NUTRITIONAL STRATEGIES TO IMPROVE UTILIZATION OF NUTRIENTS IN LACTATING DAIRY COWS EXPOSED TO HEAT STRESS

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DEDICATION

The work and research presented in this dissertation is dedicated to my wife, Jordan Kaufman, my parents (Michael and Jinger Kaufman), my family, and my furry companions (Abraham and Sampson). I also dedicate my work to the dairy industry and farmers that pour their devotion and faith into producing high quality dairy products for consumers. The efforts put forth by dairy producers has driven the need for continual research and education by researchers to improve their sustainability.

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ABSTRACT

Two experiments were conducted to evaluate levels of dietary crude protein (CP) and a novel postbiotic on nutrient utilization in heat-stressed cows. Experiment 1 consisted of evaluating the interaction effects between feeding a medium (16.1%) and low (12.5%) CP level to late-lactating cows in heat stress with or without cooling on milk production, inflammatory response, and in vitro rumen fermentation. Cooling decreased afternoon body temperature and increased yields of milk, lactose, and protein. Cooling tended to decrease the acute phase reaction. Compared with low CP, medium CP increased milk fat yield and tended to increase energy-corrected milk yield in cooled cows but not in heat-stressed cows. The medium CP diet increased milk yield but increased morning udder surface temperatures. Medium CP increased glucose and insulin and decreased free fatty acid concentrations in plasma compared with low CP. Compared with low CP, medium CP increased ammonia concentrations in rumen content of heat-stressed cows greater than cooled cows. Experiment 2 consisted of evaluating the effects of supplementing a novel postbiotic from *Aspergillus oryzae* (AO) fermentation at 3, 6, and 18 g/d compared with a control at 0 g/d on a basal diet given to heat-stressed cows in 2 periods [1) heat stress with cooling and 2) more intense heat stress with no mid-day cooling] on milk production and inflammatory response. An ex-vivo lipopolysaccharide challenge was conducted in period 2 to evaluate treatment effects on pro-inflammatory cytokine expression. In period 1, 6 g/d of AO decreased body temperature the greatest while increasing yields of milk, energy-corrected milk, and lactose and feed-use efficiency. In period 2, 6 g/d of AO increased energy-corrected milk, protein, fat, and lactose and decreased morning body temperature. The 3 g/d supply of AO decreased the acute phase reaction and cytokine expression from the lipopolysaccharide challenge. In conclusion, cooling cows increased milk production with medium CP, whereas

non-cooled, heat-stressed cows sustained milk production with lower CP. A novel AO postbiotic improved nutrient utilization for milk production and reduced the inflammatory response in heat-stressed cows. Overall, low dietary CP and postbiotic additives can be beneficial nutritional strategies for heat-stressed, lactating cows.

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CHAPTER 1.

LITERATURE REVIEW

INTRODUCTION

One of the most problematic issues in the United States' dairy industry is caused b high environmental temperatures and humidity that result in heat stress and decrease nutrient utilization for milk production. Heat stress increases excretion of urinary N by 16% (Kamiya et al., 2006), which can contribute to a decrease in efficient use of dietary protein for milk production by 41% and harmful N pollution to the environment (Kamiya et al., 2005). In addition, the use of energy substrates (e.g., glucose) is deprioritized away from the mammary gland to maintain homeostasis, the immune system, and thermoregulation during heat stress (Rhoads et al., 2009; Wheelock et al., 2010; Baumgard and Rhoads, 2013). The decrease in milk production from reduced nutrient utilization during heat stress can reduce the economic resiliency of dairy farm producers and can result in greater than $1.2 billion annual revenue loss (Key et al., 2014). Thus, strategies are important to provide solutions for increased utilization of nutrients for milk production under heat stress conditions.

Feed is the greatest cost to produce milk from dairy cows. During heat stress, lactating cow diets typically will have increased dietary protein and non-structural carbohydrate (i.e., concentrate) contents to adjust for reduced dry matter intake (**DMI**) and milk production. Most importantly, dietary protein contributes the greatest to increased feed costs and changes in nutrient utilization. Unfortunately, the current NRC (2001) recommendations for dietary protein provide excess amounts to dairy cows exposed to heat stress compared with lower dietary protein. For example, a high crude protein (**CP**) diet increased rumen ammonia-N and milk urea-N concentrations with no effects on DMI or milk yield compared with a lower CP diet in heat-stressed, mid-lactating cows (Arieli et al., 2004). Energy content of the diet affects heat-stressed cow responses. A higher energy diet with either increased fat or starch content increased milk

yield and feed efficiency in heat-stressed cows compared with a lower energy diet (Drackley et al., 2003). In addition, supplementing with feed additives based from *Aspergillus oryzae* (**AO**) decreased body temperature and increased milk production and feed-use efficiency of heat-stressed cows (Huber et al., 1994). Managing different nutritional strategies may provide beneficial metabolic and physiological changes to better support milk production in lactating dairy cows exposed to heat stress. Lowering the CP fraction of the diet will decrease feed costs and may improve nutrient utilization, and providing feed additives can benefit milk production of heat-stressed cows. The objective of this dissertation is to evaluate different nutritional strategies on improving production, metabolism, and inflammatory responses experienced by heat-stressed, dairy cows.

HEAT STRESS AND NUTRIENT USE IN LACTATING COWS

Heat Stress

Heat Stress and Assessment

Heat stress is defined as the inability of animals to successfully cope with extreme changes and thresholds of high environmental temperature and relative humidity. The threshold of heat stress where lactating dairy cows have an initial reduction in milk yield starts at a combination of environmental temperature and relative humidity equaling a temperature-humidity index (**THI**) at ≥ 68 (Cook et al., 2007; Zimbelman et al., 2009). Varying concentrations of environmental temperature and relative humidity achieve the THI of 68 threshold. For example, pairings of environmental temperature and relative humidity to reach THI of 68 range from 26.5°C and 0% to 22.0°C and 45%. Levels of heat stress above a THI of 68 will result in increasingly lower yields of milk and components (Zimbelman et al., 2009) and

can potentially lead to metabolic acidosis. Therefore, assessment of heat stress is necessary to make management decisions to limit loses on productivity in lactating dairy cows.

Individual dairy cows respond differently to heat stress; therefore, direct measurements from the animal are highly recommended to assess thermal load. The least invasive method of heat stress assessment is by counting flank movements to estimate respiration rate. Additional measurements can be done through estimations of body temperature. Body temperature is assessed in a variety of ways, but the gold standard would be internal body temperature from rectal or vaginal measurements compared with skin surface temperatures that are more variable due to external influencers (e.g., wind and moisture). Researchers compared rectal, vaginal, and skin surface temperature measurements from cows exposed to heat stress with THI estimation (Kaufman et al., 2018c). The results showed that afternoon vaginal temperatures had the best relationship (R^2 = 40%) and correlation with THI (r = 0.35) to predict a cow's thermal load during heat stress. In addition, afternoon skin surface temperature was more variable with a weaker correlation with THI (r = 0.23), but it was still a good estimator of thermal load. Skin surface temperatures should be restricted for use of confined animals and management for on-farm thermal load. In all, heat stress management can be conducted in various ways, but continuous vaginal temperature measurement is the most accurate method to determine the thermal load.

Thermoregulation

Heat stress causes an increased thermoregulatory adaptation through a thermoreceptor response by the skin. The response stimulates the somatosensory nervous system and hypothalamus (Christison and Johnson, 1972; Spiers et al., 2004), which results in vasodilation

near the skin and increased blood flow to peripheral tissues. The increase in blood flow to peripheral tissues increases heat loss via radiation and convection (Kadzere et al., 2002). Peripheral tissues consist mostly of the skin, which limits the blood flow of nutrients to internal tissues such as the rumen and digestive tract (Hales et al., 1984) and productive tissues such as the mammary gland (Baumgard and Rhoads, 2013). The NRC (2001) states that energy requirements for maintenance increase by up to 25%, which may derive from the heat exchange to relieve thermal load and the energy expenditure required. Therefore, the thermoregulatory mechanism undertaken to decrease thermal load reduces the use of nutrients for productive tissues and increases the energy requirement.

In addition to increased blood flow to peripheral tissues, lactating dairy cows will minimize internal heat production to limit the release of thermal energy. An adaptation to heat stress will decrease feed intake or consumption of total nutrients or dry matter (**DM**). For example, a peak THI of 82 decreased DMI by 6.6 kg/d compared with cows in thermoneutral conditions (Rhoads et al., 2009). Likewise, a peak THI of 76 (i.e., mild heat stress) decreased DMI by 1.4 kg/d compared with cows in thermoneutral conditions (Kassube et al., 2017). The decrease in DMI will limit heat produced through fermentation of feed in the rumen and digestion and absorption of nutrients (Fuquay, 1981). Decreased rumen digestion changes the molar ratio of acetate and propionate and decreases the total volatile fatty acid (**VFA**) concentration (Kelley et al., 1967), which can limit the supply and utilization to the cow (McGuire et al., 1989). Additionally, high producing cows are more susceptible to heat stress than lower-producing cows because of greater nutrient requirements (Bernabucci et al., 2010). Overall, heat-stressed cows have digestive and metabolic changes that help reduce the thermal load but decrease nutrient utilization for milk production.

Physiological Changes and Immunometabolism in Lactating Cows

In an effort to maximize heat loss, cows increase respiration rate in addition to surface temperature and sweating. A heat-stressed, dairy cow can have a significant increase in respiration rate by ≥ 20 to 60 breaths/min from a resting, thermoneutral rate of ~60 breaths/min. Lactating cows in hot environmental conditions at a farm had a 53 breaths/min increase in respiration rate compared with cows in cool environmental conditions (West et al., 1999). In agreement, respiration rate increased by 49 breaths/min from early-lactating cows exposed to a THI of 76 in environment chambers compared with cows in a THI of 60 (Lamp et al., 2015). The humidity forces dairy cows to rely on heavy panting. Panting causes a physiological change of the acid/base chemistry in the animal through loss of CO_2 with pulmonary ventilation, which can causes respiratory alkalosis (West, 2003). The removal of CO_2 alters the acid/base chemistry in the blood and increases blood pH, which also contributes to greater bicarbonate excretion in urine to maintain a 20:1 bicarbonate to CO_2 ratio (Kadzere et al., 2002). As a result, the buffering capacity from bicarbonate decreases in the rumen, which can contribute to rumen acidosis (Kadzere et al., 2002; West, 2003). The physiological changes from increased panting cause metabolic concerns and prevent proper utilization of nutrients.

The activation of heat shock proteins (e.g., HSP70) have been shown to stimulate nitric oxide synthesis, which is a vasodilator of blood vessels (Panjwani et al., 2002). Heat-induced increase in blood flow to the skin will result in decreased blood flow and oxygen supply to internal tissues (Hall et al., 2001; Bradford et al., 2015). Thus, heat stress can result in a deficiency of oxygen (i.e., hypoxia) reaching tissues such as the intestinal tract (Hall et al., 1999). As a potential result, tissues such as the intestinal tract will rely on anaerobic glycolysis to support energy requirements, which converts pyruvate to lactate (Berg et al., 2002). This is

highly regulated by the hypoxia-inducible factor 1 that results in translocation and presence of glucose transporters 1 and 3 (**GLUT1** and **GLUT3**) on non-insulin dependent tissues and leukocytes and downregulation of mitochondrial oxygen use (Airley et al., 2001; Papandreou et al., 2006). Thus, anaerobic glycolysis becomes the favored energy-producing pathway for cells and tissues, which produces an inefficient amount of ATP (i.e., net 2 ATP) from glucose via GLUT1 and GLUT3 compared with complete oxidative phosphorylation (Berg et al., 2002). In response, the cow requires greater concentrations of glucose for metabolism under anaerobic glycolysis compared with oxidative phosphorylation to support energy requirements during heat stress. An activated immune system is a major nutrient utilizer and can potentially contribute to greater blood flow for systemic inflammation.

The intestinal epithelium is a major oxygen-consuming tissue to perform digestion, absorption, and metabolism of nutrients; therefore, the intestinal epithelium is sensitive to low levels of oxygen (i.e., hypoxia) due to the need to sustain oxidative phosphorylation of energy substrates (Hall et al., 1999). As a result, hypoxia can damage intestinal epithelial cells during heat stress, causing an increase in intestinal permeability (Hall et al., 1999). Due to a damaged intestinal epithelium, lipopolysaccharide (**LPS**), present on the outer membrane of Gram-negative bacteria, infiltrates the lamina propria of the intestines through tight junctions and enters into systemic circulation (Koch et al., 2019) as shown in Figure 1-1. The LPS is recognized by toll-like receptor 4 (**TLR4**) on leukocytes (Muzio et al., 2000), intestinal (Abreu, 2010) and mammary epithelial cells (Ibeagha-Awemu et al., 2008), hepatocytes, adipocytes (Lin et al., 2000), and myocytes (Frisard et al., 2010). The LPS in circulation is bound to LPS-binding protein (**LBP**), an acute phase protein, and transported to TLR4 where it activates the inhibitor of κ kinase (**IKK**) and nuclear transcription factor κB proteins (**NFκB**; Figure 1-2) in the

LPS/TLR4 transduction pathway. The LPS/TLR4 transduction pathway increases the transcription of pro-inflammatory cytokines [interleukin-1β (**IL-1β**), interleukin-6 (**IL-6**), and tumor necrosis factor-α (**TNF-α**); (Lu et al., 2008)]. The LPS and cytokine presence at the liver reacts to produce large amounts of positive acute phase proteins (haptoglobin, serum amyloid A, and LBP) to propagate the detoxification of endotoxins (e.g., LPS) and bind free hemoglobin from red blood cell (**RBC**) lysis (Ceciliani et al., 2012). Thus, the inflammatory response is highly active and requires large amounts of nutrients, which limits substrate availability for milk synthesis.

In potential association with the immune response, heat stress causes hormonal changes, which alters the metabolic rate and utilization of nutrients in the animal. Insulin, catecholamines (e.g., epinephrine), and cortisol concentrations in plasma increase with exposure of heat stress in lactating cows (Collier et al., 1982; Itoh et al., 1998; Wheelock et al., 2010). Increased cortisol does not last long under chronic heat stress at an initial attempt to maintain the immune system and milk production; however, cortisol levels are reduced to limit metabolic heat production (Ronchi et al., 2001). The increase in these hormones supports homeorhetic processes for survivability and maintenance by changing energy utilization toward release of body heat and immune system activation. In addition, the hormonal response may serve to limit production of internal body heat.

