et al. 2017). Together, heat stress effects on immune function are inconclusive, thus, warrant further studies.

Heat stress induced intestinal barrier disruption is associated with local production of pro- and anti-inflammatory cytokines in growing dairy bull calves (Yu et al., 2023). Increased inflammation tone can disrupt the intestinal barrier function, leading to an increased intestinal permeability, allowing increased intestinal permeation of luminal antigens (Al-Sadi et al., 2009). It has been reported that IL-1β increased intestinal permeability in Caco-2 cells (Al-Sadi and Ma, 2007). Similar results of increased permeability were observed in IL-6 treated Caco-2 cells (Tazuke et al., 2003). Hence, changes in the expression of cytokines triggered by heat stress is associated with intestinal integrity and warrant more investigation.

Gut dysbiosis has been linked to a variety of inflammatory conditions induced by different stressors (e.g., thermal stress) (Qiu et al., 2022). Healthy gut microbiome communities remain a diverse and complex ecosystem, whereas thermal stress has been reported to reduce microbial diversity, which may contribute to inflammatory bowel diseases (Park et al., 2022).

Inflammation caused by gut dysbiosis may be mediated by microbiome metabolites. Compounds such as trimethylamine N-oxide (TMAO) can contribute to chronic inflammation (Sun et al., 2016, Chen et al., 2017) whereas short chain fatty acids can inhibit inflammation (Tan et al., 2014, van der Beek et al., 2017). The decreased abundance of bacteria that produce SCFAs and the increased abundance of bacteria that produce TMAO may be associated with intestinal inflammation in animals exposed to heat stress. Inflammation of the intestinal mucosa induced by heat stress has been reported in numerous species; however, the impact of gut microbiome dysbiosis in calves remains elusive.

Dexamethasone has been used to reduce inflammation and prevent associated tissue damages by limiting the migration of immune cells, and suppressing the production and release of various inflammatory mediators, such as cytokines, chemokines, and prostaglandins (Burton et al., 1995, Yubero et al., 2012). In cattle, dexamethasone has been used to treat a variety of inflammatory and immune-mediated conditions, such as endotoxemia (Walz et al., 2008). Given that heat stress triggers immune dysfunction and inflammation, we hypothesized that administration of a therapeutic dose of dexamethasone could reduce inflammation and, subsequently, improve intestinal barrier function in heat-stressed calves. We further hypothesized that a reduction of the inflammatory response would restore productivity in dexamethasone-treated calves exposed to heat stress. To test the hypotheses, a study was designed with the following objectives: 1) to reveal the effects of heat stress on growth performance, nutrient metabolism, inflammation, intestinal integrity, and intestinal microbiota, 2) to determine the effect of alleviating inflammation by administering dexamethasone in heat stressed dairy calves.

MATERIALS AND METHODS

Animal housing and management

All procedures were approved by the University of Tennessee IACUC (protocol# 2851-0921). Thirty-two Holstein bull calves were registered in the study (68.5 ± 1.37 kg; Mean ± SEM) based on body weight (BW), starter intake, and average daily gain (ADG) collected during two weeks pretreatment period. Calves were raised individually at the East Tennessee AgResearch and Education Center (Little River Animal and Environmental Unit) for two weeks and then were transferred to the Johnson Research and Teaching Unit (East Tennessee Research and Education Center, Knoxville, TN). Water and calf starter were fed ad libitum and milk replacer was served twice daily at 0530 and 1600 throughout the study (0.34 kg milk replacer/feeding). During the study (Figure 3-1), calves were housed individually in the environmental chamber five days prior to the treatment for acclimatization to the facilities and then were randomly assigned to one of the four treatments as follows: 1) thermoneutral with administration of saline solution on day1 and 3 (TN, saline solution volume equal to dexamethasone, ~ 20°C ambient temperature, 24 h/d); 2) thermoneutral with administration of dexamethasone on day1 and 3 (TND, 0.05 mg/kg BW, ~ 20°C ambient temperature, 24 h/d); 3) heat stress with administration of or saline solution on day1 and 3 (HS, saline solution volume equal to dexamethasone, ~40°C ambient temperature, from 0800 to 1900 h/d); 4) heat stress with administration of dexamethasone on day1 and 3 (HSD, 0.05 mg/kg BW, ~40°C ambient temperature, from 0800 to 1900 h/d). Heater was turned on from 0800 to 1900, in order to induce a 12 h/d of heat stress from day 1 through day 5 (Figure 3-1). Dexamethasone and saline solution were administrated intramuscularly at 0700 with a dosage of 0.05 mg/kg BW. Pentobarbital was used to euthanize the calves on day 6, and tissue samples were collected and processed within 15 min of auscultated cardiac arrest.

Thermal load assessment

Rectal temperature (RT; GLA M700 digital thermometer; accuracy ± 0.1°C) and respiration rate (RR, breaths per minute) were measured three times (0630, 1400, 1800) per day throughout the study. Trained students were hired to access RR, utilizing the method of quantifying the movement of the flank area within a 15-second timeframe.

Performance measurements

The BWs (Tru-test XR-3000, Datamars, Lamone, Switzerland) were recorded on days 0, 2, 4, and 6. Water and calf starter (Table 3-1) were offered ad libitum and intake was recorded daily. Calves in HS and HSD groups were checked at least 3 times daily to ensure their water bucket had plenty of water. Fecal samples were collected daily from rectum and stored in the freezer until fecal water content analysis.

Plasma metabolites

Blood samples were collected twice (0530,1800) daily from jugular venipuncture with sodium heparin tubes (BD and Co., Franklin Lakes, NJ), while two extra samples (1200,1500) were also harvested on day 1. Blood samples were centrifuged at 1,200 × *g* for 10 minutes at 4 °C and plasma were stored at -80°C. Plasma glucose (Thermo Fisher Scientific, Waltham, MA; Catlog No. TR15421), plasma urea nitrogen (PUN; Invitrogen, Waltham, MA; Catlog No. EIABUN) and plasma non-esterified fatty acid (NEFA; FUJIFILM Medical Systems, Valhalla, NY) were measured according to the manufacturer protocol. Plasma metabolite concentrations were determined through spectrophotometry (Synergy H1 Multi-Mode Reader; BioTek, Winooski, VT).

Hematology

Blood samples were also collected on days 0, 1, 5 at 0530 with EDTA vacutainers (Thermo Fisher Scientific, Waltham, MA; Catlog No. 22-040-037), and complete blood counts (CBCs) were performed at The University of Tennessee Veterinary Medical Center Clinical Pathology Laboratory with an automated hematology analyzer (Siemens ADVIA 2120, siemens healthiness, Erlangen, Germany). Blood samples were stored on ice immediately after taken, and were analyzed within 2 to 24 hours.

Intestinal permeability

To collect 10-hour urine output and intestinal permeability on days -14,1,5, plastic pallet (dimensions: 121.92 × 101.6 × 15.24 cm; dynamic capacity: 997.9 kg) was raised up with garden edges (dimensions: 121.92×15.24×5.08 cm) inside of the pen. Four plastic trays (Winco, Boise, ID; size: 66.04 × 45.72 cm) were fitted in the gap between pallet and concrete ground, covering the entire area underneath pallet. Fiberglass mesh was stabbed on the top surface of pallet to prevent feces contaminating urine samples. Calves were reared on top of pallets, utilizing a halter to limit their mobility while ensuring undisturbed access to water, starting feed, and the ability to lay down. Lactulose (0.5g/kg BW; Thermo Fisher Scientific, Waltham, MA; LotNo. M25F028) D-mannitol (0.1g/kg BW; Sigma Aldrich, St. Louis, MO; Lot No.M4125) were provided with milk replacer on days -14, 1, and 5. Blood samples were collected 1 hour before marker feeding and 2 hours after marker supplement, plasma was harvested at 1,200 × g for 10 minutes at 4 °C and stored at -80 °C. Urine output was filtered, measured and recorded after 5 and 10 hours of marker supplement. Lactulose and mannitol were extracted using liquid/liquid extraction based on the previous protocol (Armirotti et al., 2014). After the extraction, eluate was transferred to a liquid chromatography-mass spectrometry (LC-MS) vial, 5 µl of sample was injected into

column (2.1 × 100 mm column) and results were processed through software (Analyst 1.7.0, SCIEX, Framingham, MA).

Morphological analysis of the intestine

Jejunum was rinsed with 1× phosphate-buffered saline and samples were collected, snap-frozen with liquid nitrogen and stored at -80℃ until analysis. Additional jejunum samples were fixed in a 4% formaldehyde solution to perform villus height, crypt depth and ratio as described previously (Zitnan et al., 2008). Briefly, jejunum samples were initially treated with a 4% neutral formaldehyde solution to preserve their structural integrity. Following this, the samples were then rinsed with water and subjected to a gradual dehydration process using a range of ethanol concentrations followed by cleaning with benzene and embedded in paraffin to facilitate further analysis. Subsequently, 10 slices of 5 μm thickness were taken from each sample and stained with hematoxylin and eosin for further analysis. To investigate the morphological properties of villus structures, 30 villi with corresponding crypts from the intestine were selected for examination. Advanced image analysis techniques (Leica Imaging Systems Ltd., Cambridge, UK) were employed to obtain accurate measurements of the size of these structures.

Cytokine concentration

Enzyme-linked immunoassay reagent kits (Invitrogen, Waltham, MA; catalog No. ESS0027 and ESS0029) were used to measure IL-6 and IL-1β levels in serum and jejunum lysate, respectively. Jejunum lysate was prepared in accordance with previously published methods (Yu et al., 2023). A 96-well microplate (Corning, Tewksbury, MA, catalog no. 9018) was used, and standard or samples were added to each well in triplicates, incubated at room temperature for 1 hour, followed by the addition of detection antibody (detection antibody: reagent diluent = 1:100) and kept for another hour of incubation. After adding the streptavidin-

HRP reagent, the plate was kept for a 30-minute incubation at room temperature. After adding the substrate solution and letting it incubate for 20 minutes at room temperature in the dark, the reaction was terminated with the stop solution. Spectrophotometer (Biotek, Santa Clara, CA) readings were taken at 450 nm as per the manufacturer instructions, cytokine concentrations were calculated based on standard curve.

16s rRNA sequencing

DNA isolation

Total digesta contents from rumen, jejunum and colon segments were collected, snap-frozen and stored at -80℃ until bacteria community analyses were conducted. The DNA from rumen, jejunum, and colon contents was extracted with a commercial kit (Fast DNA SPIN Kit for Soil, MP biomedicals, Santa Ana, California). Briefly, up to 500 mg of sample was added to a Lysing Matrix E tube, followed by the addition of 978 µL of Sodium Phosphate Buffer and 122 µL of MT Buffer. Subsequently, sample was homogenized in a FastPrepTM 24-5G tissue-cell homogenizer (MP Biomedicals, USA) for 40 seconds at a speed setting of 6.0 followed by centrifuging at 14,000 × g for 15 minutes. Supernatant was transferred to a clean 2.0 mL microcentrifuge tube, and Protein Precipitation Solution (PPS) was added to the mixture, which was mixed by inverting the tube 10 times. Then mixture was centrifuged again at 14,000 × g for 5 minutes. After that, the Binding Matrix suspension was resuspended and 1.0 mL was added to the supernatant in the 15 mL tube. The tube then was inverted by hand for 2 minutes to allow binding of DNA, and then placed on a rack for 3 minutes to allow settling of silica matrix. About 650 µL of supernatant was removed and discarded and Binding Matrix was gently resuspended in the remaining amount of supernatant. Approximately 650 µL of the mixture was transferred to a SPIN filter and centrifuged at 14,000 × g for 1 minute. Finally, SEWS-M (500

µl) was added to the pellet and gently resuspended using the force of the liquid from the pipet tip.The mixture was centrifuged once more at 14,000 × g for 1 minute, the purified DNA remained bound to the silica. The tube was centrifuged again and was dried at room temperature, then DNase-free water was used to resuspend matrix and DNA was stored at -80°C.

DNA purification

The DNA samples were purified with a commercial kit (Zymo Research, Irvine, CA; Catalog No. D4004). Briefly, DNA sample was mixed with DNA Binding Buffer and transferred to a Zymo-Spin™ V Column placed inside a Collection Tube. The mixture was centrifuged at ≥10,000 × g for 1 minute, and the flow-through was discarded. Then Zymo-Spin™ V Column was washed twice with 600 µl DNA Wash Buffer and centrifuged at maximum speed for 1 minute after each wash. Subsequently, the residual wash buffer was removed by centrifuging the Zymo-Spin™ V Column in a Collection Tube at maximum speed for 30 seconds. Then, the Zymo-Spin™ V Column was transferred to a new 1.5 ml microcentrifuge tube, and 150 µl DNA Elution Buffer was added directly to the column matrix. After waiting for one minute to ensure full hydration of the column matrix, the DNA was eluted by centrifugation at maximum speed for 1 minute. The DNA was stored at -80°C until use.

DNA amplification

The DNA amplification started with cocktail preparation with the ratio of Phusion flash Master Mix, Ultra pure water, Cap515f and 806r primer as 12.5:10:1:1. About 22 µl of cocktail was add to each tube after UV disinfection, followed by 2 µl of DNA sample and 1 µl of barcode primer. Subsequently, thermocycler was used with procedure as described previously (Cao et al., 2021). Post PCR, the quantity of the DNA products were determined using the NanoDrop ND-3300 fluoro spectrometer (Thermo Scientific, Waltham, MA, USA).