Heat-stressed cows are in negative energy balance from decreased nutrient intake and increased energy requirements (NRC, 1989), but the additional energy requirement is not provided by mobilization of fatty acids from stored triacyglycerides in adipose tissue (Rhoads et al., 2009). High circulating insulin found during heat stress may prevent mobilization of fatty acids from adipose tissue (Wheelock et al., 2010). Researchers conducted an epinephrine

challenge in heat-stressed cows to understand lipid metabolism better. The epinephrine challenge stimulated lipolysis. However, the epinephrine challenge lacked a significant increase in free fatty acid concentrations in plasma from heat-stressed cows compared with the high increase in feed-restricted cows (Baumgard et al., 2011). Therefore, adipose tissue is not utilized to support needed energy requirements during heat stress, possibly because metabolism of fatty acids have a high heat increment (Houseknecht et al., 1998; Baumgard and Rhoads, 2007; Wheelock et al., 2010; Aggarwal and Upadhyay, 2012).

Skeletal muscle tissue has decreased insulin sensitivity during heat stress (Cole et al., 2011). Glucose uptake in the muscle is primarily insulin-dependent via glucose transporter 4 (**GLUT4**), which means muscle cells have decreased glucose uptake when there is high blood insulin concentrations. Low glucose uptake by muscle may be a method for sparing glucose for the immune system and other homeorhetic processes (Baumgard and Rhoads, 2013). Therefore, muscle cells experience insulin resistance, which is possibly derived from changes in downstream signaling to translocate GLUT4 to the cell membrane (Xie et al., 2016). The reduction in DMI during heat stress decreased the phosphorylation ratio of protein kinase B in muscle cells, which is a major initiator of GLUT4 exocytosis (Xie et al., 2016). Therefore, muscle tissue must utilize other nutrients (e.g., glycogen) to produce ATP, which can produce large amounts of lactate via lactate dehydrogenase (Baumgard and Rhoads, 2013). The lactate is then utilized for gluconeogenesis by the liver (Collins et al., 1980; Baumgard and Rhoads, 2013) and may also bind to G-protein receptors of adipocytes, which reduces lipolysis (Brooks, 2009).

Muscle is catabolized to provide amino acids (**AA**) for whole-body metabolism, which contradicts the anti-proteolytic nature of high plasma insulin levels observed during heat stress (Wheelock et al., 2010). This reinforces the idea that muscle tissue is insulin resistant during heat

stress conditions. In addition, the presence of increased cytokines (TNF-α and IL-1β) stimulates muscle catabolism to provide AA for metabolism (Zamir et al., 1992). Amino acids have a greater heat increment for metabolism compared with carbohydrates, which lessens their potential involvement in energy supply to the heat-stressed cow. The catabolism of protein most likely occurs to provide AA for gluconeogenesis and for synthesis of acute phase proteins and heat shock proteins as part of the inflammatory response (Baumgard and Rhoads, 2013; Cowley et al., 2015; Gao et al., 2017). The overall muscle metabolism during heat stress is functioning to spare glucose for the immune system (Baumgard and Rhoads, 2013; Kvidera et al., 2017b) and to rely on lactate for ATP synthesis and on AA for gluconeogenesis and protein synthesis.

Cooling Cows Exposed to Heat Stress

On farm during warm climates, dairy producers with proper management will provide single or multiple forms of cooling for their cows. Due to the inefficient ability to remove body heat, lactating cows need external cooling to decrease thermal load and improve physiological and metabolic processes. There are various methods to provide cooling to lactating cows such as shade, fans, misters, evaporative cooling or combination of those. The articles from Armstrong (1994) and Collier et al. (2006) highlight an extensive explanation of the optimal uses and strategies of cooling systems. Moreover, shade and evaporative cooling techniques do not affect the actual climate by reducing temperature and humidity (West et al., 1999) such as in the southeastern United States. Individual applications of these cooling systems are not as efficient and useful compared with when they are in combination. Therefore, cows housed under shade or cover and provided heat and humidity relief from fans and misters can improve milk production and physiological responses to a thermal load.

Application of shade easily improves thermal load with decreased body temperature and respiration rates by up to 0.9°C and 37 breaths/min and increased milk yield by 19% (Collier et al., 1981). Mister and fan cooling systems with shade reduced body temperature and respiration rates even greater and improved milk yield by 8.6% more than shade alone (Igono et al., 1987). In agreement, evaporative cooling decreased rectal temperature and respiration rate and increased milk and milk component yields greater compared with shade at a THI of 76.6 (Chen et al., 1993). Cooling from sprinklers and fans decreased rectal temperature by 0.3°C and afternoon respiration rates by 21 breaths/min and increased yields of milk fat and protein yields and feed-use efficiency (i.e., the amount of DMI producing fat-corrected milk yield) from postpartum cows experiencing a THI of 78.3 (Tao et al., 2012). In accordance with decreased thermal load and increased milk production, strong and sudden cooling downregulated genes expressed for acute phase reaction on heat-stressed cows that initiate an inflammatory response (Cheng et al., 2018). In addition, cooling heat-stressed cows increased utilization of fatty acids from adipose tissue for energy metabolism otherwise limited in non-cooled cows (Tao et al., 2012). From the previous work, lower basal insulin concentrations and response in peripheral tissues may have influenced the increase in circulating fatty acids. However, previous research demonstrated that cooling only provides a small influence on metabolic responses when cows are experiencing heat stress (Koubkova et al., 2002). Therefore, additional strategies need to address the metabolic changes that limit physiological and productive abilities directly impacted from heat stress.

Heat Stress on Nutrient Use

Rumen Fermentation

Heat stress affects rumen pH and fermentation from changes in microbial population and buffering capacity. As a result, heat stress decreases rumen pH (Mishra et al., 1970; Cowley et al., 2015), which can cause rumen acidosis. Heat stress may change the rumen microbial population, which can change the profile of VFA production from the rumen and decrease rumen pH (Table 1-1). A couple factors outside of the rumen contribute to the change in rumen pH. Saliva production and drooling increase during heat stress, which limit the supply and buffering capacity of saliva in the rumen (West, 2003). Additionally, a common dietary modification during heat stress is to increase the non-fibrous carbohydrate fraction of the diet, which gives the animal an energy-dense diet needed for increased energy requirements. An increase in readily fermentable carbohydrates changes the rumen microbes population to support starch digestion, which decreases rumen pH (Yadav et al., 2013) and saliva production. The changes in rumen fermentation of heat-stressed cows seem to minimize fermentation heat by poor forage digestion and modified VFA proportions. The rumen fermentation adaptations will decrease rumen pH and lead to acidosis.

The rumen microbiome is cooperative with the ruminant animal requirements, and the microbial population fluctuates with dietary and environmental changes. The microbial population dictates the production of VFA and additional fermentation byproducts. Volatile fatty acids are short-chain fatty acids (i.e., 1 to 5 C-length) that are commonly termed acetate, propionate, and butyrate. Additional VFA and byproducts come from rumen fermentation that include isobutyrate, isovalerate, valerate, lactic acid, ammonia, and gases (e.g., CO_2, and CH_4).

High grain diets fed to heat-stressed cows increase lactic acid production (Mishra et al., 1970). Lactic acid is a byproduct from amylolytic bacteria digestion of starch, and high concentrations seen in heat-stressed cows contribute to rumen acidosis. Amylolytic bacteria populations increase and change the molar proportions of VFA (Bernabucci et al., 2009). Therefore, the decrease in rumen pH may drive changes in VFA production (Dijkstra et al., 2012). For example, heat stress decreased rumen pH by 0.7 units compared with cows in thermoneutral conditions with a similar feed intake, which also decreased the acetate to propionate ratio (Gao et al., 2017). Compared with thermoneutral conditions at ad libitum intake, heat stress decreased acetate and propionate molar proportions of total VFA and increased butyrate proportions (Gao et al., 2017). As previously stated, energy supplied from the VFA is deprioritized from the mammary gland during heat stress. The low rumen pH increases VFA absorption across the rumen wall (Bloomfield et al., 1963; Mishra et al., 1970). Research has shown that chronic and acidotic conditions in the rumen will damage the rumen wall and increase rumen permeability, which allows for VFA and bacterial clearance (Steele et al., 2011). Overall, heat stress alters enteric microbial population, allows bacterial content release into circulation, and decreases production of substrates for milk synthesis.

Indirect Effect of Heat Stress from Reduced DMI

Heat stress indirectly affects milk production through a reduction in DMI (Table 1-1). Reduced DMI derives from the homeorhetic aspects of the animal to decrease metabolic heat production from digestion and nutrient metabolism (Fuquay, 1981; Eslamizad et al., 2015). Heat stress at a THI of 76 decreased DMI by 1.2 kg/d in an environmentally controlled chamber compared with thermoneutral cows (Kassube et al., 2017). Similarly, more severe heat stress at a THI of 82 decreased DMI by 6.6 kg/d in an environmentally controlled chamber compared with

thermoneutral cows (Rhoads et al., 2009). Previous research demonstrated that the reduction in DMI is associated with the increase in mean air temperature 2 days prior to the DMI measurement (West et al., 2003). They showed that for every unit change in the 2-day lag of mean air temperature, DMI decreased by 0.85 kg in Holstein dairy cows. Likewise, DMI decreased by 0.51 kg for every unit increase in THI (West et al., 2003). Therefore, DMI linearly decreases with increasing temperature and humidity (Johnson et al., 1962). In addition, heat stress affects the feeding behavior of lactating cows by changing feeding bouts to many small meals to very few large meals, which may also contribute to the reduction in DMI (Bernabucci et al., 2010). The very few large meals prolong rumen fill, concentrate the acid production in the rumen, lower rumen pH, and cause rumen acidosis. Overall, reduced DMI from heat stress can decrease milk production from a limitation in dietary nutrient supply and metabolic concerns.

The indirect effect of heat stress with reduced DMI results in decreased milk yield and milk components (i.e., milk quality). The response in milk yield can derive from milk lactose synthesis, as milk lactose is the major osmotic regulator of fluid milk production (Kronfeld, 1982). Wheelock et al. (2010) reported a 0.19 percentage unit decrease in milk lactose content from cows going from thermoneutral conditions to heat stress. In addition, the potential impacts on rumen fermentation and nutrient supply from reduced DMI may decrease milk protein production (Kelley et al., 1967). Heat stress decreased milk protein by 0.13 percentage units compared with when those cows were experiencing thermoneutral conditions (Rhoads et al., 2009). Consequently, decreased DMI from heat stress limits nutrient supply to the rumen and animal to meet lactation requirements.

Direct Effect of Heat Stress

Outside the presence of reduced DMI during heat stress, research demonstrated an influence on milk production directly from high environmental temperature and humidity (Table 1-1). Research methods utilize pair-feeding to remove the confounding effects of DMI between cows experiencing heat stress and thermoneutral conditions. The design allows for comparing the direct effect of heat stress on production and metabolic performance of lactating cows. For example, heat stress decreased milk yield directly by 7.5 kg/d compared with pair-fed cows in thermoneutral conditions; whereas heat stress decreased milk yield by 10.6 kg/d compared with well-fed cows in thermoneutral conditions (Rhoads et al., 2009). Therefore, heat stress directly is responsible for the 7.5 kg/d decrease in milk yield and a reduction in DMI is only responsible for the 3.1 kg/d decrease in milk yield. In agreement, heat stress at a THI of 84.5 decreased milk yield by 4.4 kg/d compared with cows in thermoneutral conditions that were pair-fed (Gao et al., 2017). Thus, heat stress can decrease milk yield directly by 17 to 29% in lactating cows, respectively. In addition, heat stress can directly affect milk protein content. Heat stress decreased milk protein percent from 2.68 to 2.57% compared with pair-fed counterparts (Gao et al., 2017). Likewise, a less severe heat stress (THI = 78) decreased total casein concentrations in milk by 3.3% compared with milk from pair-fed cows (Cowley et al., 2015). These results agree with a heat stress effect on milk protein synthesis machinery through the decreased activation of the mammalian target of rapamycin (**mTOR**) signaling pathway (Kaufman et al., 2018a), a reduction in milk casein synthesis (Bernabucci et al., 2002), and decreased blood flow and supply of AA (Gao et al., 2017). The direct heat stress effect on changes in metabolism drives the additional reduction in milk production.

Heat stress alters the metabolic response of lactating cows to limit milk production and improve removal of internal heat. Research showed previously that heat stress results in negative energy balance (Shwartz et al., 2009). Negative energy balance is a typical metabolic problem of early-lactating cows because of their inability to consume adequate nutrients to support a high nutrient requirement for milk production. However, heat-stressed cows have a reduction in milk production, but they have a greater energy need to maintain homeostasis and thermoregulation (Rhoads et al., 2009). In comparison to pair-fed cows in thermoneutral conditions, heat stress decreases glucose and free fatty acid concentrations and increases insulin and urea-N concentrations in plasma (Rhoads et al., 2009; Wheelock et al., 2010; Gao et al., 2017). Pair-fed cows and heat-stressed cows experience negative energy balance, but they have different metabolic responses. Pair-fed cows utilize fatty acids mobilized from adipose tissue for energy metabolism (Rhoads et al., 2009); however, heat-stressed cows break down muscle to supply AA for gluconeogenesis and inflammation (Gao et al., 2017). The reduction in milk protein content during heat stress may derive from a reduction in gluconeogenic AA and glucose concentrations (Gao et al., 2017) and increased glucose clearance from plasma (Wheelock et al., 2010). Muscle catabolism and dietary AA may supply the animal indirectly with energy sources to maintain homeostasis and thermoregulation and stimulate an inflammatory response (Kvidera et al., 2017a). Overall, the metabolic adjustment of heat-stressed cows deprioritizes nutrient supply to the mammary gland and milk production to prioritize homeostasis, thermoregulation, and the immune response.