Pool sample quantification

The methodology employed in our research involved the pooling of nine to ten individual samples into a single sample, followed by KAPA (Kapa Biosystems, Wilmington, MA) quantification on each of the pools. The pool samples were subsequently diluted based on the quantification results to achieve a concentration of 10 nM. The final library was then created by pooling the samples according to the number of samples in each pool. The final library was subjected to KAPA quantification and ultimately diluted to a final concentration of 4 nM. Paired-end sequencing was performed on a MiSeq (Illumina, San Diego, CA, USA) using v3 reagents, generating 300 bp reads per end, according to manufacturer instructions.

Statistical analyses

SAS v. 9.4 (SAS Inst. Inc., Cary, NC) was used to evaluate phenotype, tissue and plasma results. Prior to statistical analysis, data were log-transformed if they did not pass the normal distribution of residuals and homoscedasticity criteria. Mixed models (SAS, version 9.4, SAS Institute Inc., Cary, NC) were used to analyze the data, which included the overall mean, the fixed effect of environment (heat stress or thermoneutral), treatment (dexamethasone or saline), and time (day or hour), the interactions among main effects, the random impact of calf, and the random error. Least square means and standard deviations were presented, and effects were judged significant at $P < 0.05$, with trends examined at $P < 0.10$. QIIME2 (version 2019.4) and R (version 4.0.3) were used to analyze the sequence reads. SAS software (version 9.4; SAS Inst. Inc., Cary, NC) was used to assess the differences in alpha diversity (Shannon, Faith's PD, Evenness, and Observed ASVs). Any *P* value less than 0.05 was regarded as statistically significant. Principal coordinate analysis (PCoA) tests were performed using the weighted and unweighted Unifrac distances (Lozupone et al., 2011). The Mann-Whitney test is used to

discover substantially different abundances in LEfSe analysis, while LDA scores are used to evaluate the effect size (threshold 2).

RESULTS

Dexamethasone did not reduce body temperature in heat stress calves

Heat stress increased RT, however, against our expectation, dexamethasone increased RT in heat stress but not in thermoneutral calves (Figure 3-3, Envir×Treat, $P < 0.05$). Heat stress increased RR (Envir, $P < 0.001$); however, dexamethasone did not affect RR in heat stress calves (Envir×Treat, $P > 0.10$).

Dexamethasone restored productivity in calves exposed to heat stress

Dry matter intake (DMI) was not affected by treatments (Table 3-2, $P > 0.10$). An environment by treatment interaction indicated that dexamethasone increased the ADG in heat stress calves but not in thermoneutral calves (Table 3-3, Envir×Treat, $P < 0.05$). There was a tendency towards an environment by treatment interaction for feed efficiency (FE, Envir×Treat, $P = 0.096$) with higher FE in the dexamethasone (40.5%) than in the saline heat stress calves (23.6%). Heat stress increased water intake and urine excretion (Table 3-2, Envir, $P < 0.01$). Dexamethasone increased water intake (Treat, $P < 0.05$), and decreased fecal water content (Treat, $P < 0.05$). Dexamethasone increased BW gain (Treat, $P < 0.01$).

Dexamethasone changed nutrient metabolism in heat stress calves

Dexamethasone increased postprandial plasma glucose concentration in heat stress but not in thermoneutral calves at noon post-morning feeding on day 1 (Figure 3-4 panel A, Table 3-A1, Envir×Treat×Time, $P < 0.01$). The plasma glucose concentrations determined at 0530 (preprandial) and 1800 (postprandial) on days 2 to 5 tended to change (Figure 3-4 panel B, Table 3-A2, Envir×Treat×Day×Time, $P = 0.078$). Dexamethasone increased postprandial plasma

glucose concentration in heat stress but not in thermoneutral calves on day 2 (Figure 3-4 panel D, Table 3-A4, Envir×Treat×Day, $P < 0.05$). Dexamethasone increased PUN (Table 3-4, Treat, $P < 0.01$). Dexamethasone decreased NEFA in heat stress but not in thermoneutral calves (Envir×Treat, $P < 0.05$).

Dexamethasone changed concentrations and percents of leukocytes in heat stress calves

There was tendency towards an increase in white blood cell (WBC) count caused by dexamethasone greater in heat stress than in thermoneutral calves on day 1 (Envir×Treat×Day, $P = 0.077$). The administration of dexamethasone increased WBC count (Treat, $P < 0.001$). Dexamethasone increased blood lymphocyte counts in thermoneutral but decreased lymphocyte counts in heat stress calves on day 1 (Envir×Treat×Day, $P < 0.05$). Dexamethasone inhibited the rise in blood monocyte counts in heat stress but not in thermoneutral calves on day 1 (Envir×Treat×Day, $P < 0.05$). There was a tendency indicating that the dexamethasone rise in blood neutrophils counts was more pronounced in heat stress than in thermoneutral calves on day 1 (Envir×Treat×Day, $P = 0.077$). Dexamethasone increased blood neutrophil counts (Treat, $P < 0.001$). Dexamethasone decrease of lymphocytes percent was more pronounced in heat stress than in thermoneutral calves on day 1, and the dexamethasone reduction in lymphocytes percent was also observed on day 5 (Envir×Treat×Day, $P < 0.01$). Dexamethasone rise in neutrophils percent was more pronounced in heat stress than in thermoneutral claves on day 1 (Envir×Treat×Day, $P < 0.001$). Compared with thermoneutral, heat stress reduced eosinophils and basophils percent (Envir, $P < 0.05$).

Dexamethasone altered RBC in heat stress calves

Compared with thermoneutral, heat stress decreased RBC count (Envir, $P < 0.05$). Compared with saline, dexamethasone decreased RBC count (Treat, $P < 0.05$). Heat stress

increased mean corpuscular volume (MCV, Envir, $P < 0.001$) and mean cell hemoglobin (MCH, Envir, $P < 0.05$). Furthermore, dexamethasone decreased cellular hemoglobin concentration (CHCM) in heat stress but not in thermoneutral calves (Envir×Treat, $P < 0.05$). Heat stress and dexamethasone decreased mean cellular hemoglobin (Envir, $P < 0.01$, Treat, $P < 0.05$).

Dexamethasone lowered jejunum IL-6, intestinal permeability, and crypt depth in heat stress calves

Dexamethasone decreased crypt depth in heat stress but not in thermoneutral calves (Table 3-7, Envir×Treat, $P < 0.05$). Dexamethasone decreased jejunum IL-6 concentration in heat stress, but increased in thermoneutral calves (Table 3-8, Envir×Treat, $P < 0.001$).

Our results showed that the mean plasma lactulose concentrations did not differ among the four groups on either day 1 or day 5 (Table 3-9, Envir×Treat×Day, $P > 0.10$). However, the lactulose to mannitol ratio was significantly higher in HS calves compared to HSD calves on day 1 (Envir×Treat×Day, $P < 0.05$). This result suggests greater intestinal permeability in HS than in HSD calves.

Dexamethasone administration to alleviate heat stress altered rumen alpha diversity

Table 3-10 presents the alpha diversity measures of rumen, jejunum, and colon microbiota in calves. In the rumen, the HSD group showed significantly lower values for both Shannon index and Faith's PD compared to TND calves (Envir×Treat, $P < 0.05$). In the jejunum, there were no significant differences in alpha diversity measures between different treatments or environments ($P > 0.05$). Similarly, no significant differences were observed in any of the alpha diversity measures in the colon ($P > 0.05$). UniFrac, a phylogeny-based method, was applied to the data and Principal Coordinates Analysis (PCoA) was used to determine if any separation into

sample clusters existed. The results indicated that there was no observable separation into clusters for the rumen, jejunum or colon, as shown in Figure 3-5.

Dexamethasone administration to alleviate heat stress altered the microbiome at the phylum and genus level

In the rumen, the phylum dataset was dominated by Bacteroidetes and Firmicutes within each group, as illustrated in Figure 3-6. The less dominant phyla included Proteobacteria, Actinobacteriota, Spirochaetota, Euryarchaeota, and Cyanobacteria. *Prevotella* spp. were the most abundant at the genus level, followed by *Succinivibrionaceae*_UCG-001, *Prevotellaceae*_NK3B31_group, *Olsenella, Succiniclasticum*, and *Treponema*. However, Firmicutes and Actinobacteriota were the top two phyla in the jejunum, as shown in Figure 3-7. *Olsenella* emerged as the most highly expressed genus, while *Prevotella* became the 18th most dominant genus, in contrast to its top ranking in the rumen. In the colon, similar to the rumen, the phylum dataset was dominated by Bacteroidetes and Firmicutes, as depicted in Figure 3-8. The less dominant phyla were Actinobacteriota, Euryarchaeota, and Proteobacteria. At the genus level, *Prevotella* reclaimed its dominant position as observed in the rumen.

Dexamethasone administration to alleviate heat stress altered the abundance of specific species

When a taxon exhibits a high Linear Discriminant Analysis (LDA) score in a given group, it may serve as a potential biomarker for that group. Taxa were identified using a logarithmic LDA size effect value of 2, as shown in Figure 3-9, 10, and 11. In the rumen, Verrucomicrobiota, Campylobacter, and Campylobacteraceae were more abundant in TND group. Notably, Campylobacter and Verrucomicrobiota accounted for 0.0012% and 0.0009% of the total reads, respectively. In the jejunum, Corynebacterium pilosum was more abundant in

HSD group, with a relative abundance of 0.0025%. In the colon, Bacteroides and Lachnospiraceae were more abundant in the HSD group.

DISCUSSION

Heat stress favors intestinal permeability, tissue injury, and inflammation and, thus, we hypothesized that the administration of dexamethasone would reduce inflammation and subsequently improve nutrient metabolism, intestinal barrier function, and intestinal microbial community composition in dairy calves subjected to heat stress. A research project was devised first, to investigate the impact of heat stress on growth performance, nutrient metabolism, inflammation, intestinal integrity, and intestinal microbiota; and second, to evaluate the potential of dexamethasone in mitigating inflammation induced by heat stress in dairy calves.

All calves had similar initial RR and RT before the initiation of the study. The administration of dexamethasone resulted in an elevation of RT only in heat-stressed calves, a finding that differs from the anticipated outcome. In this study, we anticipate that the administration of dexamethasone would mitigate heat stress, hence resulting in a decrease in RT. In comparison and using a restrain-stressed rodent model, Kainuma et al., (2009) demonstrated the role of a glucocorticoid in lowering body temperature. In agreement with our study, heat stress increased RR in dairy cows (Bernabucci et al., 2010, Wheelock et al., 2010), beef cattle (Renaudeau et al., 2012), pigs (Renaudeau et al., 2012), and poultry (St-Pierre et al., 2003, Lara and Rostagno, 2013). The effect of dexamethasone in RT of HSD calves is puzzling and further research is required to understand the reasons behind the increase in RT.

Compared to TN, HS calves decreased ADG and FE, however, dexamethasone restored ADG and FE in HSD calves. In comparison and using a rodent model, dexamethasone administration increased sodium reabsorption in the kidneys, thereby resulting in water retention

(Kenyon et al., 1990). It is possible that the improved ADG and FE observed in HSD calves can be attributed to the enhanced water retention effect reported in dexamethasone treated animals. Alternatively, the restored ADG and FE may due to the decrease in IL-6 in HSD calves, indicating a lower pro-inflammatory state. Given that the active of immune system is an energetically costly event (Greer, 2008), the decrease in inflammation may shift energy from maintenance (i.e. immune system) to growth. Furthermore, in our study dexamethasone decreased crypt depth in HSD calves. In comparison and using poultry, previous studies reported improvements in growth performance when animals exhibited decreased crypt depth, or an increased ratio of villus height/crypt depth (Deng et al., 2012, Cheng et al., 2019). In our study, decreased crypt depth may be associated with the improvements in ADG and FE in HSD calves. Collectively, the improvement of growth performance in HSD calves may be due to changes in the immune response, intestinal morphology, water retention or their combinations.

The glucose concentration in plasma was elevated in HSD calves on days 1 and 2. In agreement, our previous work showed an increase in plasma glucose concentration in heat-stressed calves treated with a postbiotic with anti-inflammatory properties (Ríus et al., 2022). In comparison, the administration of hydrocortisone elicited an immediate four-fold increase in plasma glucose concentration in a restrain-stressed rodent model (Kainuma et al., 2009). The elevated glucose levels in the HSD group of our study also may be attributed to the well-established response of glucocorticoids stimulating glycogenolysis and gluconeogenesis, while inhibiting peripheral glucose transport and utilization (Sapolsky et al., 2000) and mitochondrial glucose oxidation (Kainuma et al., 2009).

The plasma NEFA concentration is used as an indicator of adipose tissue mobilization of energy reserves. The administration of dexamethasone resulted in a reduction in NEFA levels

indicating a decrease in the mobilization of energy reserves in HSD calves compared with HS calves. Heat stress increased fat tissue accretion in pigs (Stahly et al., 1979, Collin et al., 2001), rodents (Katsumata et al., 1990), and growing chickens (Lu et al., 2007) due to changes in metabolism such as increased NEFA re-esterified and increased carbohydrate dependence. The decreased plasma NEFA concentration in the HSD group might be due to changes in energy metabolism. Heat stress triggers the activation of the immune response (Cantent et al., 2022), leading to the utilization of carbohydrate oxidation as the sole energy source by active immune cells. The observed decrease in NEFA levels in the plasma, along with the concurrent elevation of circulating glucose in HSD calves, could potentially indicate a synchronized upregulation of carbohydrate metabolism. This metabolic response may serve as an adaptive mechanism to promptly accommodate alterations in immune system homeostasis. The observed reduction in plasma NEFA combined with the increase of plasma glucose may potentially be part of the metabolic response to support growth performance in HSD calves.

The increased in WBC and blood neutrophil counts in response to dexamethasone treatment was more pronounce in HSD than in TND calves on day 1 only. The return to basal levels of blood WBC and neutrophil counts on day 5 highlights the transient nature of this change. The basal levels of WBC and neutrophil counts at day 5 in heat stress calves might be due to cell migration to mount an inflammatory response in the gut. The increased jejunum IL-6 concentrations in HSS but not in HSD calves supports this notion. In rodents, dexamethasone administration reduced the expression of L-selectin and CD18 markers of cell activation, inhibiting the migration of leukocytes from circulation to sites of inflammation (Burton et al., 1995). Studies in humans and rodents showed that dexamethasone enhanced neutrophil release from bone marrow and half-life increasing neutrophil counts in circulation (Liles et al., 1995,

Nittoh et al., 1998). In rabbits, dexamethasone-induced granulocytosis resulted from heightened bone marrow release (10%), diminished migration out of the bloodstream (61%), and prolonged half-life (29%) of granulocytes (Nakagawa et al., 1998). Cortisol, an endogenous glucocorticoid, has similar but more subtle effects on L-selectin and CD18 expression in neutrophils (Burton et al., 1995). Regarding the temporal implications, this phenomenon may be attributed to the transient nature of the effects of dexamethasone on L-selectin and CD18, which are observed to endure for a maximum duration of 48 hours (Burton et al., 1995). Collectively, it is possible that in our study natural and synthetic glucocorticoids combined may have triggered the responses observed in neutrophils of heat stress calves.

Dexamethasone reduced blood lymphocytes counts in heat stress but increased blood lymphocyte counts in thermoneutral calves on day 1. Studies in bulls indicated that dexamethasone failed to stimulate migration of the alpha beta T subpopulation but promoted migration of the gamma delta T subpopulation of lymphocytes out of peripheral circulation (Burton and Kehrli, 1996). Gamma delta T-cell functional responses are induced upon the recognition of stress antigens to regulate pathogen clearance, inflammation, and tissue homeostasis in response to stress in rodents. Furthermore, in human subjects, dexamethasone induced apoptosis of circulating CD4+ and CD8+ T lymphocytes while increasing regulatory T cell activation and proliferation status and contributing to their immune suppressive activity (Aston et al., 2019). In our study, the reduced blood lymphocytes counts in HSD animals could be a consequence of increased migration of T cells to sites of inflammation.

Regarding monocytes, dexamethasone reduced the increase in monocyte blood counts observed in the heat stress group on day 1. In comparison, glucocorticoids stimulated the differentiation of human monocytes into an anti-inflammatory subtype (Ehrchen et al., 2006). In

our study, dexamethasone might have stimulated migration out of peripheral circulation and anti-inflammatory differentiation of monocytes. The reduction in jejunal IL-6 concentration in HSD calves supports this view. Further to this point, our previous work showed a jejunal increase of anti-inflammatory cytokines in Holstein calves exposed to a 7-day heat stress period, suggesting the presence of an anti-inflammatory response in the jejunum of heat-stressed calves (Yu et al., 2023). The observed reduction in blood lymphocytes and monocytes counts in HSD calves might be part of enhanced response to restore homeostasis in the intestine with increased permeability to lumen antigens.

Compared with TNS, the HSS calves had lower cellular hemoglobin concentration mean (CHCM) but HSD calves had a lower CHCM compared with the other three groups. Reduced CHCM may indicate RBC swelling (e.g., increased cell volume) or decreased hemoglobin concentration. In our study, the treatment by environment interaction was not significant on mean corpuscular volume (MCV), but heat stress alone increased MCV significantly. Moreover, it is worth noting that the administration of dexamethasone, as well as the exposure to heat stress, resulted in a reduction in the mean cellular hemoglobin (CH) levels. Our finding is consistent with previous studies showing a 6 to 7% reduction in CH in heat-stressed broilers and lambs (Barnes et al., 2021, Moustafa et al., 2021). One possible explanation for this observation is that heat stress may lead to a decrease in iron absorption and serum iron concentration (Wei et al., 2008). However, corticosteroids have also been reported to have no effects on hemoglobin concentration and RBC (King et al., 1988). Overall, our data may indicate that the treatment and environment caused RBC swelling and lower hemoglobin concentration which might affect the oxygen delivery to peripheral tissues.

Dexamethasone reduced IL-6 concentrations by 50% in jejunum in heat stress calves. In comparison, heat stress increased gut and systemic inflammation, leading to the elevation of cytokine concentrations in the intestinal mucosa of rodents and dairy cattle (Lambert, 2009, Koch et al., 2019). Previous research in broilers, demonstrated that heat stress activated the TLR/MyD88/NF-κB pathway and, in turn, upregulated pro-inflammatory cytokines (i.e., IL-1, IL-6, TNF-α) in the spleen (He et al., 2019). In our study, the decreased IL-6 concentration in jejunum is likely due to dexamethasone inhibitory effect on NF-κB activity (Auphan et al., 1995).

The use of markers of intestinal permeability showed that dexamethasone reduced intestinal permeability in heat stress calves on day 1. Mannitol is a monomer that can be absorbed through the transcellular route in the intestinal crypt, while lactulose is a dimer that can be absorbed through paracellular route mainly across intestinal villi. A decrease in the lactulose to mannitol ratio indicates lower intestinal permeability in response to reduced villi length, increased crypt depth or a combination of both (Vojdani, 2013). Heat stress has been reported to disrupt expression and location of tight junction proteins in rodents, poultry, swine, and cattle (Hall et al., 2001, Pearce et al 2013a, b, Pearce et al., 2014, Koch et al., 2019) which leads to increased intestinal permeability. In dairy calves, heat stress decreased the expression of *TJP1* as stated in chapter 2. The reduced permeability in HSD calves could be due to the observed reduction in crypt depth in HSD calves. Given that the crypt region is the main pathway for lactulose passing from the lumen into circulation, a decreased crypt depth observed may limit the absorption of lactulose, leading to a reduced lactulose/mannitol ratio (Vanuytsel et al., 2021). Furthermore, the decreased permeability observed in HSD calves may be partially associated with the drop in jejunum IL-6 concentrations discussed previously. This concept is supported by

data showing that increased concentrations of IL-6 downregulated the expression of Claudin-2 and increased between cell permeability in Caco-2 cells (Suzuki et al., 2011).

The decreased crypt depth could be associated with the reduced IL-6 concentration observed at day 5 in HSD calves. The newly formed enterocytes in the crypt migrate to the villus top in 5-7 days (Clevers, 2013). An increased in crypt depth indicates an accelerated rate of cell migration, leading to a situation where the enterocytes of the villus do not have adequate time to undergo complete differentiation and reach their maximum catalytic capability before being expelled from the tip of the villus (Jeffery et al., 2017). Shallower crypts allow more time for differentiation, hence promoting the maturation of cells and their capacity for enhanced secretion. Moreover, IL-6 knockout mice reduced crypt depth in duodenum after the exposure to cold water immersion stress effect compared with their wild type counterparts (Zhang et al., 2022). In our study, the decreased jejunal crypt depth may be linked with the reduced IL-6 concentration in this tissue. Collectively, these findings agree with our hypothesis and suggest that intestinal permeability, crypt depth, and IL-6 may play a role in enhancing the performance of calves experiencing heat stress.

The interaction of HS and dexamethasone decreased alpha diversity indicators Shannon index, Faith's PD, Evenness in rumen, representing reduced species diversity. Previous studies reported that HS tended to decrease alpha diversity of rumen microbiota in dairy cows (Chen et al., 2018, Park et al., 2022), which aligns with our results. However, the alpha diversity on ileal microbiota increased in broilers (Wang et al., 2018)or was not affected in pigs, cows, broilers, and rabbits (Kim et al., 2020, Yasoob et al., 2021, Hu et al., 2022, Wang et al., 2022) exposed to heat stress. These controversial results may relate to duration, intensity of stress, species and the gut segments in different studies. Dexamethasone treatment has been reported to reduce the

alpha diversity of wild birds and squirrels (Noguera et al., 2018, Petrullo et al., 2022). This suggests a communication between glucocorticoids and gut microbiome composition which agrees with our results. The rumen has the most abundant bacteria compared to jejunum and colon in ruminants (Lin et al., 2023), and the region specific change we observed may be because there is a delay of the effects on other segments. In PCoA plots, closer points indicate a more similar microbial community in sequence composition. In our study the interaction between heat stress and dexamethasone did not change the PCoA. Bacteroidetes and Firmicutes were most abundant phylum in rumen and colon which agrees the results in dairy cows (Chen et al., 2018, Baek et al., 2020). While in jejunum, Firmicutes and Actinobacteriota were the dominant phyla.

Heat stress and dexamethasone increased the abundance of *Corynebacterium pilosum* in jejunum, which is the dominant species in skin of lactating dairy cows, and were also observed in the calf gastrointestinal content (Yeoman et al., 2018). Corynebacterium spp has been isolated from beddings, water buckets, walking areas on-farm (Woudstra et al., 2023). This may facilitate passage of Corynebacterium spp between host and environment niches.

Bacteroides coprocola levels were higher in the HSD group. Bacteroides colonization in the gut benefits the host because of these microorganisms facilitate the absorption of polysaccharides in the mucosa and the release enzymes to promote sugar transport and utilization (Wexler, 2007). Heat stress rats and chickens showed an increase in bacteroides (Ducray et al., 2019, Chen et al., 2021),. Bacteroides possess a unique environmental sensing system that allows them to detect and respond to changes in their environment via the extracytoplasmic function factor (Wexler, 2007). The difference we observed in our study could be attributed to the restoration and adaptation of commensal bacteria.

CONCLUSIONS

Heat stress led to the initiation of localized inflammation and disruption of intestinal function. However, the administration of dexamethasone mitigated the inflammatory response. Additionally, dexamethasone restored the structure and function of the intestines by reducing intestinal permeability and crypt depth in heat stressed calves. Consequently, these changes in intestinal health and immune response affected the metabolism of nutrients in calves, ultimately leading to enhanced growth performance. The alterations in bacterial communities observed in HSD calves may serve as a potential contributing component in the reduction of inflammation and restoration of intestinal function.

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APPENDIX

Table 3-1. Milk replacer and calf starter chemical composition consumed in study (% of DM or stated otherwise)

Items	Starter	Milk replacer
Crude Protein	18	26
Crude Fat	2	20
Crude Fiber	15	0.15
Acid Detergent Fiber	21	-
Calcium	1.2	0.75
Phosphorus	0.55	0.7
Salt	0.85	-
Potassium	0.80	-
Selenium	0.3 ppm	-
Vitamin A	28,660 IU/kg	6,820 IU/kg

Table 3-2. Dry matter (milk replacer plus starter)and water intake in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection.

Parameter	Day	TN		HS		SEM	*P*-value[1]			
		S N=8	D N=8	S N=8	D N=8		E	T	E×T	E×T×D
Dry matter intake (kg/d)	1	1.68	1.78	1.72	1.89	0.119	0.422	0.351	0.575	0.313
	2	1.51	1.71	1.71	1.85					
	3	1.59	1.80	1.78	2.05					
	4	1.62	1.65	1.82	1.88					
	5	2.00	2.05	1.91	1.42					
Water intake (kg/d)	1	2.50	2.73	5.35	5.75	0.404	<.0001	0.023	0.675	0.811
	2	2.68	3.01	6.36	6.87					
	3	2.69	3.51	6.46	8.24					
	4	2.89	3.61	6.77	7.85					
	5	4.17	4.75	6.20	6.30					
Urine output[2] (ml)	1	793.3	1421	1266	1925	229.4	0.006	0.181	0.643	0.267
	5	949.8	1053	1874	1574					
Fecal water content (%)	1	75.8	74.4	75.5	73.8	1.30	0.659	0.034	0.613	0.665
	2	75.2	75.0	74.2	74.5					
	3	78.6	74.5	77.7	73.7					
	4	75.5	76.3	77.3	74.1					
	5	80.9	78.6	81.6	79.4					

[1]E: environment effect of heat stress (40°C, from 0800 to 1900) or thermoneutral (20°C, 24 h/day); T: treatment effect of dexamethasone or saline solution; E×T: interaction of environment and treatment effects; E×T×D: interaction of environment, treatment, and day.
[2]Urine was collected consistently for 10 hours on day1 and day5.

Table 3-3. Growth performance in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection.

Item	TN		HS		SEM	*P*-value[1]		
	S N=8	D N=8	S N=8	D N=8		E	T	E × T
Initial BW (kg)	66.7	65.7	70.9	70.6	2.75	0.105	0.822	0.902
Final BW (kg)	70.3	69.7	73.4	74.8	2.78	0.151	0.880	0.709
BW gain (kg)	2.68	3.44	2.04	4.31	0.740	0.838	0.009	0.167
BW gain (%)	3.92	5.32	2.85	6.17	0.839	0.900	0.009	0.256
ADG (kg/d)	0.694^{x}	0.649^{xy}	0.449^{y}	0.745^{x}	0.152	0.369	0.137	0.048
FE (%)	36.96	38.47	23.61	40.54	5.953	0.217	0.049	0.096

^{xy}Difference letters in the same row indicated difference in E × T interaction, $P < 0.05$.