PROTEIN AND NITROGEN UTILIZATION

Heat Stress on Protein and Nitrogen-Use Efficiency

Rumen Utilization of Protein

Heat stress affects the use of dietary protein in the rumen. Protein digestibility in the rumen varies depending on chemical structure, the microbial population, energy content of the diet, and passage rate (Satter and Roffler, 1975; Bach et al., 2005). The rumen microbes deaminate the majority of dietary protein from feed particles via secretion of proteolytic enzymes by at least 50% of the microbial population (Prins et al., 1983). The rumen microbes then utilize the free ammonia for growth, proliferation, and synthesis of microbial CP. Rumen microbes have a large requirement for ammonia to synthesize microbial CP. However, ammonia concentrations in the rumen increase from heat stress. For example, heat stress at a THI of 78 for 7 d increased ammonia-N concentrations by 28% compared with cows experiencing a THI of < 70 (Cowley et al., 2015). Subsequently, after cooling for an additional 7 d, the heat-stressed cows still had a numerical increase in ammonia-N concentrations compared with pre-heat stress conditions at < 70 THI. Likewise, heat stress increased ammonia-N concentrations in the rumen by 42% compared with cows in thermoneutral conditions and pair-fed and numerically increased ammonia-N by 38% compared with those same cows when well-fed in thermoneutral conditions (Gao et al., 2017). The results from these studies symbolize poor microbial CP synthesis and inefficient use of protein and ammonia in the rumen (Cowley et al., 2015; Gao et al., 2017). Heat stress may limit the capability of rumen microbes to utilize efficiently the ammonia from digestion for microbial CP synthesis. Overall, the degradable protein in the rumen is not

supplying optimal microbial CP to the cow, which can provide > 50% of the AA for protein synthesis in the cow.

Nitrogen-Use Efficiency

Heat stress limits the efficient use of N from the diet to support milk protein synthesis. Milk protein, milk urea N, urinary N excretion, and plasma concentrations of urea N, creatinine, and 3-methylhistidine are all estimators of N-use efficiency for milk protein (Schneider et al., 1988; Jonker et al., 1998; Kamiya et al., 2006). Heat stress can increase the amount of urinary N excreted in relation to N consumed by 12 percentage units (Kamiya et al., 2005). In addition, heat stress can decrease the amount of N consumed being captured as milk N by 3.0 percentage units compared with cows in thermoneutral conditions when both groups had similar DMI (Kamiya et al., 2005). As muscle catabolism biomarkers to provide AA for metabolism, heat stress increased plasma 3-methylhistidine concentrations and milk urea N concentrations (Kamiya et al., 2006). In agreement, past research demonstrated that heat stress increased urinary N excretion and plasma creatinine excretion compared with thermoneutral conditions (Schneider et al., 1988). The measurements establish that N consumed from the diet is not effectively utilized to synthesize milk protein. Therefore, heat stress drives N utilization toward extramammary tissue metabolism or increases the wasting of N via urine.

Amino Acid Availability

The effect on N utilization has direct implications on AA availability for milk production. However, research is limited for the full understanding of how and why heat stress shunts AA away from the mammary gland for milk production and how well those AA are absorbed from digestion. Recent research showed that heat stress increased AA transporter activity and

decreased activation of the mTOR signaling pathway in immortalized mammary epithelial cells (Kaufman et al., 2018a). An increase in AA transporter activity with a decrease in activation of the pathway that regulates protein synthesis translation may symbolize that AA are utilized for mammary epithelial cell integrity and cell mass instead of milk protein synthesis (Kaufman et al., 2018a). An additional explanation for the reduced availability of AA for milk protein synthesis is the AA consumption to synthesize heat shock proteins. Within the acute phase of heat stress, gene expression for heat shock protein synthesis increased, which function to fix damaged proteins by limiting misfolding of proteins, and expression for milk casein synthesis decreased (Hu et al., 2016). In addition, one may speculate that AA are being mobilized from muscle to support synthesis of acute phase protein and heat shock protein in association with the inflammatory response (Cowley et al., 2015; Gao et al., 2017). Research also demonstrates that AA are being prioritized for gluconeogenesis instead of productive parameters by reporting decreased systemic circulating glucogenic AA (e.g., Cys, Glu, Gly, Asp, and Arg; Gao et al., 2017). Therefore, heat stress decreases the availability of AA in multiple aspects, but research on absorption of those AA from the small intestine is minimal and would improve the understanding of how the AA from the diet are utilized.

Protein in the Diet and Nitrogen-Use Efficiency

RDP and RUP Digestion and Use

Dietary CP provided to ruminants can be divided into two rough estimates of degradability, which is demonstrated in Figure 1-3 for use toward milk protein synthesis. The two divisions are rumen degradable (**RDP**) and undegradable protein (**RUP**), and the combination of the two at the small intestine is called metabolizable protein (**MP**), which is the

accumulated absorbable true protein and AA. Rumen degradable protein is the protein and non-protein N (**NPN**) proportions of feed ingredients that are degradable in the rumen by microbes. The extent of degradability varies depending on feed ingredients, but the NPN sources (e.g., urea) are highly degradable. The rumen microbes utilize the RDP fraction of CP to deaminate protein and use the resulting ammonia to synthesize microbial CP. The microbial CP is digested and absorbed as AA and small peptides with the digesta as one proportion of MP (Bach et al., 2005). The current recommendations for RDP are to provide approximately between 10 and 11% of DM for high-producing cows that will optimize production of microbial CP (NRC, 2001). However, the current recommendations may overestimate RDP requirements for rumen microbes (Cyriac et al., 2008), which results in decreased rumen efficiency to convert ammonia from RDP into microbial CP. As a result, the ammonia is underutilized, absorbed in portal blood, and sent to the liver to synthesize urea for excretion in urine (Hristov et al., 2004; Mutsvangwa et al., 2016). Lowering RDP from 10.1 to 8.8% of DM increased the efficiency of conversion into microbial CP, which is observed from a 15% increase in N-use efficiency for capture as milk N in the mammary gland and an 18% decrease of predicted urinary N excretion (Cyriac et al., 2008). However, the influence of lower RDP has inconsistent results to sustain (Bahrami-yekdangi et al., 2016; Mutsvangwa et al., 2016) or decrease (Kalscheur et al., 2006; Cyriac et al., 2008) milk yield and components compared with recommended RDP levels. Balancing for RDP is an important aspect of a lactating dairy cow ration for proper rumen microbiome function and synthesis of > 80% of MCP and > 50% of the MP supply (NRC, 2001; Bach et al., 2005).

Rumen undegradable protein is the CP fraction that bypasses the rumen and avoids microbial degradation. The RUP proportion of MP can supply an easily measurable profile of AA to meet specific essential AA (**EAA**) limitations present from the ration (Bach et al., 2005).

The degradation efficiency of RUP can vary, but absorption of the resulting AA as part of MP is about 80% (Satter and Roffler, 1975; Owens et al., 2014). Similar to RDP, the digestibility and availability of RUP in the abomasum and small intestine varies greatly on the type of feed ingredient and processing of those ingredients. The feed ingredients that provide RUP are fibrous plant protein (e.g., alfalfa and cottonseed), non-fibrous plant protein (e.g. soybean, canola, and corn gluten meals), and animal protein of non-bovine specificity (e.g., blood and fish meals). The non-fibrous plant protein sources are processed commonly to decrease degradability in the rumen through chemicals, mechanical manipulation, and heating. Each source of RUP has a different AA profile with animal protein sources providing the highest levels of EAA needed for milk protein synthesis, while the plant sources vary (Erasmus et al., 1994). The NRC (2001) reported that milk yield responded linearly with increasing levels of RUP. However, the current recommendations for RUP are to provide approximately between 7 and 8% of DM for high-producing cows to provide sufficient AA to support protein synthesis (NRC, 2001). Research has shown that increasing RUP content from 4.5 to 7.9% of DM increased milk yield linearly by increasing the MP supply to the cows (Wang et al., 2007); however, there are more results that state no change in milk yield (Christensen et al., 1993b; Kalscheur et al., 1999; Bahrami-Yekdangi et al., 2014). Excess RUP has been shown to effect microbial CP synthesis (Christensen et al., 1993a) and result in large amounts of urinary N excretion and poor N-use efficiency for milk protein (Wang et al., 2007). Consequently, higher RUP content of the diet decreased milk protein yield compared with low RUP at ~5% of DM (Kalscheur et al., 1999; Flis and Wattiaux, 2005; Wang et al., 2007). Higher RUP was slightly beneficial in a low dietary CP diet at ~15% by increasing N-use efficiency for early-lactating cows but not for later stages of lactation (Kalscheur et al., 1999). Overall, dietary RUP can be fed at low amounts in the diet,

which will maintain milk production and improve N utilization as long as the AA profile meets individual AA requirements of the lactating cow.

Dietary protein has direct implications on the need for N recycling in lactating dairy cows. Nitrogen provided below requirements results in recycling of N from the blood and tissues to saliva or the rumen wall (Calsamiglia et al., 2010). However, N provided above requirements results in excess ammonia provided to liver for urea synthesis, which drives the need for AA catabolism to supply amino groups for urea synthesis (Castillo et al., 2001). Thus, the main source of recycled N is urea, which is used as NPN to support rumen microbial needs. A review of previous research has stated that 15 to 40% of N intake can be recycled if needed to meet requirements (Lapierre and Lobley, 2001). Improving capture of recycled N to the rumen would reduce urea excretion in urine, feces, and milk and decrease the need to provide a high RUP content, which would save on feed costs (Castillo et al., 2001; Bach et al., 2005). Therefore, increasing dietary RDP and RUP to increase milk production responses will decrease use of ammonia in the rumen and use of N for milk protein synthesis. For example, high RDP in excess of lactating cow requirements had no improvement in microbial CP synthesis but increased rumen ammonia concentrations by 25% and decreased milk N yield as a percent of N intake by 15% compared with a diet meeting RDP requirements (Hristov et al., 2004). In addition, a diet with low CP from low RUP (13% and 5.4% of DM basis, respectively) sustained milk protein yield from mid-lactating cows with greater N intake conversion into milk protein yield by 15% compared with a 15.4% CP diet with 7.9% RUP (Wang et al., 2007). Therefore, a lower RUP content can be possible to achieve increased use of N for milk protein synthesis when there is potentially greater recycling of N to the rumen from a low CP diet. Overall, N recycling is a

unique aspect in ruminant animals that the diet can modify and optimize with proper balancing of RDP and RUP.

Recommendations of RDP and RUP for Heat-Stressed Cows

Heat-stressed cows can benefit from reduced dietary RDP and RUP through increased dietary N captured as milk N in the mammary gland, sustained milk production, and changes in nutrient utilization. The current NRC (2001) recommendations are not ideal for heat-stressed cows as protein metabolism has a higher heat increment compared with carbohydrates (Noblet et al., 2001), and excess removal of urea has an additional energy expenditure (Reed et al., 2017). Past research demonstrated that lactating cows exposed to heat stress decreased milk yield and the amount of consumed N captured as milk protein when fed high dietary protein (Higginbotham et al., 1989; Huber et al., 1994; Arieli et al., 2004). Thus, lowering dietary protein will serve better to meet the homeorhetic processes to lower body heat and predictably increase the utilization of N and support an improvement on milk production responses. Recent research conducted on multiparous cows fed one of four diets with varying levels of RDP and RUP (10 or 8% RDP with 8 or 6% RUP) during warm climates to determine the utilization of nutrients for milk production. Lowering RDP and RUP together in the diet from 10 and 8% to 8 and 6%, respectively, at a THI of 81.8 sustained energy-corrected milk yield and increased N-use efficiency by 20% (Kaufman et al., 2017). This research showed that a RDP content reduction from 10 to 8% did not limit protein supply for microbial CP synthesis, but at 10% RDP (i.e. recommended level), a RUP content reduction from 8 to 6% limited MP supply and milk production responses. The sustained and improved production responses from lowering dietary RDP and RUP for heat-stressed cows derive from protein-driven changes in nutrient utilization. The reduction of RDP and RUP repartitioned N toward milk production and away from urinary

excretion (Kaufman et al., 2017). In addition, the 8% RDP and 6% RUP diet limited AA availability to peripheral tissues, which were utilized to support greater milk protein synthesis with an increase in milk protein yield efficiency by 27% compared with the 10% RDP and 8% RDP diet (Kaufman et al., 2018b). The low RDP and RUP diet also decreased 3-methylhistidine concentrations in plasma, showing a decrease in muscle protein catabolism, and limited urea synthesis (Kaufman et al., 2018b). Altogether, the low RDP and RUP supply to heat-stressed cows improved N partitioning to the mammary gland for milk production responses and increased utilization from the diet. Therefore, lactating cows exposed to heat stress require different dietary content of RDP, RUP, and MP compared with thermoneutral cows and lower than 10% RDP but greater than 6% RUP should be provided. An adequate supply of RUP should be considered under heat stress conditions to improve AA supply to the animal because of decreased N recycling and rumen MCP synthesis. However, the information on how the lower dietary RDP and RUP are interacting with the environmental conditions is currently limited.

ENERGY AND FEED ADDITIVE UTILIZATION

Energy Components in the Diet of Heat-Stressed Cows

Carbohydrate Feeding Recommendations during Heat Stress

The metabolism of dietary carbohydrates for milk lactose and de novo milk fat synthesis is shown in Figure 1-4. Fiber content in the diet of lactating dairy cows maintains the acid/base balance through saliva stimulation and slowing passage rate from the rumen (NRC, 2001). The neutral detergent fiber (**NDF**) and acid detergent fiber (**ADF**) proportions of the diet signify the fiber portion and result in acetate and butyrate production from the rumen. Those VFA are important for synthesizing milk fat. The common practice for heat-stressed cows is to decrease

the NDF and ADF portions of the diet from forages to increase supply of more energy-dense ingredients (West et al., 1999). A greater energy-dense diet supports the increased maintenance requirements during heat stress and improves the decrease on energy utilization (NRC, 1981). Although, research has demonstrated that increasing levels of NDF (from 30 to 42% of DM) did not affect DMI in heat-stressed cows compared with cows in thermoneutral conditions (West et al., 1999). The fiber portion may not be responsible for decreased DMI and milk production during heat stress and will benefit the acid/base balance and digestion of the diet. Non-forage byproducts can provide a more readily fermentable NDF source compared with forages that may provide fiber but not limit energy availability. Low replacement of immature corn silage with beet pulp (i.e. a non-forage fiber source) increased milk and milk protein yield and sustained DMI and milk fat in cows exposed to mild heat stress (Naderi et al., 2016). Researchers demonstrated changes in NDF to non-fibrous carbohydrates (**NFC**) amounts, VFA ratio in the rumen, and additional supply of RUP from forage fiber replacement with non-forage fiber sources (Naderi et al., 2016). Likewise, NDF reduction provided from forage by replacing with soy hulls decreased body temperature and increased DMI and milk yield in heat-stressed cows (Miron et al., 2008). Therefore, inadequate energy content of the diet is another aspect driving decreased DMI and milk production from heat-stressed cows seen in research (West et al., 1999). High-quality fiber content as NDF through non-forage fiber sources should be provided to heat-stressed cows in accordance with sufficient net energy of lactation to support production requirements and dietary protein utilization.

Non-fibrous carbohydrates in the diet supply readily usable energy (i.e. glucose) for rumen microbes and the main production of propionate (i.e. the core glucogenic precursor in ruminants) for the cow. Readily usable energy in the rumen helps increase microbial CP

synthesis and is needed in large amounts when the diet has high RDP to handle the excess ammonia (Broderick, 2003). As stated previously, high NFC content is common in the diet for heat-stressed cows to satisfy increased maintenance requirements (NRC, 1981). Higher concentrate levels compared with forage levels in the diet lowered body temperature and increased DMI potentially from a decrease in heat produced from rumen fermentation (Coppock et al., 1964; Reynolds et al., 1991). However, the readily usable energy worsens low rumen pH already experienced from heat stress through a decrease in saliva production, which causes concerns for rumen acidosis (West et al., 2003). Cumulative data suggests heat-stressed cows should be maintained on > 18% ADF and 28% NDF (DM basis) when providing higher NFC levels to optimize rumen function and health (West, 1999; Conte et al., 2018). Rations for heat-stressed cows require proper balance between fiber and concentrate levels as to maintain rumen pH and provide adequate energy.

Fat Feeding Recommendations and Supplements during Heat Stress

The metabolism of dietary fat for milk production is shown in Figure 1-5. Fat is an energy-packed nutrient per amount consumed by lactating cows. In addition, dietary fat has low heat increment compared with fiber and starch, which also support the energy content of the diet. With a lower heat increment, dietary fat provides a potential strategy to improve energy supply, nutrient-use efficiency, and body temperature in heat-stressed cows. Although, reported benefits of feeding dietary fat have been inconsistent for heat-stressed cows. Dietary fat at 6% (DM basis) impacted DMI and milk protein percent negatively in heat-stressed cows compared with a higher NFC and lower fat content diet with similar energy value; however, higher fat supply decreased morning respiration rates and rectal temperatures (Drackley et al., 2003). In addition, the high fat diet numerically decreased milk fat percent by 0.19 percentage units compared with the control

diet. Whereas, a 5% fat supplementation to a control diet did not affect DMI and milk production in heat-stressed cows (Knapp and Grummer, 1991). Supplementing with 5% fat (DM basis) numerically increased milk production for cows that calved during heat stress (Skaar et al., 1989). The inconsistent results from dietary fat may derive from the chemical nature of the fatty acids. Rumen fibrolytic bacteria have a low tolerance for unsaturated fatty acids (i.e., containing C double bonds), and the fatty acids go through biohydrogenation to be saturated by mainly *Anaerovibrio lipolytica* and *Butyrivibrio fibrosolvens* bacteria in the rumen (NRC, 2001). Almost the entirety of saturated fatty acids then leaves the rumen for absorption. However, incomplete saturation occurs, especially with a lower rumen pH, and results in the production of *trans*-10, *cis*-12 conjugated linoleic acid, which can depress milk fat (Moore et al., 2005). Therefore, ruminally inert unsaturated and saturated fatty acids are commercially available, as used by Skaar et al. (1989), to supply better fatty acid profiles to lactating cows beyond the rumen.

Fat supplements designed to bypass the rumen show benefits to lactating dairy cows exposed to heat stress when they consist mostly of saturated fatty acids. A dried fat supplement containing ~89% saturated fatty acids and ~9% unsaturated fatty acids designed to bypass the rumen decreased afternoon rectal temperature and increased yields of milk, fat, protein, and lactose from heat-stressed dairy cows compared with no fat supplement (Wang et al., 2010). Researchers state that the bypass fat supplement lowered heat of fermentation and metabolic heat and increased energy intake by replacing NFC in the diet. Likewise, the same saturated fatty acid supplement was fed to cows in hot climate and increased milk yield as reviewed by Huber et al. (1994). In agreement, similar results were observed by Skaar et al. (1989) providing a prilled fat supplement to heat-stressed cows. The rumen bypass fat may limit incomplete biohydrogenation in the rumen from producing conjugated linoleic acid that causes milk fat depression. In addition,

the greater energy intake supported efficient utilization of nutrients for milk production in heat-stressed cows. Therefore, rumen bypass fat may be an excellent nutritional strategy to supplement for the increased energy requirements of lactating dairy cows experiencing heat stress. The concern is the amount of research conducted to verify the use of these supplements long-term for heat-stressed cows.

Feed Additive Supplementation of Heat-Stressed Cows

Antimicrobials as Microbial Population Modifiers

Energy is typically the most limiting nutrient in lactating dairy cow diets, and reduced DMI and increased energy requirements exacerbate the limitation under heat stress conditions. Feed additives are external substances from the diet that enhance nutrient availability and efficiency. Antimicrobials such as monensin, commonly known as the brand name Rumensin (monensin salt) by Elanco, are ionophores that change the rumen microbiome, enhance ruminal fermentation and improve conversion of feed into milk and muscle (Tyler et al., 1992). Monensin increases the production of propionate from the rumen (Schelling, 1984), which may provide greater glucose supply from the liver to heat-stressed cows. Ionophores decrease Gram-positive bacteria in the rumen by changing their membrane potential with greater influx of Na^+ and H^+ and outflow of K^+. This inevitably reduces lactate and methane production in exchange for propionate. As a result, lactose synthesis will increase and consequently drive osmoregulation for increased fluid milk yield. Rumensin supplemented at 450 mg/d increased glucose production per amount of DMI in lactating cows exposed to THI of 82 (Baumgard et al., 2011). These results agree with an increase in propionate production from the rumen that increases hepatic gluconeogenesis (Schelling, 1984). However, heat-stressed cows supplemented with Rumensin

did not improve milk yield compared with cows not supplemented, but feed-use efficiency was increased by improving the amount of DMI used to produce fat-corrected milk yield (Baumgard et al., 2011). Therefore, heat stress has a large metabolic need for glucose to tissues other than the mammary gland. Research also showed that monensin increases feed-use efficiency and molar propionate proportion, protein digestibility, and N retention in the rumen (Salles et al., 2008). Holstein calves fed 85 mg/d of monensin increased the amount dietary N being used in the body during heat stress (Salles et al., 2008). The increase in protein digestibility and N retention contributed toward improved feed-use efficiency. Therefore, supplemental monensin or antimicrobial products can improve rumen fermentation to provide glucogenic precursors and enhance glucose and protein utilization, which leads to greater feed-use efficiency and energy availability in ruminants exposed to heat stress.

Fungal Feed Additives

Additional feed additives that originate from microorganisms can help improve energy supply and utilization in heat-stressed cows. Cultures from fungi (e.g, yeasts, and molds) promote a healthy rumen microbe population to increase fibrous plant material utilization and provide antioxidant properties and additional nutrients with the most common being from *Saccharomyces cerevisiae* and *Aspergillus oryzae*. The feed additives designed from these cultures can vary on formulation, usability, and function (e.g., probiotic or direct-fed microbials, prebiotic, postbiotic, plant extracts, or exogenous enzymes). Research has shown that fungal cultures benefit cows that are experiencing heat stress. For example, 30 g/d of *S. cerevisiae* culture increased yields of milk, protein, and lactose from heat-stressed cows (Bruno et al., 2009). Additionally, *S. cerevisiae* extract or fermentation product increased conversion of DMI into energy-corrected milk (**ECM**) yield in heat-stressed cows (Schingoethe et al., 2004; Zhu et

al., 2016). A dried and live *S. cerevisiae* supplement (i.e., probiotic) increased DMI, milk production, and feed-use efficiency and decreased rumen ammonia concentrations of heat-stressed cows (Moallem et al., 2009). A live yeast supplemention from *S. cerevisiae* decreased plasma HSP70 concentrations in transition dairy cows exposed to heat stress (Nasiri et al., 2019). Mold extract from *A. oryzae* decreased body temperature and increased feed-use efficiency and milk production of heat-stressed cows in a couple studies (Gomez-Alarcon et al., 1991; Chiou et al., 2002). However, not all research with similar fungal-derived products show beneficial changes for heat-stressed cows (Higginbotham et al., 1994; Yu et al., 1997; Shwartz et al., 2009; Boyd et al., 2012). The problem is that the mode of action is not clear, but research is making strides to uncover how fungal cultures and extracts benefit cows in heat stress. Therefore, fungal cultures and extracts show to have potential benefits for feed-use efficiency, milk production, body temperature, and immune response of heat-stressed cows, but research needs to reveal more physiological and metabolic changes that are occurring.

IMPROVEMENT ON NUTRIENT-USE EFFICIENCY IN HEAT-STRESSED DAIRY COWS

Lactating cows exposed to heat stress change their physiological and metabolic functions to survive the thermal load, which deprioritizes the mammary gland and favors thermoregulation. Heat stress worsens the current dairy industry revenue problems through decreased milk production and feed-use efficiency and increased health concerns and costs from feed and medical care. Nutritional strategies may have the potential to change the physiology and metabolism of heat-stressed cows to improve utilization of nutrients from the diet. From previous research, providing heat-stressed cows with lowered RDP and RUP improved metabolic responses to favor the mammary gland. However, most commercial farms most likely provide a

form of heat stress management (i.e., fans, misters, or shade). Therefore, it is essential to understand the dietary protein requirements better for heat-stressed cows under cooled-off conditions in order to address the problems through dietary protein management. Additionally, new fungal-derived products that supply fermentation products beyond the rumen may better support digestion, absorption, and milk production of heat-stressed cows. These feed additives have potential to benefit the challenges experienced during warm climates with greater understanding on how they influence inflammatory responses in cows. The following chapters hypothesize and test these nutritional strategies on improving the utilization of nutrients for lactating dairy cows experiencing heat stress.

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APPENDIX

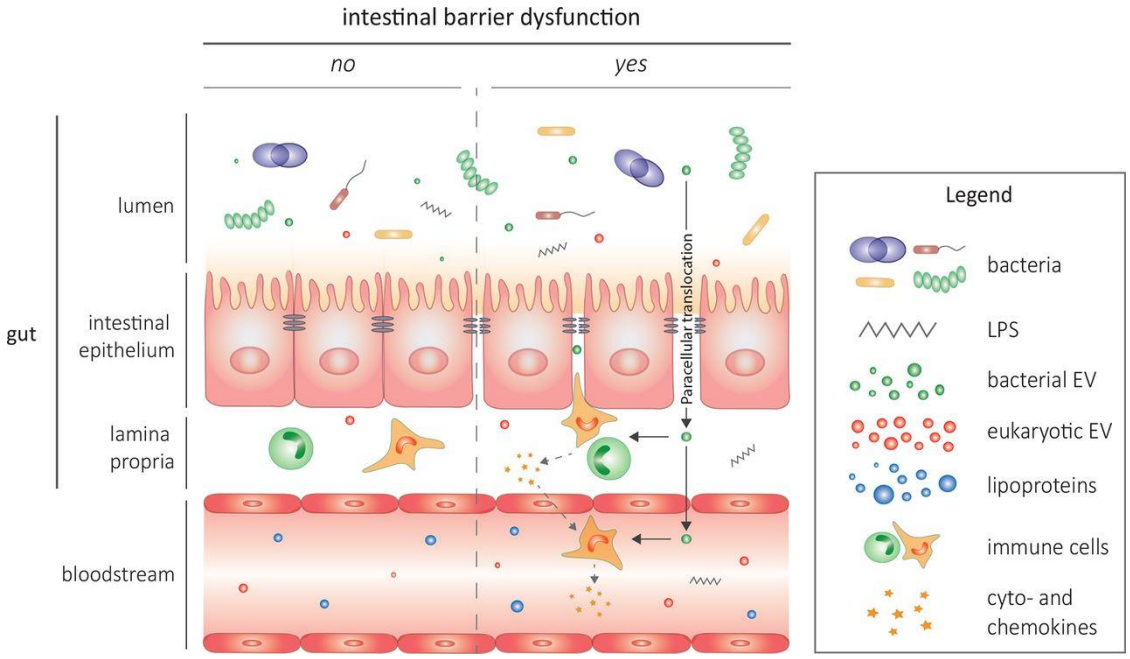

Figure 1-1. Damaged intestinal epithelium and barrier dysfunction with infiltration of lipopolysaccharide (LPS) and other bacterial components (EV = extracellular vesicles) and activation of immune cells. Gray bars in triplicate between intestinal epithelium are tight junctions. Displayed from Tulkens et al., 2018.

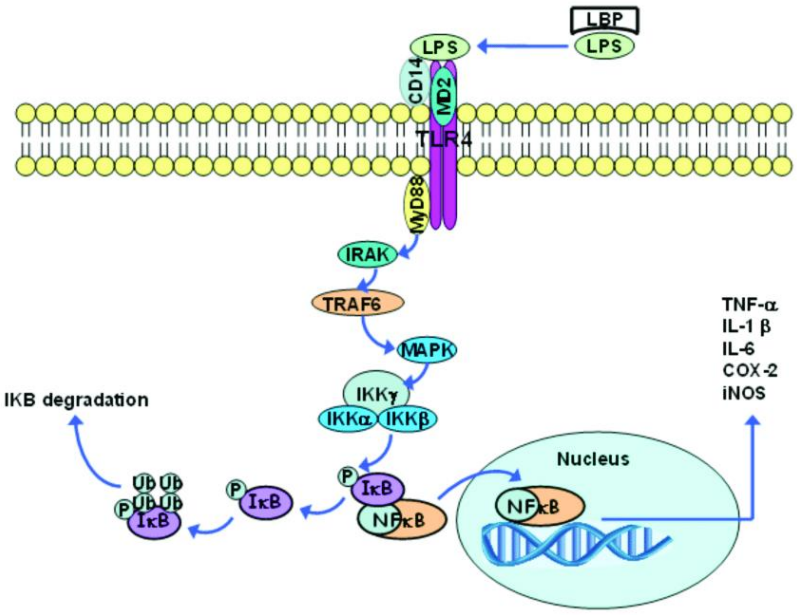

Figure 1-2. Toll-like receptor 4 (TLR4) signaling induced from lipopolysaccharide (LPS) bound to LPS-binding protein (LBP) that is activating the nuclear transcription factor κB (NFκB). CD14 = cluster of differentiation 14; MD2 = lymphocyte antigen 96; MyD88 = myeloid differentiation primary response 88; IRAK = interleukin-1 receptor associated kinase; TRAF6 = TNF receptor associated factor 6; MAPK = mitogen activated protein kinase; IKK = inhibitor of nuclear factor kappa-B kinase (α, β, γ); IκB = inhibitor of κB; iNOs = inducible nitric oxide synthase; COX-2 = cyclooxygenase 2; IL-1β = interleukin 1β; IL-6 = interleukin 6; TNF- α = tumor necrosis factor α. Displayed from Rogero and Calder, 2018.