[1]E: environment effect of heat stress (40°C, from 0800 to 1900) or thermoneutral (20°C, 24 h/day); T: treatment effect of dexamethasone or saline solution; E×T: interaction of environment and treatment effects.

Table 3-4. Plasma metabolites in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection.

Item	Day	Time	TN		HS		SEM	*P*-value[1]			
			S N=8	D N=8	S N=8	D N=8		E	T	E×T	E×T×D
PUN (mg/dL)	1	1500	15.8	17.1	15.8	19.3	2.49	0.574	0.001	0.550	0.576
		1800	15.9	16.6	18.9	22.2					
	3	1800	18.3	22.4	16.9	22.2					
	5	1800	16.2	18.4	13.7	15.9					
NEFA (mg/dL)	1		0.134	0.237	0.188	0.141	0.0323	0.571	0.001	0.038	0.182
	2		0.167	0.114	0.226	0.158					
	3		0.193	0.103	0.185	0.120					
	4		0.202	0.194	0.193	0.118					
	5		0.255	0.204	0.267	0.136					
	1-5		0.191^{xy}	0.171^{yz}	0.212^{x}	0.135^{z}					

^{xyz}Different letters indicate different in E ×T interaction, $P < 0.05$.

[1]E: environment effect of heat stress (40°C, from 0800 to 1900) or thermoneutral (20°C, 24 h/day); T: treatment effect of dexamethasone or saline solution; E×T: interaction of environment and treatment effects; E×T×D: interaction of environment, treatment, and day.

Table 3-5. Hematology in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection.

Parameter1	Day	TN		HS		SEM	*P*-value[2]			
		S N=8	D N=8	S N=8	D N=8		E	T	E×T	E×T×D
WBC, 10^3 /µL	1	7.51	13.54	8.89	17.83	1.23	0.108	<.0001	0.823	0.077
	5	6.68	8.54	7.03	6.54					
Lymphocytes,10^3 /µL	1	4.54^{b}	5.53^{a}	4.16^{bc}	2.06^{e}	0.317	<.0001	0.002	<.0001	0.047
	5	3.78^{bcd}	3.67^{cd}	4.37^{bc}	3.29^{d}					
Monocytes, 10^3 /µL∗	1	0.20^{c}	0.23^{bc}	0.29^{ab}	0.17^{c}	0.037	0.495	0.312	0.099	0.013
	5	0.32^{a}	0.36^{a}	0.29^{ab}	0.30^{ab}					
Neutrophils, 10^3 /µL∗	1	2.22	7.02	4.18	15.25	0.772	0.055	<.0001	0.965	0.077
	5	2.08	4.06	2.10	2.52					
Eosinophils, 10^3 /µL∗	1	0.34	0.55	0.19	0.33	0.096	0.110	0.107	0.686	0.384
	5	0.25	0.25	0.18	0.27					
Basophils, 10^3 /µL	1	0.11	0.15	0.11	0.10	0.013	0.217	0.211	0.078	0.522
	5	0.09	0.11	0.10	0.09					
LUC, 10^3 /µL∗	1	0.02	0.03	0.03	0.03	0.010	0.531	0.460	0.093	0.497
	5	0.04	0.06	0.05	0.05					
Lymphocytes, %	1	60.26^{ab}	44.50^{c}	47.55^{c}	12.51^{d}	3.45	0.010	<.0001	0.097	0.006
	5	57.21^{ab}	44.98^{c}	62.78^{a}	51.07^{bc}					
Monocytes, %	1	2.70	2.22	3.31	0.91	0.68	0.440	0.030	0.814	0.060
	5	5.55	4.25	4.23	5.17					
Neutrophils, %	1	29.4^{d}	47.68^{b}	46.48^{bc}	85.08^{a}	3.491	0.001	<.0001	0.270	0.001
	5	30.44^{d}	45.38^{bc}	29.53^{d}	37.63^{cd}					
Eosinophils, %∗	1	4.52	3.47	2.26	1.57	0.68	0.008	0.581	0.881	0.599
	5	3.51	2.77	2.45	1.81					
Basophils, %	1	1.50	1.21	1.18	0.64	0.10	0.047	0.004	0.760	0.107
	5	1.45	1.29	1.44	1.44					
LUC, %	1	0.34	0.33	0.31	0.12	0.12	0.214	0.183	0.119	0.280
	5	0.55	0.73	0.70	0.72					

[abcde]Values within the same variable with different superscripts indicate significant differences in E×T×D, $P < 0.05$.

∗Data were log transformed to pass the normality and homeodesticity test and non-transformed LSM were reported.

[1]WBC=white blood cells; LUC=Large unstained cell counts.

[2]E: environment effect of heat stress (40°C, from 0800 to 1900) or thermoneutral (20°C, 24 h/day); T: treatment effect of dexamethasone or saline solution; E×T: interaction of environment and treatment effects; E×T×D: interaction of environment, treatment, and day.

Table 3-6. Routine complete blood count in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection.

Parameter[1]	Day	TN		HS		SEM	*P*-value[2]			
		S N=8	D N=8	S N=8	D N=8		E	T	E×T	E×T×D
RBC, 10^6 /µL	1	9.34	9.27	9.08	8.80	0.14	0.010	0.046	0.605	0.527
	5	8.93	8.66	8.71	8.43					
HCT, %	1	30.02	29.68	30.05	29.31	0.62	0.645	0.068	0.941	0.528
	5	28.55	27.21	28.16	27.10					
MCV, fL	1	32.10	32.04	33.13	33.58	0.55	0.0001	0.787	0.259	0.880
	5	31.98	31.44	32.36	32.28					
MCH, pg	1	11.31	11.34	11.51	11.66	0.07	0.015	0.738	0.205	0.572
	5	11.37	11.23	11.33	11.38					
MCHC, g/dL	1	35.15	35.04	35.19	34.63	0.59	0.397	0.230	0.480	0.450
	5	35.48	35.42	35.38	35.30					
CHCM, g/dL	1	35.67^{x}	35.49^{xy}	35.03^{y}	33.95^{z}	0.60	<.0001	0.006	0.025	0.442
	5	36.15^{x}	36.15^{xy}	35.88^{y}	35.43^{z}					
CH, pg	1	11.40	11.42	11.36	11.30	0.04	0.003	0.025	0.814	0.062
	5	11.55	11.37	11.38	11.30					
RDW, %	1	21.35	21.22	21.33	21.08	0.14	0.505	0.077	0.601	0.068
	5	21.30	21.01	21.11	21.14					
HDW, g/dl	1	2.82	2.81	2.68	2.60	0.66	0.0001	0.952	0.539	0.660
	5	2.73	2.78	2.62	2.65					
Hgb, g/dl	1	10.62	10.52	10.50	10.29	0.18	0.111	0.071	0.863	0.440
	5	10.19	9.78	9.86	9.65					
Platelet, 10^3/ µL	1	420.97	444.67	463.13	392.60	33.63	0.573	0.081	0.399	0.168
	5	405.10	497.05	425.38	537.35					

[xyz]Values within the same variable with different superscripts indicate significant differences in E×T interaction, $P < 0.05$.

[1]RBC=red blood cells; HCT=hematocrit red blood cell percentage; MCV=mean corpuscular volume; MCH=mean cell hemoglobin; MCHC=mean corpuscular hemoglobin concentration; CHCM=cellular hemoglobin concentration mean; CH=mean cellular hemoglobin; RDW=red cell distribution width; HDW=Hemoglobin distribution width; Hgb=hemoglobin

[2]E: environment effect of heat stress (40°C, from 0800 to 1900) or thermoneutral (20°C, 24 h/day); T: treatment effect of dexamethasone or saline solution; E×T: interaction of environment and treatment effects; E×T×D: interaction of environment, treatment, and day.

Table 3-7. Villus height, crypt depth and their ratio in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection in jejunum.

Parameter	TN		HS		SEM	*P*-value[1]		
	S N=8	D N=8	S N=8	D N=8		E	T	E×T
Villi height (μm)	555.5	622.7	612.9	508.3	76.9	0.705	0.804	0.259
Crypt depth (μm)	474.4^{xy}	540.7^{xy}	550.7^{x}	457.2^{y}	66.0	0.908	0.661	0.015
Villi height/Crypt depth	1.232	1.220	1.197	1.039	0.189	0.522	0.612	0.662

^{xy}Values within the same variable with different superscripts indicate significant differences in E×T interaction, $P < 0.05$.
[1]E: environment effect of heat stress (40°C, from 0800 to 1900) or thermoneutral (20°C, 24 h/day); T: treatment effect of dexamethasone or saline solution; E×T: interaction of environment and treatment effects.

Table 3-8. Cytokine concentration in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection.

Parameter	Day	TN		HS		SEM	*P*-value[1]			
		S N=8	D N=8	S N=8	D N=8		E	T	E×T	E×T×D
Jejunum IL-1β (pg/ml)[2]	6	3314	2148	4829	2764	935.2	0.117	0.196	0.869	
IL-6 (pg/ml)	6	34.84^{y}	88.35^{x}	72.78^{x}	35.82^{y}	20.84	0.530	0.477	0.0005	
Serum IL-6 (pg/ml)	1	24.35	181.5	109.3	90.84	85.50	0.361	0.989	0.191	0.710
	2	115.3	156.0	224.1	189.2					
	5	56.25	54.19	190.7	52.24					

^{xy}Values within the same variable with different superscripts indicate significant differences in E×T interaction, $P < 0.05$.
[1]E: environment effect of heat stress (40°C, from 0800 to 1900 h) or thermoneutral (20°C, 24 h/day); T: treatment effect of dexamethasone or saline solution; E×T: interaction of environment and treatment effects; E×T×D: interaction of environment, treatment, and day.
[2]*P*-value was reported for logarithmic transformation of data (TN=7.675±0.3042; TND=7.323±0.3042; HS=8.217±0.3042; HSD=7.764±0.3042).

Table 3-9. Intestinal permeability markers (lactulose and mannitol) in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection.

Parameter	Day	TN		HS		SEM	*P*-value[1]			
		S N=8	D N=8	S N=8	D N=8		E	T	E×T	E×T×D
Lactulose (pmol/μL)	1	29.80	28.51	30.17	20.90	0.16	0.395	0.469	0.161	0.983
	5	30.46	35.95	45.67	35.57					
Mannitol (pmol/μL)	1	14.55	13.49	12.57	14.27	2.51	0.845	0.465	0.730	0.069
	5	18.69	22.81	20.35	20.23					
Lactulose :mannitol	1	1.96^{ab}	2.07^{ab}	2.44^{a}	1.53^{b}	0.27	0.693	0.113	0.347	0.025
	5	2.09^{a}	2.06^{a}	2.28^{a}	2.03^{a}					

^{abc}Different letters in the same row indicate different least-square means, $P < 0.05$.
[1]E: environment effect of heat stress (40°C, from 0800 to 1900) or thermoneutral (20°C, 24 h/day); T: treatment effect of dexamethasone or saline solution; E×T: interaction of environment and treatment effects; E×T×D: interaction of environment, treatment, and day.

Measurements	TN		HS		SEM	*P*-value[1]		
	S N=8	D N=8	S N=8	D N=8		E	T	E×T
Rumen								
Observed ASVs	218	262	233	198	21.0	0.241	0.844	0.067
Shannon	5.59^{xy}	5.97^{x}	5.84^{xy}	5.39^{y}	0.18	0.352	0.853	0.026
Faith's PD	21.9^{xy}	25.4^{x}	22.7^{xy}	20.9^{y}	1.28	0.159	0.487	0.042
Evenness	0.722^{xy}	0.749^{x}	0.744^{xy}	0.708^{y}	0.015	0.507	0.771	0.042
Jejunum								
Observed ASVs	194	195	220	218	32.3	0.455	0.986	0.956
Shannon	5.37	5.27	5.22	5.36	0.28	0.906	0.931	0.671
Faith's PD	18.2	19.0	19.8	19.5	18.9	0.777	0.943	0.977
Evenness	0.709	0.697	0.677	0.698	0.021	0.455	0.831	0.429
Colon								
Observed ASVs	373	372	358	424	31.7	0.545	0.289	0.282
Shannon	7.10	7.07	6.93	7.26	0.143	0.946	0.258	0.197
Faith's PD	26.7	26.2	25.5	27.6	1.16	0.931	0.433	0.248
Evenness	0.834	0.832	0.821	0.833	0.007	0.396	0.412	0.302

^{xy}Values within the same variable with different superscripts indicate significant differences in E×T interaction ($P < 0.05$)
[1]E: environment effect of heat stress (40°C, from 0800 to 1900) or thermoneutral (20°C, 24 h/day); T: treatment effect of dexamethasone or saline solution; E×T: interaction of environment and treatment effects.