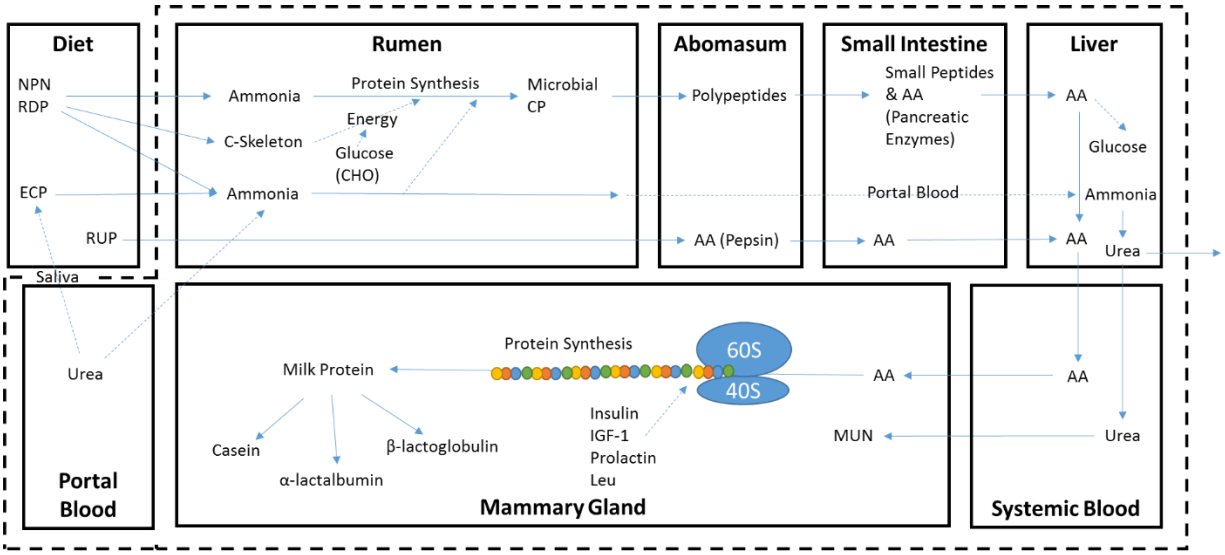

Figure 1-3. Dietary protein metabolism in dairy cows for milk protein synthesis. NPN = non-protein nitrogen; RDP = rumen degradable protein; RUP = rumen undegradable protein; ECP = endogenous crude protein; CHO = carbohydrates; CP = crude protein; AA = amino acids; MUN = milk urea nitrogen; IGF-1 = insulin-like growth factor 1.

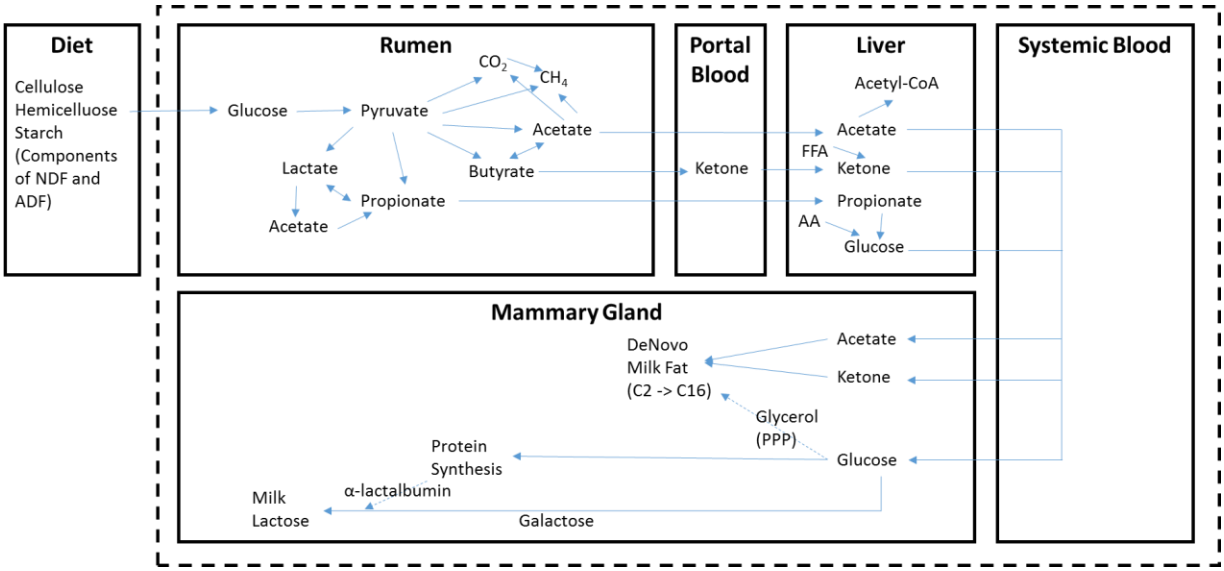

Figure 1-4. Dietary carbohydrate metabolism in dairy cows for milk lactose synthesis and de novo milk fat synthesis. CO_2 = carbon dioxide; CH_4 = methane; FFA = free fatty acids from adipose tissue; AA = amino acids from muscle; PPP = pentose phosphate pathway.

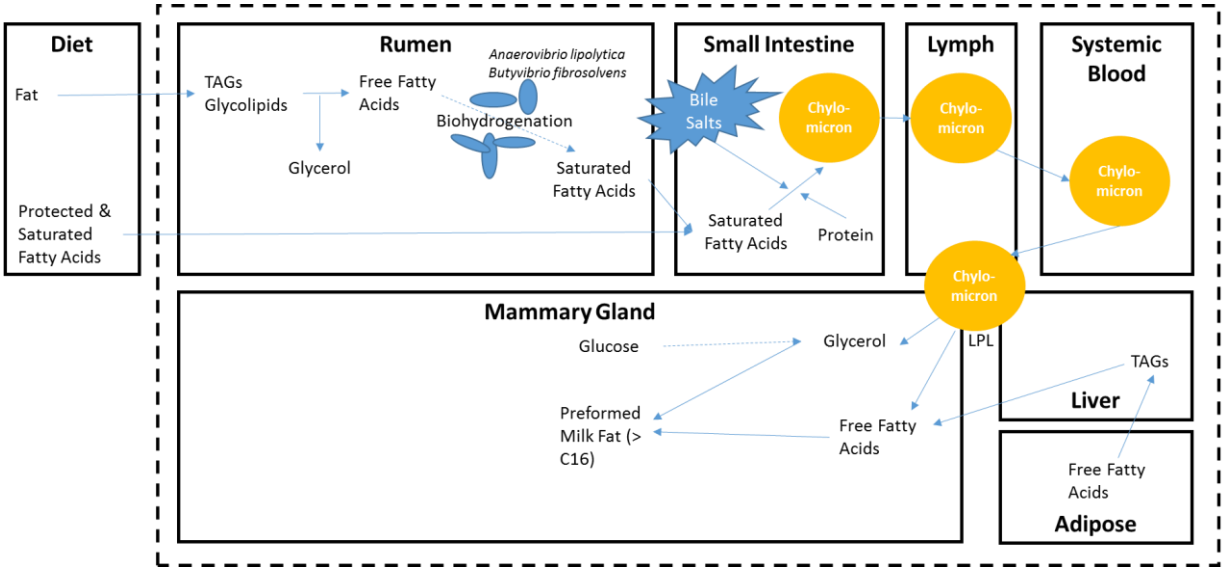

Figure 1-5. Dietary fat metabolism in dairy cows for milk fat synthesis. TAGs = triacyglyercides; LPL = lipoprotein lipase.

Table 1-1. Summary table of metabolite, hormone, and immune response directional changes [no change (-), increase (↑), and decrease (↓)] in lactating dairy cows from the rumen, small intestine, plasma, and mammary gland when under pair-fed and heat stress conditions compared with well-fed thermoneutral conditions

Item	Thermoneutral to Pair-Fed	Thermoneutral to Heat Stress	Pair-Fed to Heat Stress	Reference[10]
Rumen				
Acetate	↓	↓	↑	3
Propionate	↓	↓	↑	3
Butyrate	↓	-	↑	3
Microbial CP	-/↓	-/↓	-/-	2/3
Ammonia	-/↓	↑/↑	-/↑	2/3
pH	↑	-	↓	2, 3
Small Intestine				
Cytokine	?	?	↑	4
Plasma				
Glucose	↓/↓/-	↓/↓/-	↓/-/-	3, 9/5, 6, 8/2
Lactate	-	-	-	2
Total AA	-	↓	↓	3
Urea	↑/-	↑/↑	↑/↑	3, 8/2, 4, 5
Free Fatty Acids	↑	-	↓	2, 3, 5, 6, 8
Insulin	↓/-	↑/-	↑/↑	8/9
Epinephrine	-	↑	↑	5
Cortisol	?/-	↓/↓	?/↓	1/7
Prolactin	-	↑	↑	7
Cytokines	?	↑	?	1
Mammary Gland				
Lactose Yield	-/↓	↓/↓	↓/-	3/2, 9
Protein Yield	-/↓	↓/↓	↓/↓	3/9
Urea	-	↑	↑	3
Fat Yield	-/↓	↓/↓	↓/-	3/9
SCC	-	↓	↑	9

[1]Chen et al., 2018 (Only thermoneutral vs. heat stress); [2]Cowley et al., 2015; [3]Gao et al., 2017; [4]Koch et al., 2019 (Only pair-fed vs. heat stress); [5]Lamp et al., 2015; [6]Rhoads et al., 2009; [7]Ronchi et al., 2001; [8]Wheelock et al., 2010; [9]Xie et al., 2016.

[10]The slash between directional changes demonstrates which references correspond to that change.

CHAPTER 2.

THE USE OF COOLING AND DIETARY CRUDE PROTEIN AFFECTED MILK PRODUCTION ON HEAT-STRESSED DAIRY COWS

A version of this chapter was submitted for publication in the Livestock Science journal by: Jeffrey D. Kaufman, Hannah R. Bailey, Amanda M. Kennedy, Frank E. Löffler, and Agustín G. Ríus.

J. D. Kaufman conducted and organized the experiment, analyzed the data, and wrote the manuscript. H. R. Bailey collected samples and edited the manuscript. A. M. Kennedy analyzed rumen fluid samples and edited the manuscript. F. E. Löffler oversaw the analysis of rumen fluid data and edited the manuscript. A. G. Ríus directed the project, acquired the funding, and edited and approved the manuscript as the corresponding author.

ABSTRACT

The objective of this study was to determine the effects of cooling and heat stress at two dietary crude protein (CP) contents on milk production and indicators of energy and health status of lactating dairy cows. Thirty-six multiparous Holstein cows were randomly assigned to 1 of 4 treatments in a 2 × 2 factorial arrangement (9 cows/treatment). Treatments consisted of cooling (CO) or heat stress (HS) and diets containing medium CP content (MCP; 16.1% CP of dry matter) or low CP content (LCP; 12.5% CP of dry matter). The CO treatment provided heat abatement with sprinklers and fans that came on at > 20°C, whereas the HS treatment did not provide sprinklers and fans. Cows were housed in pens in a freestall barn, and treatments were imposed for 21 d during July and August. Compared with the CO treatment, HS increased afternoon rectal and vaginal temperatures (0.60 and 0.70°C) and afternoon respiration rate (27.6 breaths/min). Compared with the LCP treatment, MCP increased milk fat yield (23.0%) and tended to increase energy-corrected milk yield in CO but not in HS cows. Relative to the LCP treatment, MCP increased milk yield by 7.2%. Heat stress decreased yields of milk, lactose, and

protein (> 11%) compared with CO treatments. Rumen inoculum fermentation had a ~3-fold total gas production decrease in HS compared with the CO treatment. Relative to the LCP treatment, MCP increased glucose and insulin and decreased total free fatty acids in circulation. There was a trend indicating that HS increased plasma concentration of haptoglobin. In summary, CO cows responded to a CP stimulus by increasing energy-corrected milk and fat yield but HS cows did not respond. We propose that HS is associated with changes in rumen microbial activity and post-absorptive metabolism, which leads to losses in milk production.

INTRODUCTION

Heat stress in the southeastern United States results from a combination of high environmental temperature and humidity, which is largely one of the major issues facing this region's dairy industry (Tao et al., 2011). Studies have shown that physiological and metabolic changes in cattle exposed to heat stress are associated with reductions of milk synthesis (Rhoads et al., 2009; Wheelock et al., 2010). Nutritional interventions have been implemented to overcome negative effects of heat stress on dairy cattle, such as feeding nutrient-dense diets (e.g., energy, crude protein, minerals, and electrolytes). Some studies reported a decline on milk protein content or yield in heat-stressed cows compared with their thermoneutral counterpart (Cowley et al., 2015; Gao et al., 2017; Kassube et al., 2017), but this response was not observed consistently (Rhoads et al., 2009; Wheelock et al., 2010). Significant reductions in the availability of AA to the udder appear to be partially responsible for the effect of heat stress on milk protein content and yield (McGuire et al., 1989; Cowley et al., 2015; Gao et al., 2017; Ríus, 2019).

We recently reported that increasing dietary CP content from 14% to 18% [dry matter (DM) basis] did not affect yields of milk protein and energy-corrected milk in heat-stressed, multiparous cows (Kaufman et al., 2017). However, the assessment of dietary CP content on cooled cows was not conducted in this prior study. The evaluation of increasing CP content on cooled cows would provide preliminary information to determine the effectiveness of this approach to promote productivity (i.e. protein, fat, and energy-corrected milk yield) on heat-stressed cows. These outcomes would be beneficial to dairy producers worldwide.

Reports indicated that impairment of rumen function and microbial activity may contribute to losses in production of cattle exposed to heat stress (Johnson and Attebery, 1969; Warren et al., 1974; Schneider et al., 1988). However, recent studies reported that rumen concentrations of VFA and rumen total microbial growth were not affected in lactating cows during heat stress (Cowley et al., 2015; Gao et al., 2017). An increase of dietary CP content is expected to promote fermentation of organic matter and increase microbial activity and rumen VFA concentration, which would have positive consequences on milk protein synthesis and production (NRC, 2001). Therefore, the main objective of this study was to determine the effects of cooling and heat stress at two dietary CP contents on productivity and indicators of energy and health status of dairy cows. A second objective was pursued to characterize fermentability of rumen digesta associated with the treatments. We hypothesized that cooled but not heat-stressed lactating cows would increase productivity in response to increasing dietary CP content. To test this hypothesis, the effects of cooling and heat stress at two contents of dietary CP on milk components, plasma energy metabolites associated with carbohydrates and lipid metabolism, and biological indicators of animal health were measured in multiparous dairy cows.

MATERIALS AND METHODS

Animals, Housing, and Treatments

All experimental procedures were reviewed and approved by the University of Tennessee Institutional Animal Care and Use Committee. Thirty-six multiparous Holstein cows in late-lactation were used from the East Tennessee Research and Education Center, Little River Animal and Environmental Unit (**ETREC-LRD**) dairy herd in Walland, TN, USA. Cows were stratified based on DIM (219 ± 27.0 SD) and milk yield (38.3 ± 0.3 kg/d) and randomly assigned to 1 of 4 treatments (9 cows/treatment) arranged as a 2 × 2 factorial in a completely randomized design. This arrangement of treatments consisted of two environmental treatments [cooling (**CO**) and heat stress (**HS**)] and two dietary CP levels, adequate (17.6% CP, DM basis) and deficient (14.0% CP, DM basis) for 21 d. Cows were housed in two half-divided, adjacent pens (12 stalls/pen) in a sand-bedded freestall barn at the ETREC-LRD with free access to water and exposed to prevailing temperature and humidity of July and August.

Pens for the CO cows were equipped with sprinklers (over the feed bunk and activated every 10 min for 30 s) and fans (over feed bunk and stalls), which both turned on automatically with an ambient temperature > 20°C. The HS cows did not receive heat abatement from fans and sprinklers. Environmental treatments were imposed following the work conducted by research groups that successfully attained CO and HS treatments in cattle located in the southeastern U.S. (Weng et al., 2018; Fabris et al., 2019).

Dietary CP contents were achieved by replacing soybean hulls and corn grain with sources of RDP and RUP (i.e., urea, soybean meal, canola meal, and blood meal) and aimed to minimize alteration of EAA proportion in digestible protein (Kassube et al., 2017; Kaufman et

al., 2017). The diets were formulated to contain 17.6 and 14.0% CP; however, after chemical analysis of feedstuffs, we found lower contents of CP. Therefore, CP concentrations included 12.5% [low (**LCP**)] and 16.1% [medium (**MCP**)] on DM basis. To achieve adequate availability of fresh feed throughout the day, an equal amount of TMR was delivered at 0700, 1100, and 1600 h, and 9 cows were housed in each 12-stall pen. Feed bunk refusal of the HS treatment was removed daily at 0600 h, weighed, and used to determine feed delivered to cows in the CO pens. The daily amount of TMR consumed by all cows in the HS pens was determined and used to determine feed delivered to all cows in the CO treatment. If available, refused TMR on the CO pens was removed and weighed; however, this amount was negligible. The individual DMI was not measured in the present study, and arithmetic means of individual feed intake were determined using the difference between feed offered and refused as reported in lactating cows previously (Kay et al., 2013). This approach was appropriate to test the effects cooling and dietary CP contents had on heat-stressed dairy cows (Schank and Koehnle, 2009). For each treatment, the average DMI per cow (kg of DM/d) was estimated as the difference between feed offered and refused divided by the number of cows per treatment. Estimated mean DMI for individual cows were CO/LCP = 20.5 kg/d, CO/MCP = 19.9 kg/d, HS/LCP = 19.8 kg/d, and HS/MCP = 20.2 kg/d (± 1.70 SD). The daily feeding regime and the low stocking rate ensured availability of TMR to minimize cow variability in DMI. Diets were formulated for a late lactation cow with 725 kg of BW, 3.0 BCS, and 38.6 kg/d of milk yield with 3.5% fat and 3.2% protein (NRC, 2001). Diets contained 43% forage and 57% concentrate on a DM basis, corn and ryegrass silages, energy and protein concentrates, and a vitamin/mineral mix (Table 2-1).

Prior to the start of the study, cows received the MCP diet and were managed following ETREC-LRD guidelines for lactating dairy cows in summer climate (i.e. use of sprinklers

activated every 10 min for 30 s, fans, and three times a day feeding). These data were used to determine TMR allocated to the feed bunks and initial cow measurements. Cows were milked at 0600 and 1700 h in a double-eight herringbone parlor where milk yield was automatically recorded at each milking. Body weights and BCS, assessed by the same experienced participant (1 = emaciated to 5 = extremely obese explained by Edmonson et al., 1989), were recorded once weekly immediately after the 0600 h milking event.

Environmental Management and Assessment

In 10 min intervals, environmental temperature (°C) and relative humidity (%) were measured using Hobo External Temp/RH Data Logger (UX100-023; Onset Computer Corporation, Bourne, MA). Temperature-humidity index values were determined based on the Dikmen and Hansen (2009) equation (Figure 2-1), where T is the environmental temperature (°C) and RH is the relative humidity (%):

$$THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)].$$

Rectal and udder surface temperatures and respiration rates were measured individually in 6 cows/treatment at 0900 and 1600 h throughout the 21 d treatment period (Kaufman et al., 2017). Rectal temperatures were conducted using a Sharptemp V digital thermometer (Cotran Corporation, Portsmouth, RI; accuracy ± 0.10°C). Udder surface temperatures were conducted 15 cm from a clean, shaved patch of the left rear udder using an infrared imaging gun (FLIR TG165 Imaging IR Thermometer; Wilsonville, OR; accuracy ± 1.5°C). Vaginal temperatures were measured continuously on d 14 to 21 at 10 min intervals. Vaginal temperatures were conducted using water-resistant, temperature loggers (Thermochron iButton Device, Maxim Integrated, San Jose, CA; accuracy ± 0.01°C) inserted into a modified blank internal drug release

device (Elanco, Greenfield, IN) as previously described (Kaufman et al., 2017). Respiration rates were measured by counting flank movements for 15 s and reported as breaths/min.

Sample Collection and Analyses

Corn and ryegrass silage and grain mixes were sampled three times weekly, dried at 55°C, and ground through a 1-mm screen (Wiley mill; Arthur H. Thomas, Philadelphia, PA). Composite pooled samples of forages were chemically analyzed (Cumberland Valley Analytical Services, Waynesboro, PA) for starch, lignin, and crude fat using near-infrared spectroscopy (Foss 500; Foss North America; Table 2-2). Forage samples were chemically analyzed for total N (AOAC International, 1999; method 990.03) using a CN628 Carbon/Nitrogen Determinator (LECO; Saint Joseph, MI) and for minerals (AOAC International, 1999) using inductively coupled plasma spectrometry (Thermo iCAP 6300; Waltham, MA). Composite grain mix samples were chemically analyzed for total N (AOAC International, 1999; method 990.03) using a CN628 Carbon/Nitrogen Determinator (LECO); for starch (Hall, 2008); for ether extract (AOAC International, 1999; method 2003.05); and for minerals (AOAC International, 1999) using inductively coupled plasma spectrometry (Thermo iCAP 6300). All feed ingredients were analyzed for NDF (Van Soest et al., 1991) and for ADF (AOAC International, 1999; method 973.18).

Milk samples from individual cows were collected from morning and afternoon milkings on d -4, -3, -2, and -1 prior to treatments and on d 18, 19, 20, and 21 of the study. Milk samples were analyzed for fat, protein, lactose, SNF, and MUN by infrared analyses (Foss MilkoScan, Eden Prairie, MN; United DHIA, Radford, VA). Milk samples were analyzed for SCC by flow

cytometry (Foss Fossomatic FC; United DHIA). Energy-corrected milk was calculated with the equation provided by Tyrrell and Reid (1965).

Blood samples from individual cows were collected immediately after the morning milking by coccygeal venipuncture on d -1 prior to treatments and on d 21 of the study. Blood was collected in blood tubes with 140 IU of sodium heparin (Becton Dickinson and Co., Franklin Lakes, NJ). Plasma was separated from each sample via centrifugation at 1,500 × *g* for 20 min at 4°C and immediately stored in -80°C until metabolite and insulin analyses were conducted (Kaufman et al., 2018). Plasma urea-N (**PUN**; Infinity; Thermo Fisher Scientific Inc., Middletown, VA), total free fatty acids (Wako Diagnostics, Mountain View, CA), and BHBA (MilliporeSigma; Burlington, MA) analyses were conducted using commercial enzymatic assays on all plasma samples as previously described (Kaufman et al., 2018). Carbohydrate metabolism was assessed by measuring glucose and insulin concentrations on plasma samples. Acute phase proteins [haptoglobin (**HPT**) and serum amyloid A (**SAA**)] were analyzed on plasma samples using enzyme-linked immunosorbent assay according to manufacturers' protocols (bovine HPT: Immunology Consultants Laboratory, Inc., Portland, Oregon; multispecies SAA: Tridelta Development, Maynooth, County Kildare, Ireland). The intra- and inter-assay coefficients of variation for BHBA were 9.0 and 9.2%, for glucose were 2.3 and 2.2%, for fatty acids were 6.8 and 5.7%, and for PUN were 9.7 and 16.5%, respectively. The intra-assay coefficient of variation for insulin was 4.2%, for HPT was 20.5%, and for SAA was 7.1%.

In Vitro Gas Production and Fermentation of Rumen Content

Rumen fluid was collected on d 19 of the study by esophageal stomach tubing after morning milking and before feeding at 0700 h in 3 cows/treatment (n = 12 cows) and used to

conduct an in vitro analysis of total gas production, VFA, ammonia, and CH_4 concentrations. The first ~200 mL of collection was discarded to minimize saliva contamination. Rumen fluid was collected in pre-warmed glass bottles and kept in a 39°C water bath followed by immediate in vitro analysis. The in vitro analysis was conducted using radio-synced devices with pressure sensors to measure cumulative gas production during a 48 h incubation (ANKOM RF gas production system; ANKOM Technology, Macedon, NY) as previously described (McFarlane et al., 2017; Paula et al., 2017). Briefly, each bottle was filled with 0.5 g of dried, ground TMR sample that corresponded with each treatment. Artificial saliva buffer (McDougall, 1948) was used in a 4:1 ratio with rumen fluid and purged with CO_2/N_2 continuously. Aliquots of rumen fluid were purged to keep anoxic conditions and then filtered through 4 layers of cheesecloth. After addition of 40 mL of rumen fluid to the bottles, each bottle was flushed with CO_2 in three series to remove oxygen and create anoxic conditions. All bottles were enclosed in an air-ventilated shaker incubator (MaxQ 6000; Thermo Scientific, Waltham, MA) at 39°C and agitated at 50 rpm. The software used for data acquisition (Gas Pressure Monitor; Ankom Technology) was set to record the cumulative pressure every 10 min for 48 h. Valves were set to release pressure until the pressure reached 10 kPa. Cumulative gas production was determined at 3, 6, 9, 12, 24, and 48 h. After 48 h, CH_4 measurements were taken and determined using gas chromatography (Agilent 3000A MicroGC; Agilent Technologies, Santa Clara, CA) with argon as the carrier gas. Methane was not quantified in the liquid, as the headspace contains 95% of the CH_4 (Wiesenburg and Guinasso Jr, 1979). Following gas measurements, pH was determined. At the end of incubation, fluid was filtered and acidified by adding H_2SO_4 to a final concentration of 4 mM. Volatile fatty acids were analyzed using high performance liquid chromatography (Agilent 1200; Agilent Technologies; column temperature of 30°C and eluent flow rate of 0.6

mL min-1), and ammonia was analyzed using ion chromatography [Dionex ICS-1100; Sunnyvale, CA; column temperature of 30°C, eluent (20 mM H_2SO_4) flow rate of 1.0 mL min-1, and 4 mm suppressor at a current of 117 mA].

Statistical Analyses

The means ± SEM of pen THI were calculated using Proc Means of SAS 9.4 (SAS Institute Inc., Cary, NC). Data collected on d 14 to 21 were used in the statistical analysis of animal production, metabolism, and in vitro fermentation using the Mixed model procedure of SAS (Zanton, 2016). The analysis was conducted using individual cow as experimental unit as highlighted by Schank and Koehnle (2009) and studies testing the effect of cooling on heat-stressed cattle (Tao et al., 2011; Fabris et al., 2019). Measurements of body temperature and respiration rate were split into morning and afternoon and analyzed separately using the same approach reported on previous studies (Kaufman et al., 2017; Kaufman et al., 2019). Analysis of plasma concentrations of glucose, insulin, HPT, and SAA were conducted using data collected on d 21. If statistically significant ($P \leq 0.05$), a covariate adjustment was included in the analysis using data from d -7 to -1 prior to treatments for milk yield, from d -4 to -1 for milk components, and on d -1 for plasma measurements. Continual data measurements were analyzed as repeated measures in time. Repeated measures were included in the model to address non-random time constraints in the dependent variables. The autoregressive variance-covariance structure was used. The model included:

$$Y_{ijkl} = \mu + E_i + D_j + A \times (E \times D)_{ijk} + T_l + \text{“interactions”} + \beta(\chi)_{ijkl} + e_{ijkl},$$

where μ = the overall mean, C_i = the fixed effect of ith environmental (Env) treatment (i = CO and HS), D_j = the fixed effect of jth dietary CP (Diet) treatment (j = LCP and MCP), A × (E ×

$D)_{ijk}$ = the random effect of kth animal (A) within the ith and j^{th} treatment, T_l = the fixed effect of lth day (T) as repeated measures, "interactions" = the fixed effects of all E, D, and T interactions, $\beta(\chi)_{ijkl}$ = the covariate effect, and e_{ijkl} = the random error. Least significant differences were reported when Env × Diet interactions were significant. When not significant, the treatment interactions were removed from the model and main effects were reported. Significant differences were declared at $P \leq 0.05$ and trends were declared at $0.05 < P \leq 0.10$. All results are reported as LSM (± SEM).