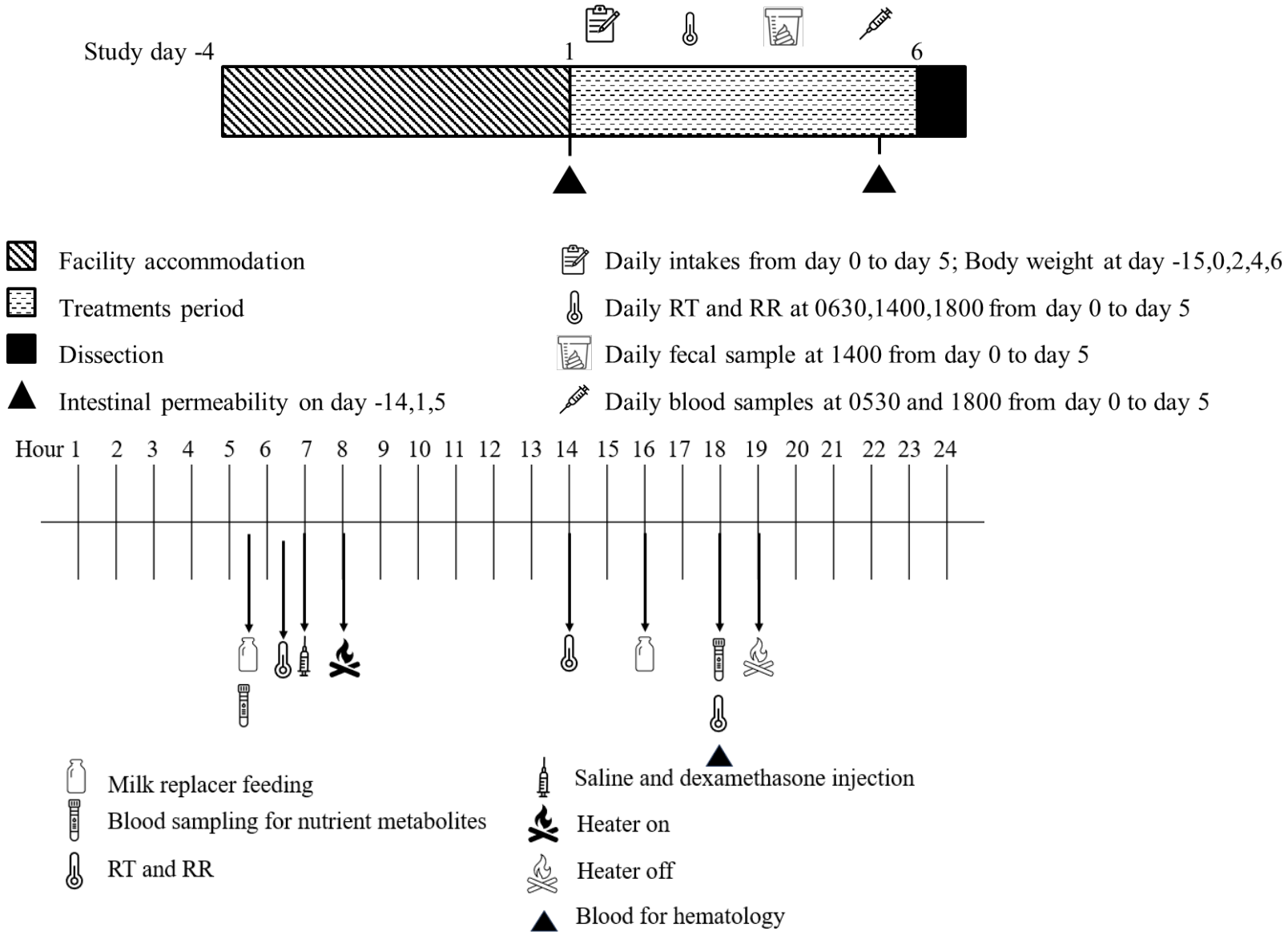


Figure 3-1. Experimental timeline (upper panel) and major daily activities in heat stress room (lower panel)

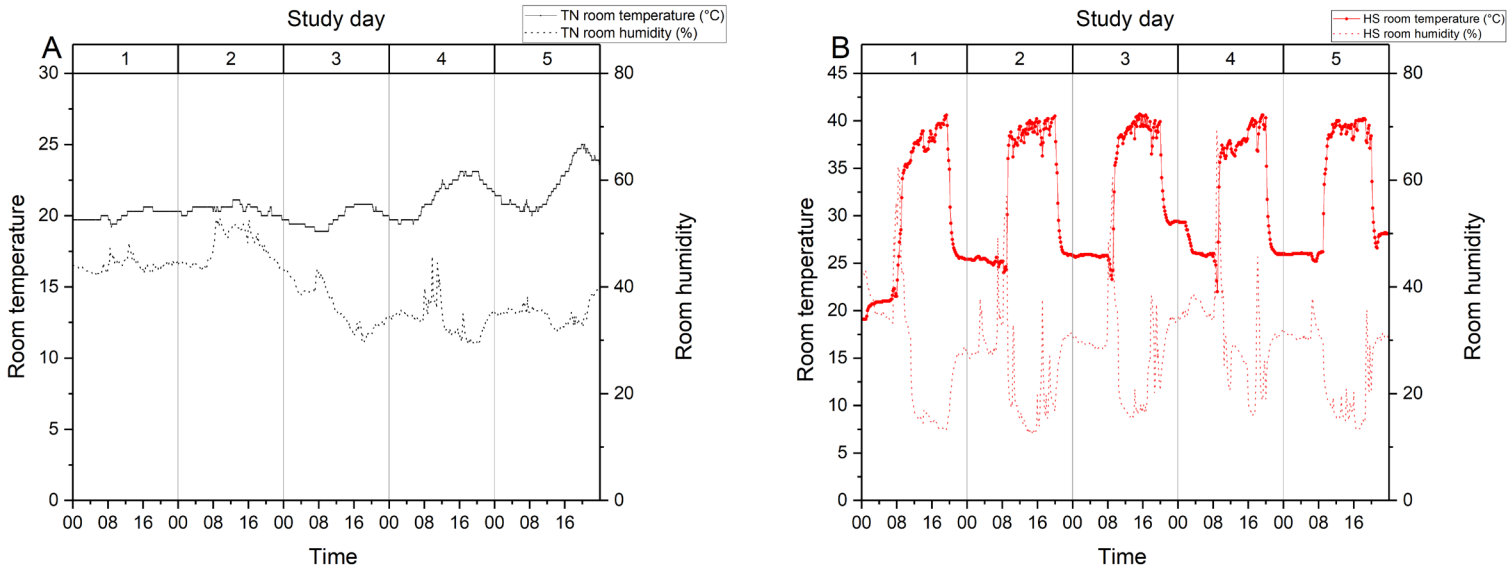


Figure 3-2. Ambient temperature and humidity in thermoneutral (panel A) and heat stress room (panel B) from day 1 to 5

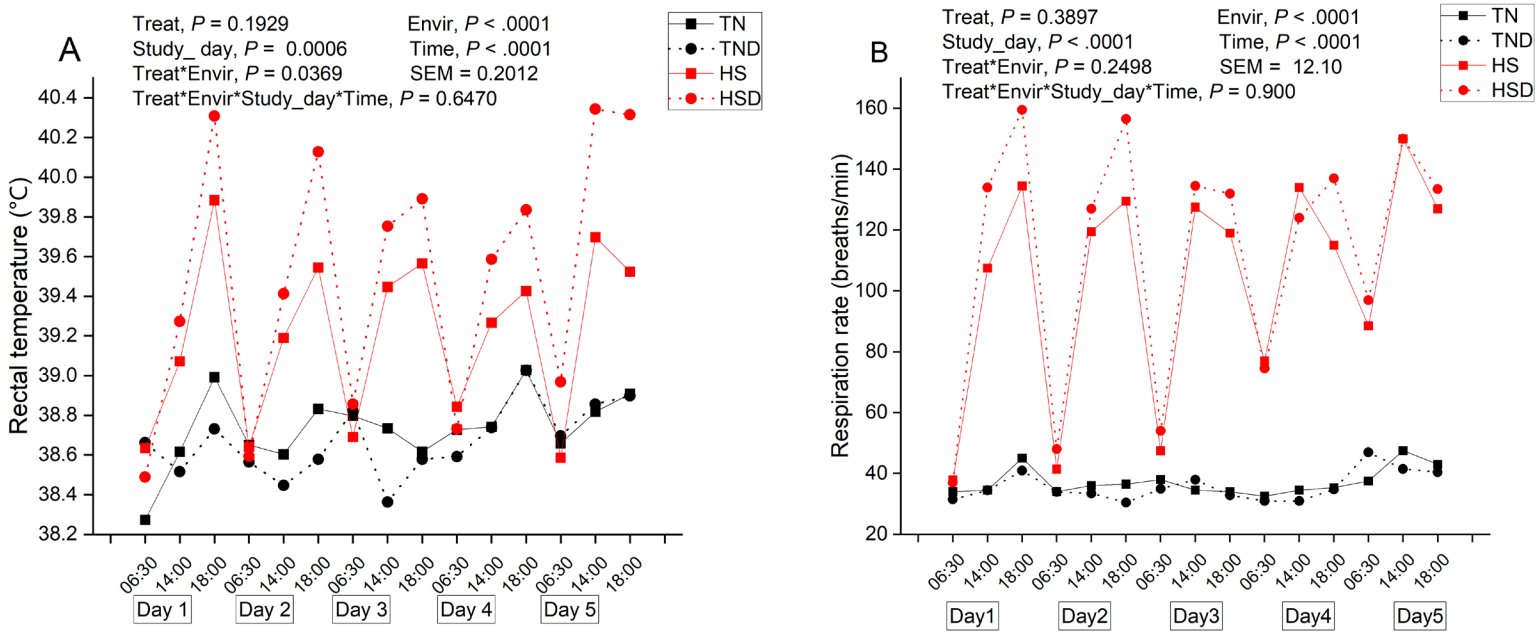


Figure 3-3. Rectal temperature (panel A) and respiration rate (panel B) in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection.

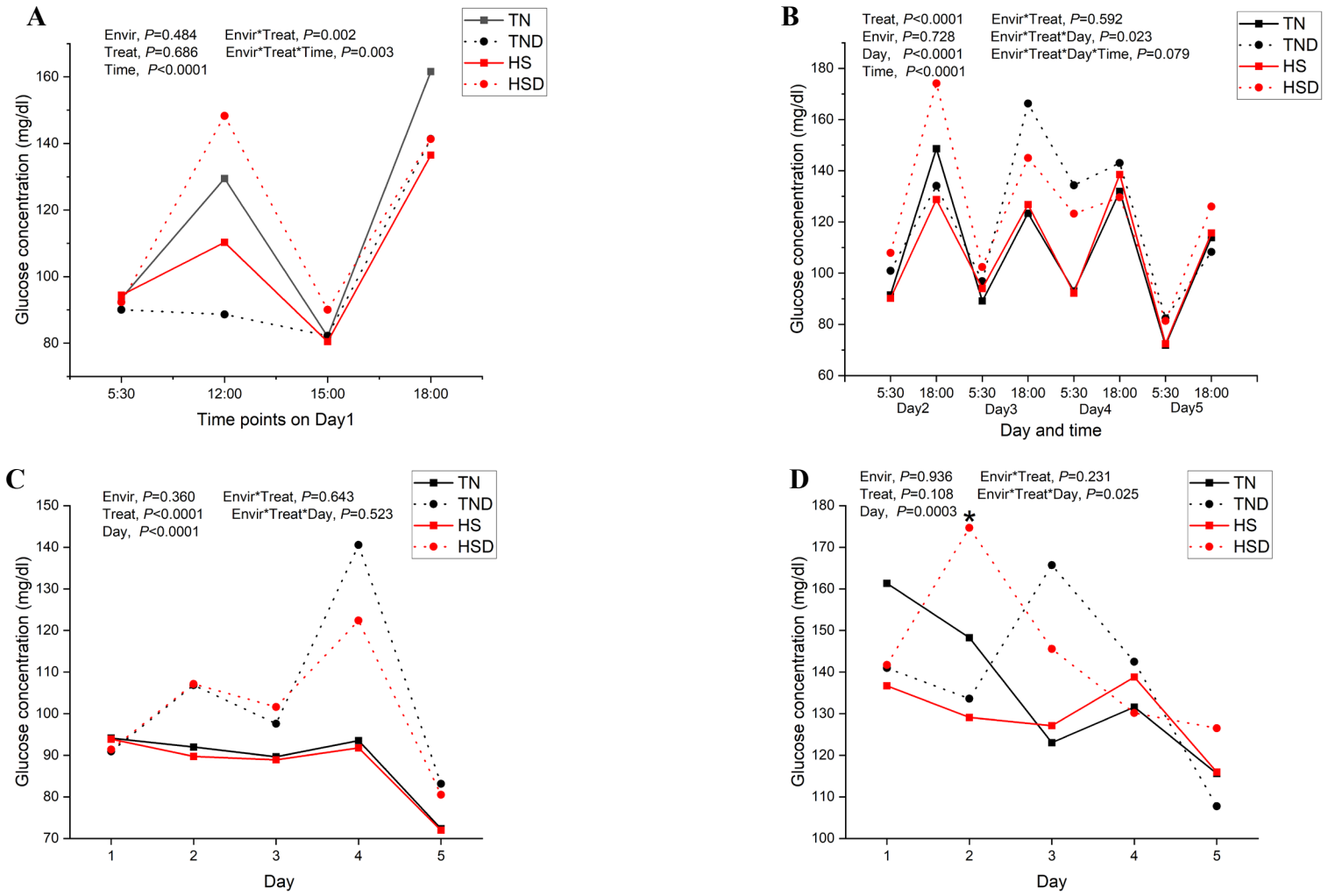


Figure 3-4. Glucose concentration in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection on study day 1 (panel A) or study day 2 to 5 (panel B). Pre-feeding (panel C) and after feeding (panel D) glucose concentration in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) and saline (S) solution injection from study day 1 to 5.
* Indicates a significant difference between HS and HSD.