RESULTS

Environment and Heat Stress Assessment

Pens had similar THI, which was expected because the cooling system in the current experiment was designed to cool the animals and not the environment (Figure 2-1; do Amaral et al., 2009; Tao et al., 2011; Fabris et al., 2019). For the CO treatment, the environment ranged from 73.8 to 78.3 THI at 1000 to 2000 h and from 66.7 to 72.5 THI at 2000 to 1000 h. The environment for the HS treatment ranged from 74.2 to 79.1 THI at 1000 to 2000 h and from 67.5 to 73.5 THI at 2000 to 1000 h. Heat stress increased (P < 0.01) afternoon rectal (38.4 vs. 39.0 ± 0.09°C) and vaginal temperatures (38.6 vs. 39.3 ± 0.09°C), morning and afternoon udder surface temperatures (32.5 vs. 34.9 ± 0.18°C and 34.8 vs. 37.7 ± 0.20°C, respectively; P < 0.01), and morning and afternoon respiration rates (37.2 vs. 46.3 ± 1.41 breaths/min and 48.6 vs. 76.2 ± 2.22 breaths/min, respectively; P < 0.01) compared with the CO treatment (Table 2-Table ***2-3***). Compared with the MCP treatment, LCP decreased (P = 0.04) morning udder surface temperature (34.0 vs. 33.4 ± 0.18°C).

Feed Composition

Observed CP contents of the diets differed from the initial formulations. Dietary CP values were lower than formulated (12.5 vs. 14.0% in LCP and 16.1 vs. 17.9% in MCP), which reduced contents of RDP from 8.5 to 7.4% (LCP) and 11.4 to 10.5% (MCP). Actual dietary CP decreased by 10.7% and 10.1% compared to the formulated value for the LCP and MCP, respectively. The values of CP decreased in LCP and MCP diets, but the LCP diet remained deficient and the difference between dietary treatments remained as designed at ~3.7% CP.

Milk Production

Compared with the CO treatment, HS decreased milk yield (32.1 vs. 28.2 ± 0.64 kg/d; $P < 0.01$; Table 2-4), lactose yield (1.51 vs. 1.33 ± 0.05 kg/d; $P < 0.01$), lactose percentage (4.81 vs. 4.73 ± 0.03%; $P = 0.05$), protein yield (0.97 vs. 0.86 ± 0.03 kg/d; $P < 0.01$), SNF yield (2.74 vs. 2.43 ± 0.08 kg/d; $P < 0.01$), and MUN (7.85 vs. 6.74 ± 0.41 kg/d; $P = 0.01$). Medium CP increased milk yield (29.1 vs. 31.2 ± 0.64 kg/d; $P = 0.03$; Table 2-4) and MUN (4.98 vs. 9.61 ± 0.41 kg/d; $P < 0.01$) compared with the LCP treatment. There was an Env × Diet interaction trend ($P = 0.08$) observed such that increasing dietary CP increased ECM yield (32.1 vs. 35.9 ± 1.08 kg/d) in the CO cows but not in the HS cows. Compared with the LCP treatment, MCP increased milk fat yield (1.13 vs. 1.39 ± 0.64 kg/d) in the CO cows but not in the HS cows (Env × Diet interaction, $P < 0.01$). Compared with the LCP treatment, MCP decreased milk protein percentage (3.15 vs. 3.01 ± 0.03%) for the CO treatment; however, MCP increased milk protein percentage (2.99 vs. 3.08 ± 0.03%) for HS (Env × Diet interaction, $P < 0.01$). Compared with the MCP treatment, LCP decreased milk fat percentage (4.35 vs. 3.74 ± 0.12%) for the CO treatment; however, LCP increased milk fat percentage (4.00 vs. 4.65 ± 0.12%) during HS (Env

× Diet interaction, $P < 0.01$). Compared with the MCP treatment, LCP increased milk SNF percentage (8.64 vs. 8.89 ± 0.04%) for the CO treatment but did not affect milk SNF percentage during HS (Env × Diet interaction, $P < 0.01$).

Plasma Concentrations

There was a trend for HS to increase ($P = 0.06$) plasma concentrations of total free fatty acids (164 vs. 199 ± 12.3 μEq/L) and HPT (0.30 vs. 1.73 ± 0.45 mg/dL) compared with the CO treatment but did not affect glucose, BHBA, PUN, insulin, and SAA (Table 2-5). Low CP decreased plasma concentrations of glucose (3.12 vs. 2.97 ± 0.06 mmol/L; $P = 0.05$) and insulin (28.2 vs. 14.8 ± 2.03 μU/mL; $P < 0.01$) compared with the MCP treatment. Additionally, LCP increased ($P = 0.05$) plasma concentrations of free fatty acids compared with the MCP treatment (163 vs. 199 ± 12.3 μEq/L).

In Vitro Gas Production and Fermentation of Rumen Content

An Env × Diet interaction ($P = 0.02$) was observed such that increasing CP increased in vitro ammonia concentration in the HS treatment (12.7 vs. 1.45 ± 0.94 mM) greater than that in the CO treatment (10.4 vs. 4.17 ± 1.20 mM; Table 2-6). Compared with the HS treatment, the CO treatment increased cumulative gas production after 48 h of incubation in the rumen liquor (Hour × Env interaction, $P = 0.02$; Figure 2-2; $P < 0.01$, 80.3 vs. 18.5 ± 17.1 mL/g of DM, Table 2-6). The HS treatment did not affect concentrations of VFA, lactate, and methane after in vitro fermentation of rumen liquor. Compared with the LCP treatment, increasing CP did not affect cumulative gas production and concentrations of propionate, butyrate, and lactate. Compared with the LCP treatment, the MCP treatment increased ($P < 0.01$) CH_4 production in vitro (15.4

vs. 20.9 ± 1.20 g/mL) and tended to increase (P = 0.10) acetate concentrations (26.1 vs. 32.8 ± 3.55 mM).

DISCUSSION

In this study, cows were exposed to housing and managerial conditions employed by dairy producers worldwide. The impossibility to control climate conditions may alter the responses to HS and CO treatments (Collier et al., 1982; Fabris et al., 2019). To this end, testing the hypothesis that CO but not HS cows would increase productivity in response to additional CP stimulus requires confirmation that HS and HS-abatement (i.e., CO) treatments were achieved. The THI data indicated that the barn THI exceeded 68 on average ≥ 10 h/d in this study. Elevated body temperature and respiration rates in the afternoon confirmed the heat strain from the HS treatment. As expected, the decrease of afternoon rectal, vaginal, and udder surface temperatures and respiration rates from the CO treatment suggests that the cooling system effectively alleviated the heat strain on these cows compared with the HS treatment. Previous studies conducted under similar housing conditions showed results of body temperature in agreement with those observed in the present study (do Amaral et al., 2009; Tao et al., 2011; Weng et al., 2018; Fabris et al., 2019).

The chemical analyses of the feed ingredients indicated that the concentrate mix of the LCP and MCP treatments had lower CP contents than target values. Similar results have been reported in previous studies that manipulated dietary CP (Rius et al., 2010; Kaufman et al., 2017). The protein supplements (i.e. soybean meal, protected soybean meal, and canola meal) likely had lower CP content than tabular values (NRC, 2001); however, the difference in CP content between the two dietary treatments was achieved (~3.7%). As planned, the LCP

treatment was deficient in RDP and RUP, whereas the MCP treatment provided adequate levels of RDP and RUP for high-producing dairy cows. The ~95% increase in MUN concentrations (from ~5 to 9 mg/dL) provides further evidence that an adequate level of CP was present in the MCP treatment and not in the LCP treatment (NRC, 2001). The adequate and deficient contents of CP can be estimated because the concentration of MUN is tightly associated with dietary CP content [R^2 = 0.84; (Broderick and Clayton, 1997)]. In summary, dietary CP contents were adequate to determine the effects of providing high and low CP in the diet of cooled and heat-stressed cows.

An increase of dietary CP supported greater ECM yield by 12% and milk fat yield by 23% from cows assigned to CO but not to HS. Thus, our hypothesis that CO but not HS cows would increase productivity in response to additional CP stimulus was accepted. Milk protein yield did not increase with greater dietary CP content; however, milk protein yield was increased by implementing heat abatement (i.e., CO treatment). Milk protein percent declined in CO cows that were fed the MCP treatment relative to their counterparts fed the LCP treatment, indicating a dilution effect. Milk protein percent increased in HS cows that were fed the MCP treatment relative to their counterparts fed the LCP treatment, but milk protein yield was not different between MCP and LCP cows. In comparison to the current study, previous work showed that increasing CP from 14 to 18%, by manipulating RDP and RUP contents, did not result in greater ECM and milk protein yields in multiparous cows exposed to heat stress (Kaufman et al., 2017). Previous studies have shown an impaired availability of AA in heat-stressed, lactating cows (McGuire et al., 1989) and pigs (Morales et al., 2016), although the precise mechanism elicited by HS remains unknown. Therefore in the current study, it is possible that the absorption of AA was reduced, which limited substrate availability for milk protein synthesis.

The responses elicited by the MCP treatment were associated with distinct changes in post-absorptive metabolism of carbohydrates and lipids. Compared with the LCP treatment, MCP increased glucose and insulin while reduced total free fatty acids in circulation. Quantitatively important substrates for hepatic gluconeogenesis are propionate and AA. Hepatic AA availability and gluconeogenesis increased possibly in response to the MCP treatment, and the subsequent rise in glucose promoted elevated plasma concentrations of insulin. The MCP treatment was associated with greater glucose production and subsequently may have supported lactose synthesis, a major osmotic regulator of milk volume (Kronfeld, 1982). The elevated levels of insulin may have inhibited lipolysis in adipocytes and reduced mobilization of fatty acids (Zechner et al., 2012) because the MCP cows showed a decline in plasma concentrations of total free fatty acids. Circulating levels of insulin and free fatty acids associated with the LCP treatment may have mediated the use of free fatty acid in the udder of cows in the HS treatment. Previous studies showed that an increase of dietary protein content from 14 to 16% increased plasma concentrations of insulin and reduced mobilization of fatty acids (Kaufman et al., 2018). Therefore, the post-absorptive metabolism of energy was altered in response to CP content of the diet, which then contributed to changes in synthesis of milk components.

Cooling cows exposed to HS tended to reduce plasma concentrations of HPT. Plasma concentrations of HPT is used as a marker of inflammation because rising levels of this acute phase protein are associated with systemic inflammatory response (Lomborg et al., 2008; Giannetto et al., 2012; Bradford et al., 2015). Previous research in heat-stressed pigs is in agreement with the HPT results reported in our study (Pearce et al., 2013). Consistent with prior reports on heat-stressed animals, our results showed that HPT can be a plasma determinant for inflammation with onset of heat stress experienced by cows. An increased immune function

promotes the oxidation of nutrients (e.g., glucose) that would be used otherwise toward milk synthesis (Bradford et al., 2015). In the present study, cooling cows was associated possibly with a reduced systemic inflammatory response, which may result in greater use of nutrients to sustain synthesis of milk components.

Increasing CP did not result in greater cumulative total gas production. Cooling elicited a ≥ 3-fold increase in cumulative total gas production suggesting that the formation of CO_2 and hydrogen, among other gases, may have been increased. Total gas production in vitro is indicative of rumen microbial fermentation activity, primarily carbohydrates; thus, this result suggests that the fermentability may be improved in cows receiving the CO treatment. The analysis of products of in vitro fermentation of rumen liquor inoculum suggests that dietary CP and HS did not exert independent effects on ammonia concentration. In vitro fermentation of rumen liquor inoculum showed that the LCP treatment was associated with lower ammonia concentrations in the HS treatment compared with that in the CO treatment. Low concentrations of ammonia at this level are a concern because fiber-digesting bacteria use ammonia as the main source of nitrogen (Russell et al., 1992; Atasoglu et al., 2001; Belanche et al., 2012). Low ammonia concentration may affect fermentability of fiber, which may reduce acetate production. The LCP treatment was associated with a numerical decline in acetate concentrations, suggesting a possible impairment of fibrolytic microorganisms. In addition, the LCP treatment was associated with lower CH_4 concentrations, suggesting that fibrolytic fermentation was reduced and less hydrogen was available to methanogenic archaea (Robertson and Waghorn, 2002; Beauchemin et al., 2008). Collectively, our results suggest that MCP and cooling affect the rumen microbiome (activity, population, or both) and improve fermentation.

CONCLUSIONS

As expected, CO reduced cows' body temperature, and MCP increased the content of dietary protein. Cooled but not HS cows increased ECM and milk fat yield in response to increased dietary CP content. Increasing dietary CP increased levels of insulin in circulation, which then may have promoted the utilization of nutrients on insulin sensitive tissues and inhibited mobilization of fatty acids. The CO treatment promoted total gas production of rumen inoculum in vitro. We propose that changes in the activity of rumen microbiota and post-absorptive metabolism of energy contributed to the observed changes in milk production.

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APPENDIX

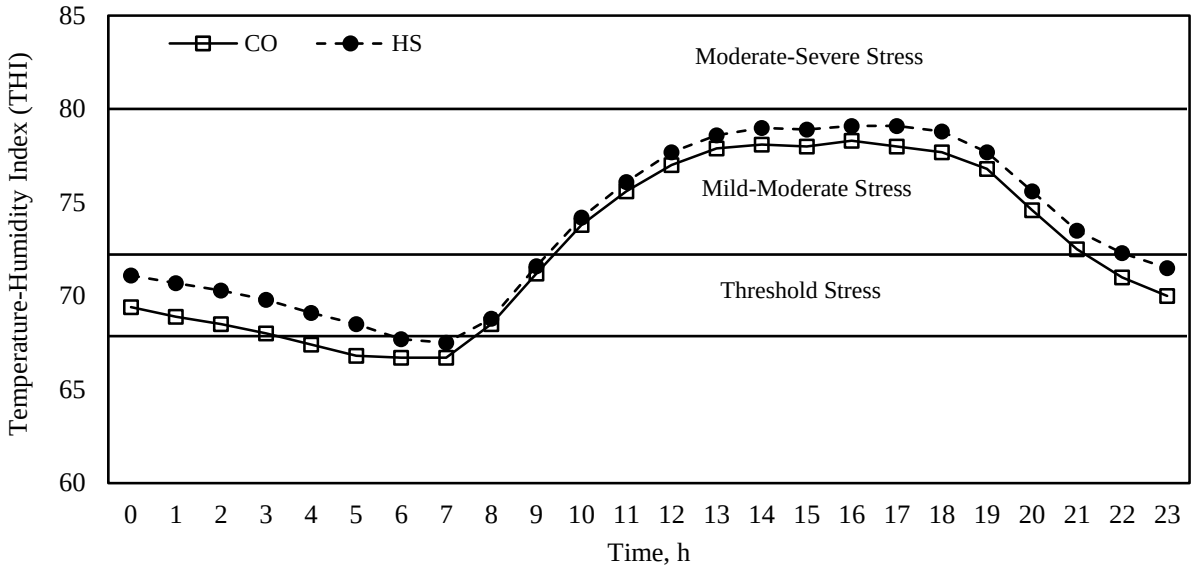

Figure 2-1. Temperature-humidity index (THI) averaged over the hour for the last 7 d of the treatment period (± 3.44 SD). Cows from cooling treatments experienced a circadian pattern of daily summer temperatures and relative humidity. Cooled (CO) treatment received consistent heat abatement consisting of fans and sprinklers. Heat-stressed (HS) treatment did not receive heat abatement throughout the treatment period.