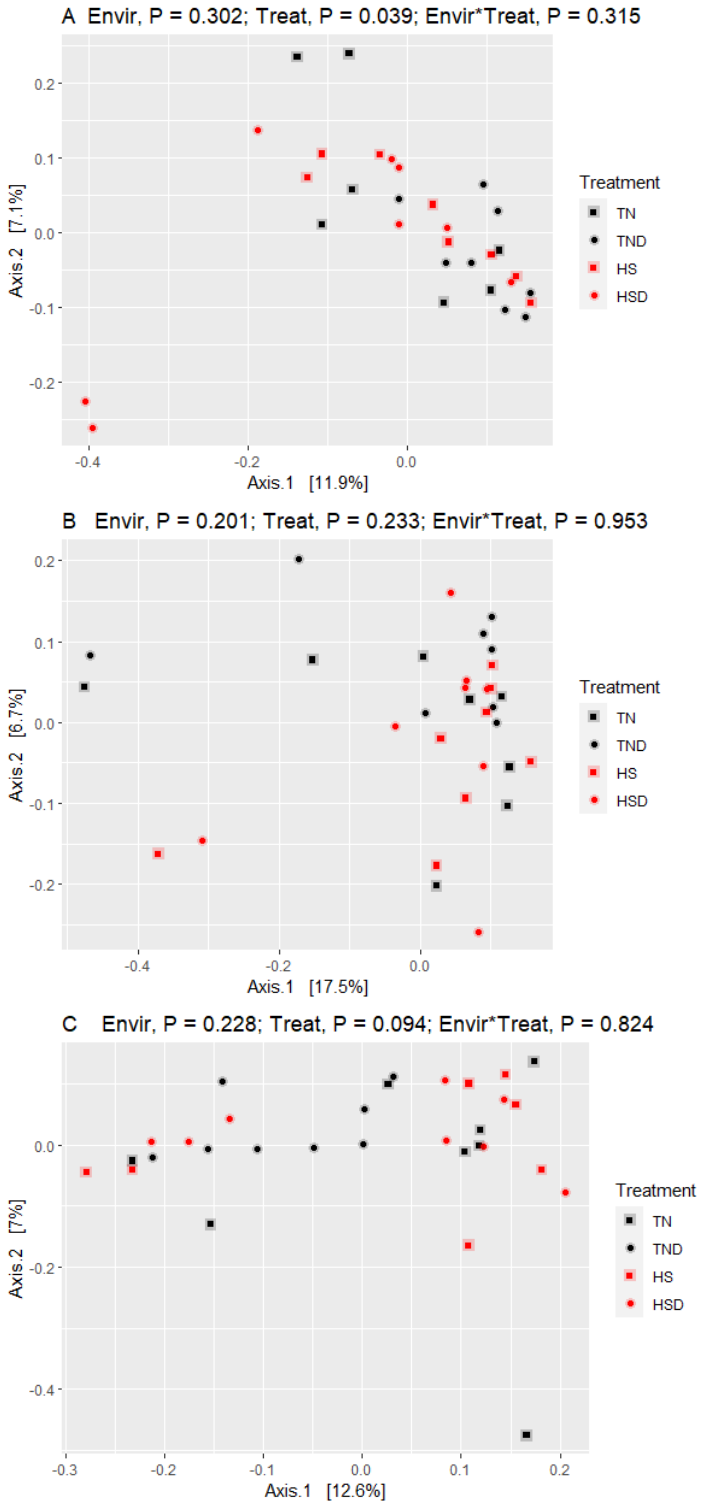
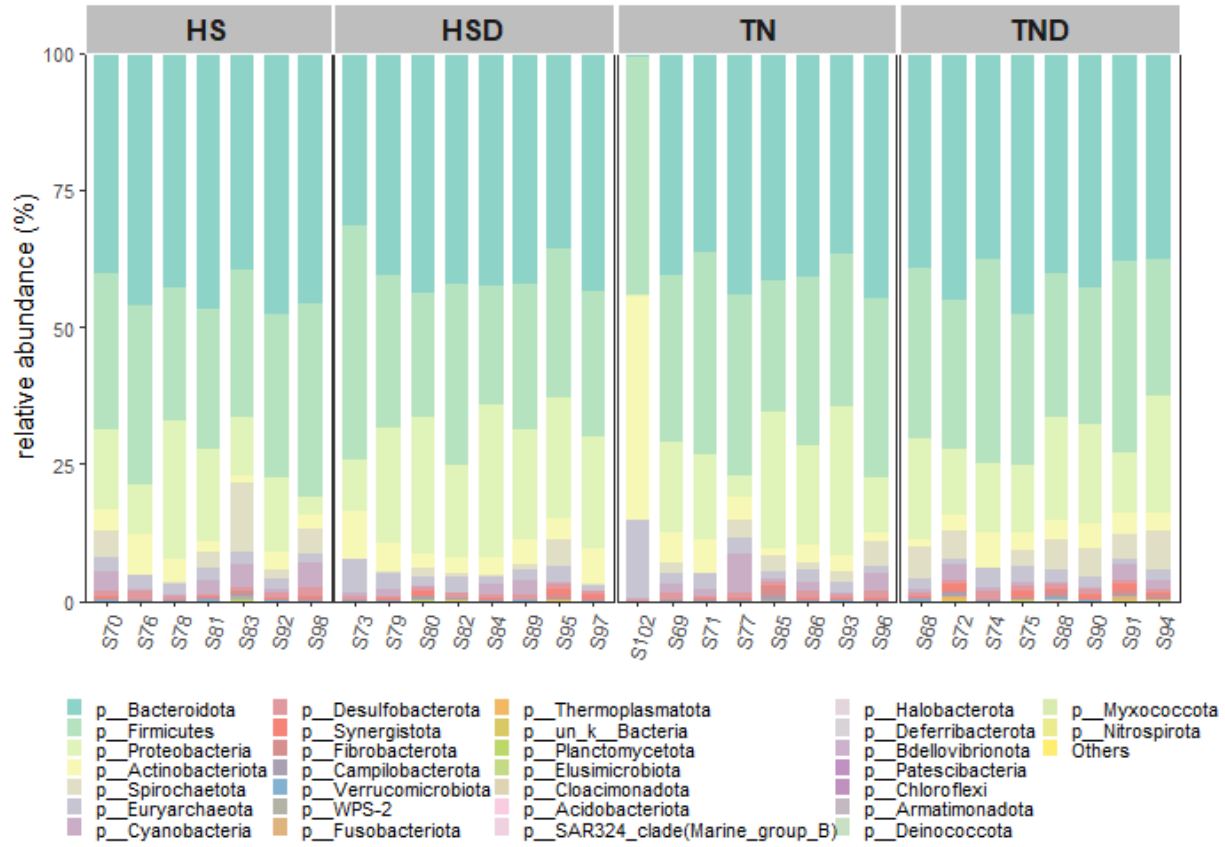


Figure 3-5. The plot of principal coordinates analysis (PCoA) of rumen (panel A), jejunum (panel B), and colon (panel C) microbiota based on unifrac distance matrices. HS, heat stress; TN, thermoneutral; D, dexamethasone; S, saline solution. Envir represents the effect of heat stress and thermoneutral.Treat represents the effect of dexamethasone or saline solution. *P*-Values were based on PERMANOVA.

A



B

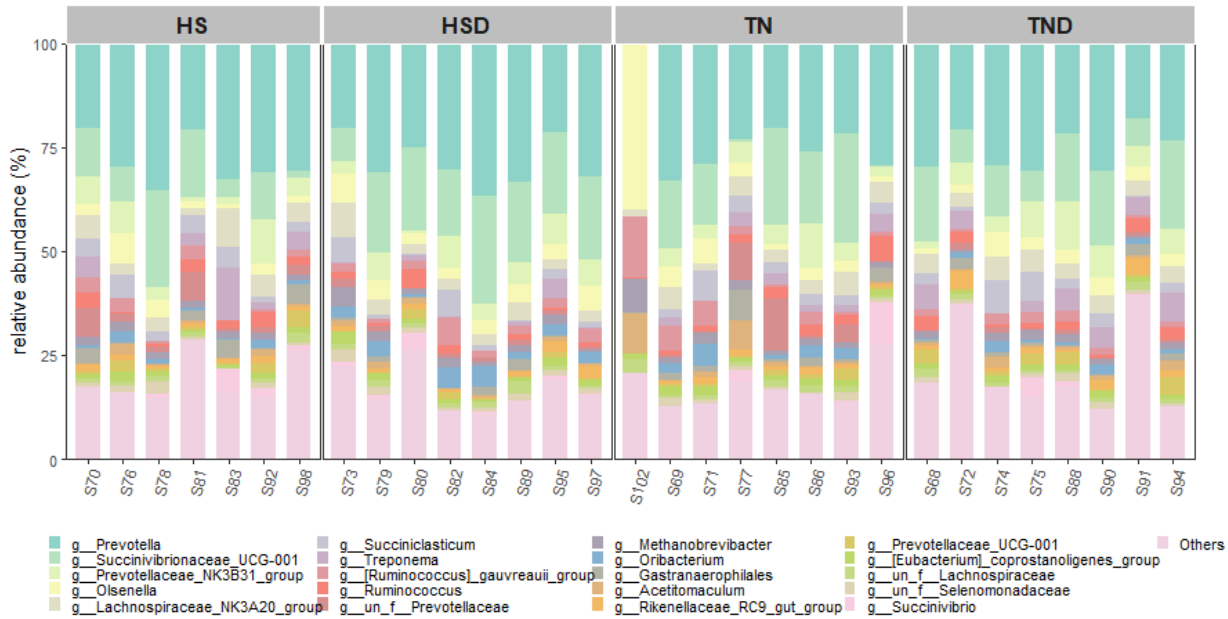
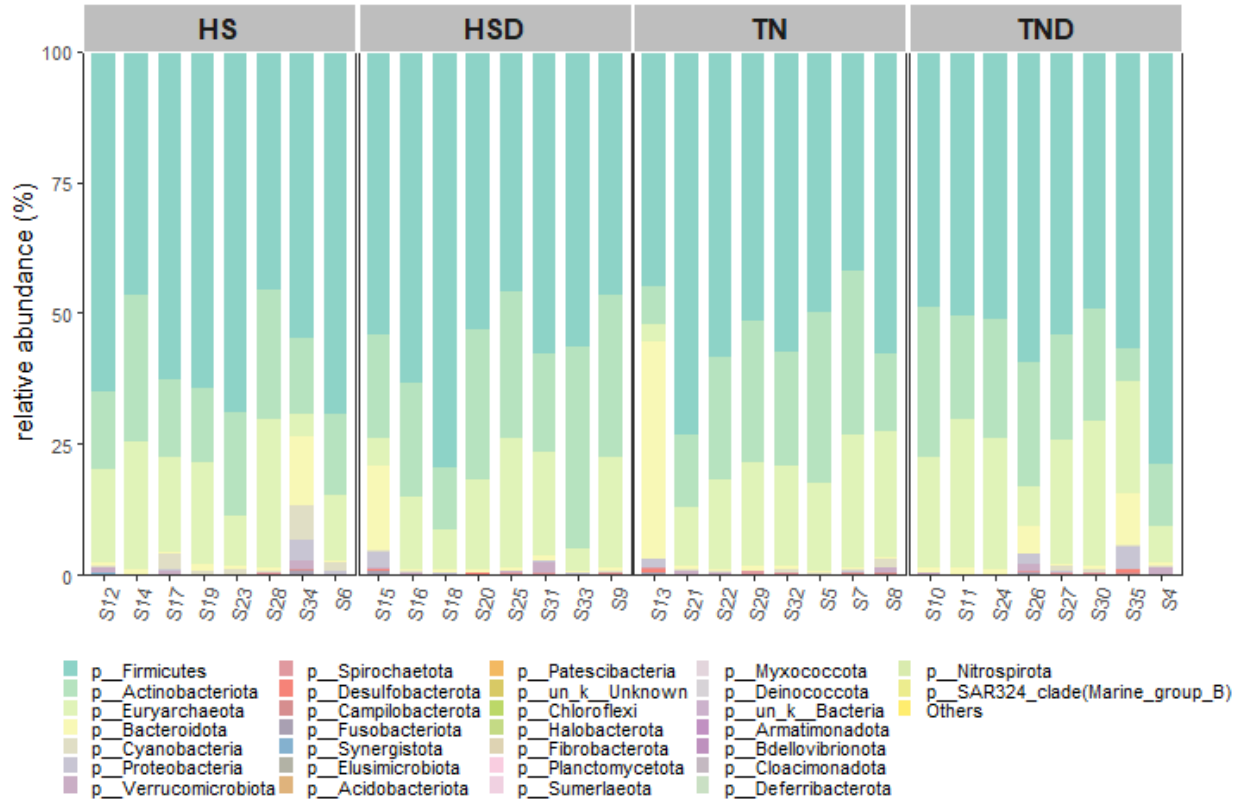


Figure 3-6. The taxonomic profiles for the relative phylum-level (A) and genus-level (B) abundance of each group in rumen. HS, heat stress; TN, thermoneutral; D, dexamethasone; S, saline solution.

A



B

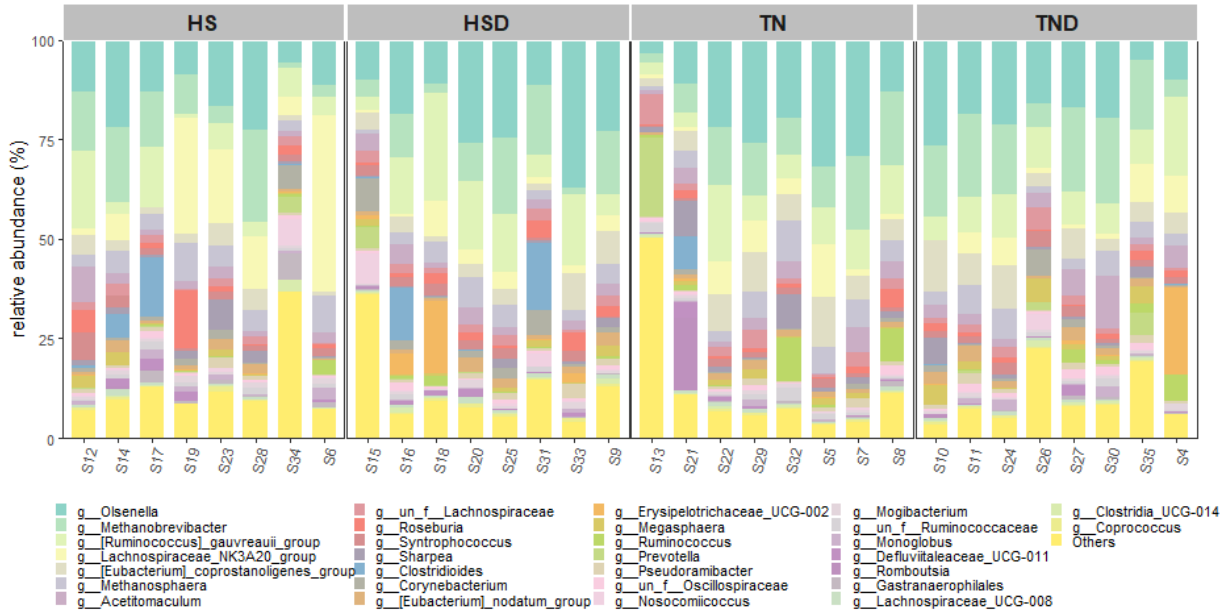
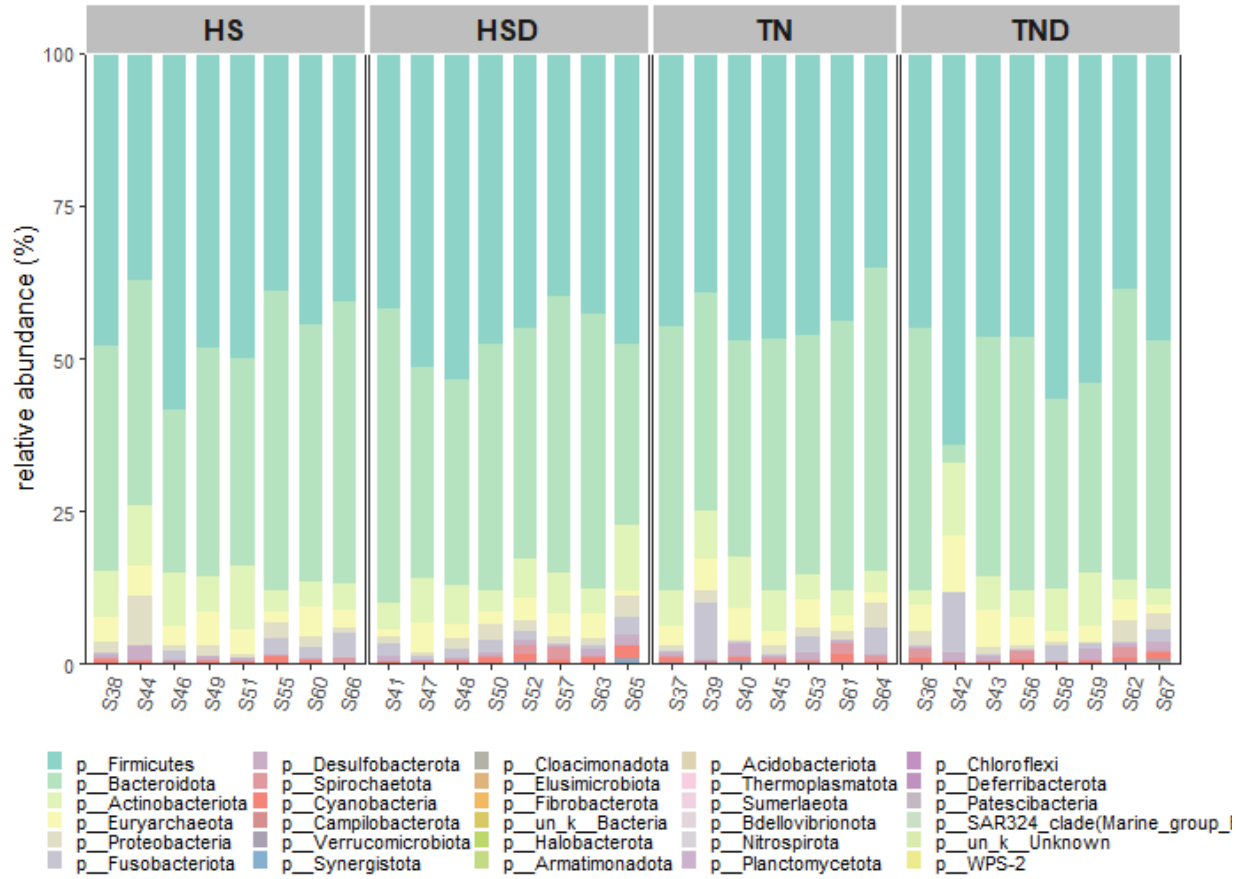


Figure 3-7. The taxonomic profiles for the relative phylum-level (A) and genus-level (B) abundance of each group in jejunum. HS, heat stress; TN, thermoneutral; D, dexamethasone; S, saline solution.

A



B

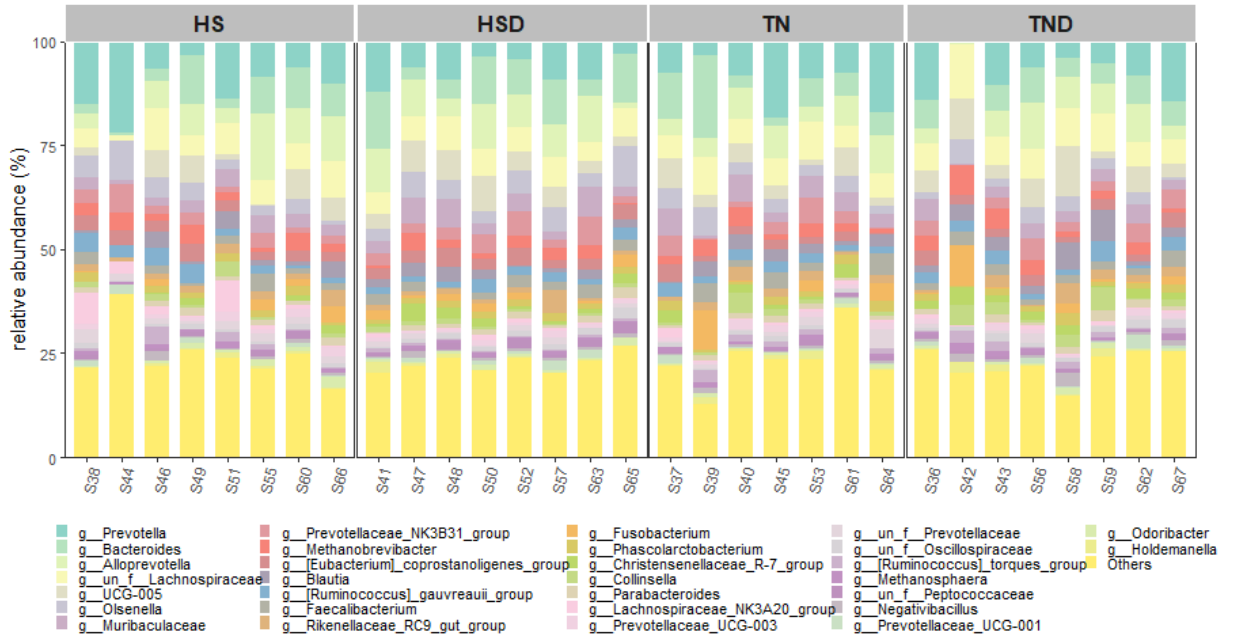


Figure 3-8. The taxonomic profiles for the relative phylum-level (A) and genus-level (B) abundance of each group in colon. HS, heat stress; TN, thermoneutral; D, dexamethasone; S, saline solution.

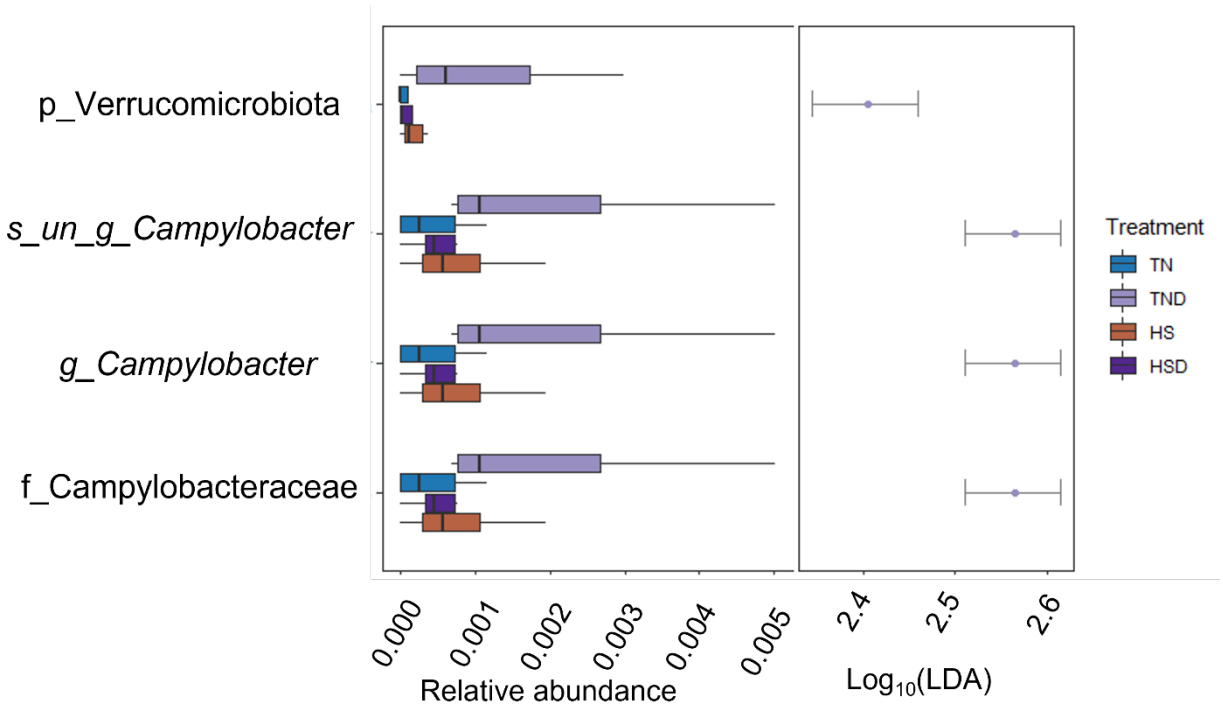


Figure 3-9.Linear discriminant analysis Effect Size (LEfSe) analysis on selected OTUs among the four groups in rumen. HS, heat stress; TN, thermoneutral; D, dexamethasone; S, saline solution. Only lineages with Least Discriminant Analysis (LDA) values >2.0 were displayed. The multiclass analysis strategy was less strict (one-against-all).

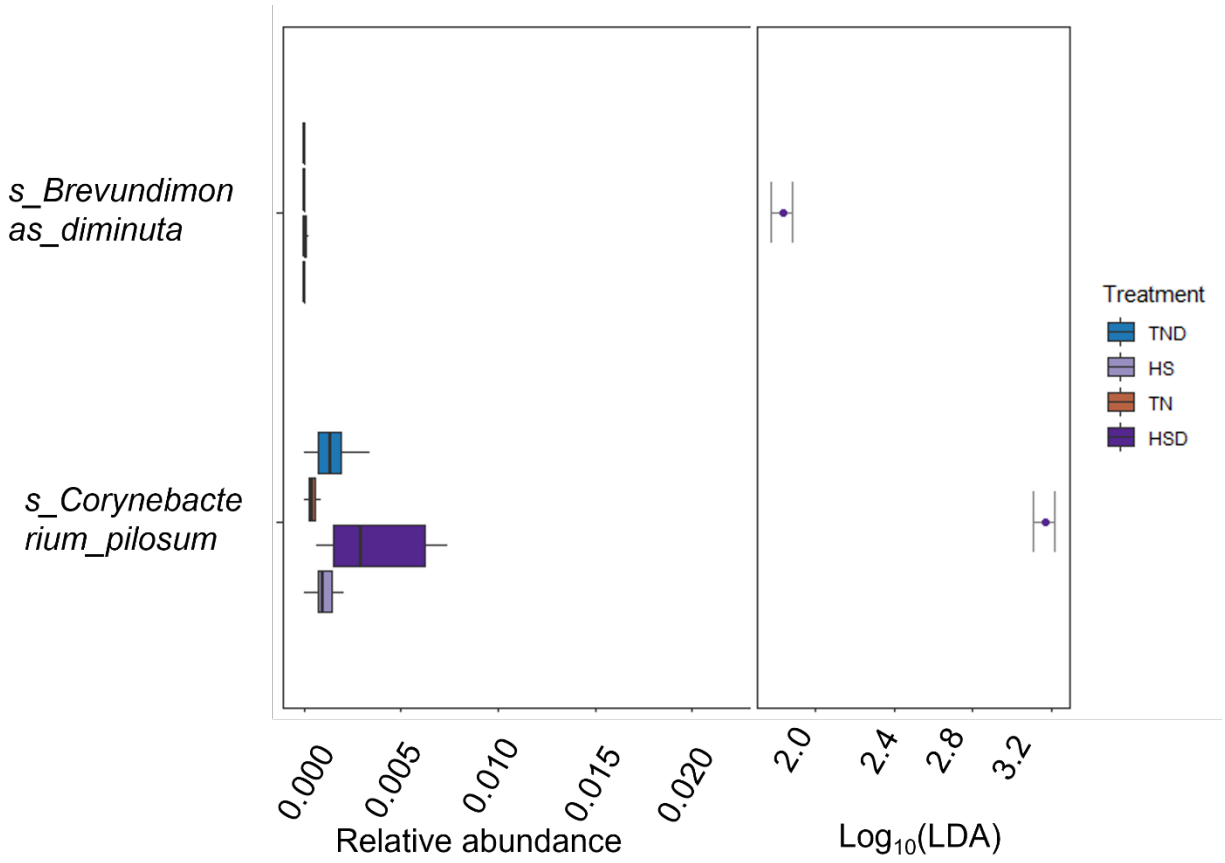


Figure 3-10. Linear discriminant analysis Effect Size (LEfSe) analysis on selected OTUs among the four groups in jejunum. HS, heat stress; TN, thermoneutral; D, dexamethasone; S, saline solution. Only lineages with Least Discriminant Analysis (LDA) values >2.0 were displayed. The multiclass analysis strategy was less strict (one-against-all).

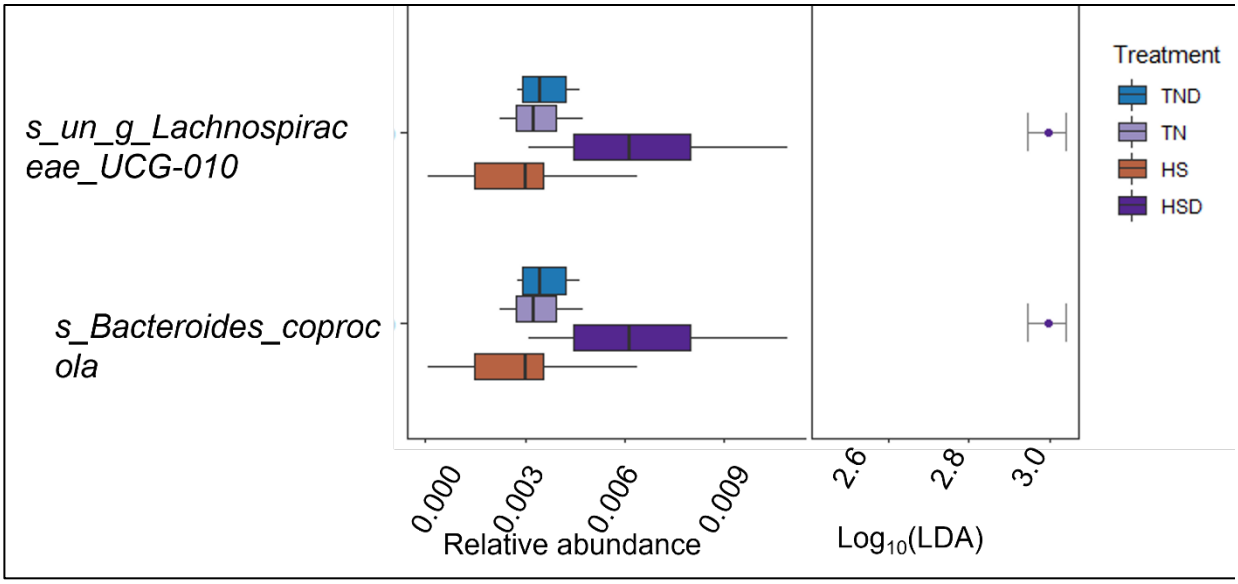


Figure 3-11. Linear discriminant analysis Effect Size (LEfSe) analysis on selected OTUs among the four groups in colon. HS, heat stress; TN, thermoneutral; D, dexamethasone; S, saline solution. Only lineages with Least Discriminant Analysis (LDA) values >2.0 were displayed. The multiclass analysis strategy was less strict (one-against-all).

SUPPLEMENTARY

Table 3-A1. Glucose concentration in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection at different time points on day 1.

Time	TN		HS		SEM	*P*-value				
	S N=8	D N=8	S N=8	D N=8		E	T	Time	E×T	E×T×Time
0530	93.5^{de}	90.1^{de}	94.4^{de}	92.3^{de}	7.91	0.484	0.686	<.0001	0.002	0.003
1200	129.4^{bc}	88.6^{de}	110.3^{cd}	148.3^{ab}						
1500	82.0^{e}	82.2^{e}	80.5^{e}	90.0^{de}						
1800	161.6^{a}	141.4^{ab}	136.5^{b}	141.3^{ab}						

^{abcde}Values within the same variable with different superscripts indicate significant differences in E×T×Time, $P < 0.05$.

Table 3-A2. Preprandial (time point 0530) glucose concentration in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection on day 1 to 5.

Day	TN		HS		SEM	*P*-value				
	S N=8	D N=8	S N=8	D N=8		E	T	D	E×T	E×T×D
1	94.1	90.9	93.8	91.4	5.68	0.360	<.0001	<.0001	0.643	0.523
2	92.0	106.9	89.7	107.1						
3	89.6	97.6	88.9	101.6						
4	93.6	140.5	91.8	122.4						
5	72.4	83.1	72.0	80.5						

Table 3-A3. Postprandial (time point 1800) glucose concentration in heat stress (HS) or thermoneutral (TN) calves with dexamethasone (D) or saline (S) solution injection on day 1 to 5.

Day	TN		HS		SEM	*P*-value				
	S N=8	D N=8	S N=8	D N=8		E	T	D	E×T	E×T×D
1	161.3^{abc}	141.0^{bcde}	136.7^{cdef}	141.7^{bcde}	10.51	0.936	0.108	0.0003	0.231	0.025
2	148.3^{abcd}	133.6^{def}	129.1^{def}	174.7^{a}						
3	123.0^{ef}	165.7^{ab}	127.1^{def}	145.6^{bcde}						
4	131.6^{def}	142.5^{bcde}	138.8^{bcde}	130.2^{def}						
5	115.6^{f}	107.8^{f}	116.0^{ef}	126.5^{def}						

^{abcdef}Values within the same variable with different superscripts indicate significant differences in E×T×D, $P < 0.05$.

CHAPTER 4 CONCLUSIONS

The studies in this dissertation were conducted to understand the role of inflammation in heat stress induced pathogenesis. Chapter 2 focused on identifying heat stress-associated alterations of inflammatory signals, tight junction proteins, and intestinal dysbiosis. Chapter 3 focused on testing whether the administration of a therapeutic dose of dexamethasone could reduce the potential inflammatory tone, improve intestinal morphology and functions, alter microbial composition and restore productivity in heat stressed calves.

Compared with saline, dexamethasone reduced intestinal permeability in HS calves. The decreased permeability observed in HSD calves may be associated with the drop in jejunum IL-6 concentrations. Previous work in heat stress calves showed an increase on intestinal permeability (Ríus et al., 2022).These findings validate our hypothesis that intestinal barrier malfunction attributed by heat stress may be alleviated by dexamethasone, that likely restored intestinal permeability through reduced inflammation.

We also observed that heat stress led to a numerical increase in neutrophil counts in plasma. This increase in neutrophil counts could have resulted in the inhibition of neutrophil migration to the inflammation site, specifically the intestine. Furthermore, the levels of myeloperoxidase (MPO), which is released by neutrophils, did not differ significantly between thermoneutral and heat-stressed calves in the jejunum, ileum, or colon, as discussed in Chapter 2, this may support the limited migration of neutrophil migration under heat stress.

We also hypothesized that thermal stress would increase pro-inflammatory cytokines, our results in Chapter 2 showed a decrease in multiple pro-inflammatory cytokines and an increase in anti-inflammatory IL-36RA in either plasma or jejunum after 7 days of heat stress. While in Chapter 3, heat stress increased the IL-6 in jejunum after 5 days of heat exposure but did not significantly change the IL-6 level in plasma. This could be attributed to the self-healing process

after a relatively long period of heat stress. Stress induced production of cortisol from the adrenal gland as a result of the cascade of events in the HPA axis can suppress the immune system by modulating cytokine secretion. Cortisol is elevated by acute heat stress, but not chronic heat stress (McBride et al., 2020). In line with that, the cortisol levels of heat-stressed dairy cattle were shown to be higher in comparison to their thermoneutral counterparts until day 5 (Marins et al., 2021). Furthermore, there was a discrepancy in the severity of heat stress between two chapters. In Chapter 2, the calves were subjected to a temperature of 37.8 °C for a duration of 10 hours. Conversely, in Chapter 3, the ambient temperature was maintained at 40°C for a period of 11 hours. The variance of heat stress severity in two studies may be attributed to the different responses (i.e., IL-6 concentration).

The abundance of *Butyrivibrio spp*, *Bacteroides spp*, and *Corynebacterium spp* bacteria increased in HS and HSD calves. The butyrate-producing characteristic of *Butyrivibrio spp* may contribute to the maintenance of gut integrity. *Bacteroides spp* possess a distinctive environmental sensing system that enable them to endure thermal duress. *Corynebacterium*, on the other hand, is frequently associated with udder health which is primarily located in water containers and walking areas. The increased abundance of *Corynebacterium* may be related to an increase in water consumption and time spent standing. Overall, thermal stress and dexamethasone administration altered the microbiome to promote the adaptive process.

Our research had limitations and shortcomings. Our objective in the first study was to compare the feed intake of two groups, but it turned out that heat-stressed calves consumed more feed than thermoneutral calves, and we did not measure the ZO-1 abundance at the protein level despite a significant difference at the mRNA level. Since the jejunum content was not collected during the initial investigation, sequencing for microbiome of the jejunum content was not

feasible. The primary limitation of the second study is in the complex effects of dexamethasone on several aspects of immune cells, cytokine transcription, nutrition metabolism, and epithelial proliferation. These multiple functions of dexamethasone pose challenges in accurately determining the primary factor responsible for the observed alterations in our results. Furthermore, the withdrawal time of dexamethasone is approximately 3-3.5 days, which would be economically inefficient if farmers administered dexamethasone injections during periods of heat stress.

For future studies, we suspect that the region-specific effects induced by heat stress are related to mucus thickness; gene-knockout rodents could be used to examine how mucus protects intestinal integrity from heat stress. Also, we hypothesize that the observed changes in intestinal permeability and histology are related to intestine stem cell proliferation; thus, future research may investigate how thermal stress affects intestine stem cell proliferation and the underlying mechanism. Furthermore, we only measured the change in microbiome abundance in our study, which is insufficient information to derive a conclusion. Another prospective study may be the effects of heat stress on changes in microbiome abundance and microbiota metabolites, as well as the relationship between these metabolites on immune response and intestinal integrity. Moreover, due to the multifaceted impact of dexamethasone on the immune system, it becomes challenging to determine the primary factor responsible for the alterations observed in our findings. Drawing from our results and recent investigations (Qing et al., 2020), it appears that IL-6 may exert a pivotal influence on stress-induced intestinal dysfunction, immune responses, and nutrient metabolism. Hence, the use of IL-6 receptor antibody, specifically Tocilizumab, may serve to inhibit the immune response regulated by IL-6 during instances of heat stress.

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VITA

Zhantao Yu was born and raised in Qinduying village, Xinye county, Henan province, China on February 3rd,1993 (Lunar calendar). He graduated from Xinye County No. 1 Senior High School. The following September, he entered Northwest A&F University and started his college life. He earned his Bachelor in Animal Science in 2016. Subsequently, he spent three years at China Agricultural University studying Ruminant Nutrition under professor Dr. Binghai Cao and Dr. Huawei Su. He then pursued a Doctor of Philosophy degree in Animal Science under the mentorship of Dr. Agustin Rius at The University of Tennessee, Knoxville.