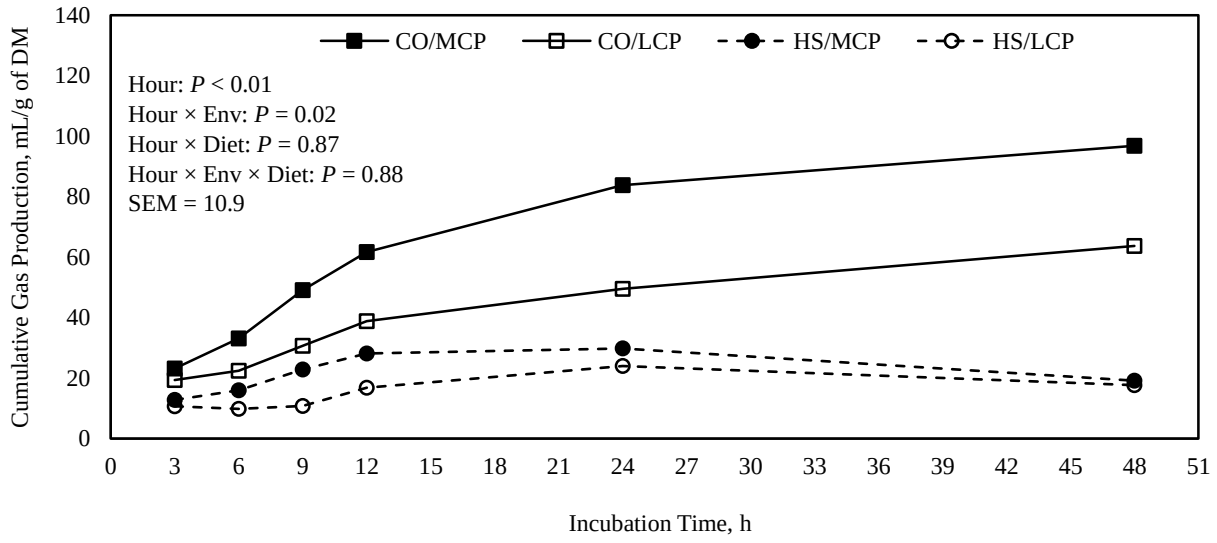

Figure 2-2. Cumulative in vitro gas production after 48 h of rumen content from cows in two environmental conditions (cooled; CO and heat stress; HS) on diets with two levels of CP (12.5% CP; LCP and 16.1% CP; MCP) as %DM. The CO treatment was feed restricted to match intake of the HS treatment. Values are LSM ± pooled SEM.

Table 2-1. Dietary feed ingredient inclusion and nutrient composition on % of DM basis (unless stated otherwise) for low (LCP) or medium (MCP) CP diets estimated with actual wet chemistry values using NRC (2001)

	Experimental Diet	
Item	LCP	MCP
Ingredient		
Corn Silage	37.0	37.0
Ryegrass Silage	6.00	6.00
Corn Grain, ground, dry	16.0	13.2
Corn Hominy	6.70	6.80
Soybean Meal, solvent (48% CP)	2.30	6.50
Soybean Hulls	15.4	11.1
Protected Soybean Meal[1]	3.70	3.20
Canola Meal, mech. extract	2.50	8.10
Molasses, sugarcane	1.70	0.60
Urea	0.00	0.50
Blood Meal, ring dried	0.70	0.00
MetaSmart, dry powder[2]	0.30	0.00
BergaFat[3]	1.20	0.70
Salt	0.30	0.30
Ammonium Phosphate (di-)	0.20	0.00
Sodium Bicarbonate	1.80	1.80
Potassium Carbonate	0.50	0.50
Calcium Carbonate	1.70	1.70
Magnesium Oxide	1.50	1.50
Trace Mineral and Vitamin Mix[4]	0.50	0.50
Nutrient Composition[5,6]		
DM, %	43.3	43.5
CP	12.5	16.1
RDP	7.40	10.5
RUP	5.10	5.60
MP	7.84	9.03
NDF	35.1	34.2
ADF	21.5	20.8
NFC	39.5	37.3
Starch[7]	27.7	27.8
Crude Fat	4.50	4.10
NE_L, Mcal/kg	1.60	1.60

[1]SoyPLUS, West Central Cooperative (Ralston, IA).
[2]Adisseo (Alpharetta, GA); Brand Contains 50% RDP and 50% RUP as pelletable form of Met.
[3]Berg+Schmidt Feed (Libertyville, IL); Rumen-stable fat powder.
[4]AgCentral Cooperative (Athens, TN).
[5]LCP: 12.3×10^3 IU/kg of Vitamin A, 3.1×10^3 IU/kg of Vitamin D, 124 IU/kg of Vitamin E, 1.2 mg/kg of Co, 33.4 mg/kg of Cu, 2.6 mg/kg of I, 313 mg/kg of Fe, 157 mg/kg of Mn, 225 mg/kg of Zn, and 0.73 mg/kg of Se.
[6]MCP: 13.6×10^3 IU/kg of Vitamin A, 3.4×10^3 IU/kg of Vitamin D, 137 IU/kg of Vitamin E, 1.3 mg/kg of Co, 35.7 mg/kg of Cu, 2.8 mg/kg of I, 274 mg/kg of Fe, 172 mg/kg of Mn, 245 mg/kg of Zn, and 0.84 mg/kg of Se.
[7]Rate of inclusion of each feed ingredient and nutrient content was used to estimate starch.

Table 2-2. Observed chemical composition of the feed ingredients used in the low (LCP) or medium (MCP) CP diets (% of DM)

	Ingredient			
Item	Corn Silage	Ryegrass Silage	Conc. Mix LCP	Conc. Mix MCP
DM, % of feed	32.7	26.8	87.6	87.3
CP	9.10	9.90	14.6	21.6
NDF	41.6	64.2	29.7	26.9
ADF	25.0	43.4	18.7	15.9
Starch	30.3	3.40	28.5	28.8
Lignin	2.97	5.54	1.66	1.98
Crude Fat	3.62	3.08	4.45	4.32
NE_L, Mcal/kg	1.68	1.28	1.63	1.70
Calcium	0.26	0.57	1.77	1.68
Phosphorus	0.26	0.27	0.39	0.51
Magnesium	0.23	0.23	1.89	0.44
Potassium	1.25	1.87	1.67	1.95
Sodium	0.03	0.18	1.18	1.04

Table 2-3. Body temperature variables for cooled (CO) or heat-stressed (HS) lactating Holstein cows supplied low (LCP) or medium (MCP) CP in a.m. and p.m. values1 (LSM ± SEM)

	Treatment					Effect (*P*-Value)		
	CO		HS					
Item	LCP	MCP	LCP	MCP	SEM	Env[2]	Diet[3]	Env × Diet
Rectal, °C								
a.m.	38.1	38.0	38.2	38.2	0.10	0.13	0.78	0.91
p.m.	38.4	38.4	39.0	39.1	0.13	<0.01	0.72	0.77
Vaginal, °C								
a.m.	38.6	38.5	38.6	38.6	0.08	0.18	0.48	0.36
p.m.	38.6	38.6	39.2	39.3	0.12	<0.01	0.68	0.52
Udder Surface, °C								
a.m.	32.2	32.8	34.6	35.2	0.26	<0.01	0.04	0.86
p.m.	34.9[c]	34.8[c]	37.3[b]	38.2[a]	0.28	<0.01	0.17	0.09
RR[4], breaths/min								
a.m.	38.4	36.0	46.4	46.2	2.00	<0.01	0.53	0.59
p.m.	47.8	49.4	74.9	77.5	3.14	<0.01	0.50	0.87

[a-c]Values within a row with differing superscripts denote Env by Diet interactions ($P \leq 0.05$) and trend ($0.05 < P \leq 0.10$).
[1]Differences amongst a.m. and p.m. temperatures were significant for rectal, vaginal, and udder surface temperatures and respiration rates ($P < 0.01$).
[2]Env = Environmental effect of CO or HS.
[3]Diet = Dietary effect of LCP or MCP.
[4]RR = Respiratory rate.

Table 2-4. Milk production and composition, BW, and BCS for cooled (CO) or heat-stressed (HS) lactating Holstein cows supplied low (LCP) or medium (MCP) CP (LSM ± SEM)

	Treatment					Effect (*P*-Value)		
	CO		HS					
Item	LCP	MCP	LCP	MCP	SEM	Env[1]	Diet[2]	Env × Diet
Milk, kg/d	31.3	33.0	26.9	29.4	0.91	<0.01	0.03	0.63
ECM[3], kg/d	32.1[b]	35.9[a]	31.6[b]	31.4[b]	1.08	0.03	0.11	0.08
Lactose, %	4.85	4.76	4.72	4.74	0.03	0.05	0.34	0.12
Lactose, kg/d	1.53	1.49	1.28	1.38	0.05	<0.01	0.15	0.57
True Protein, %	3.15[a]	3.01[bc]	2.99[c]	3.08[ab]	0.03	0.12	0.45	<0.01
True Protein, kg/d	0.96	0.97	0.82	0.90	0.03	<0.01	0.19	0.30
Fat, %	3.74[c]	4.35[ab]	4.65[a]	4.00[bc]	0.12	0.02	0.85	<0.01
Fat, kg/d	1.13[c]	1.39[a]	1.28[ab]	1.16[bc]	0.05	0.39	0.14	<0.01
SNF, %	8.89[a]	8.64[b]	8.62[b]	8.69[b]	0.04	0.02	0.07	<0.01
SNF, kg/d	2.71	2.77	2.33	2.53	0.08	<0.01	0.13	0.42
MUN, mg/dL	5.39	10.3	4.56	8.91	0.41	0.01	<0.01	0.47
SCC, $\times 10^3$ cells/mL	81.2	99.0	333	177	50.2	<0.01	0.46	0.18
Initial BW, kg	692	644	709	689	21.3	0.15	0.12	0.51
Final BW, kg	695	646	702	687	21.0	0.26	0.14	0.42
Initial BCS	2.90	2.77	2.86	2.80	0.14	0.98	0.52	0.81
Final BCS	2.79	2.76	2.88	2.92	0.13	0.33	0.96	0.80

[a-c]Values within a row with differing superscripts denote Env by Diet interactions ($P \leq 0.05$) and trend ($0.05 < P \leq 0.10$).

[1]Env = Environmental effect of CO or HS.

[2]Diet = Dietary effect of LCP or MCP.

[3]Energy-corrected milk calculated in equation derived from Tyrrell and Reid (1965).

Table 2-5. Plasma metabolites, hormone, and acute phase protein concentrations of cooled (CO) or heat-stressed (HS) lactating Holstein cows supplied low (LCP) or medium (MCP) CP (LSM ± SEM)

	Treatment					Effect (*P*-Value)		
	CO		HS					
Item[1]	LCP	MCP	LCP	MCP	SEM	Env[2]	Diet[3]	Env × Diet
Glucose, mmol/L	2.89	3.14	3.04	3.11	0.08	0.48	0.05	0.25
Insulin, µU/mL	16.5	28.4	13.2	28.1	2.87	0.55	<0.01	0.59
Fatty Acid, µEq/L	185	143	213	184	17.4	0.06	0.05	0.70
BHBA[4], mmol/L	1.14	1.06	1.12	1.01	0.06	0.60	0.13	0.86
PUN[4], mmol/L	2.41	2.38	2.62	2.44	0.29	0.65	0.73	0.80
HPT, µg/mL	0.56	0.052	1.46	2.00	0.64	0.06	0.99	0.11
SAA, µg/mL	48.1	44.9	46.7	45.8	4.33	0.95	0.64	0.80

[1]BHBA = β-hydroxybutyrate; PUN = Plasma urea nitrogen; HPT = Haptoglobin; SAA = Serum amyloid A.

[2]Env = Environmental effect of CO or HS.

[3]Diet = Dietary effect of LCP or MCP.

[4]Covariate removed ($P > 0.05$).

Table 2-6. In vitro fermentation and gas production from rumen content of cooled (CO) or heat-stressed (HS) lactating Holstein cows incubated at 39°C with a diet of low (LCP) or medium (MCP) CP for 48 h (LSM ± SEM)

	Treatment					Effect (*P*-Value)		
	CO		HS					
Item	LCP	MCP	LCP	MCP	SEM	Env[1]	Diet[2]	Env × Diet
pH	7.04	6.94	7.20	7.10	0.03	<0.01	0.02	1.00
Total VFA, mM	48.5	54.9	43.4	54.3	6.02	0.65	0.19	0.72
Acetate, mM	27.8	34.0	24.3	31.5	3.55	0.41	0.10	0.89
Propionate, mM	14.8	14.3	13.4	15.7	2.13	0.97	0.69	0.53
Butyrate, mM	5.80	6.59	5.77	7.17	0.99	0.78	0.30	0.77
Acetate:Propionate	1.87	2.43	1.96	2.00	0.24	0.51	0.24	0.31
Lactate, mM	20.4	21.7	15.3	16.7	4.25	0.30	0.77	0.99
NH_3, mM	4.17[b]	10.4[a]	1.45[c]	12.7[a]	0.94	0.84	<0.01	0.02
48 h Gas Production, mL/g of DM	63.7	96.8	17.7	19.2	17.1	<0.01	0.34	0.38
CH_4, g/mL	16.3	20.3	14.4	21.4	1.20	0.74	<0.01	0.19

[a-c]Values within a row with differing superscripts denote Env by Diet interactions ($P \leq 0.05$).

[1]Env = Environmental effect of CO or HS.

[2]Diet = Dietary effect of LCP or MCP.

CHAPTER 3.

POSTBIOTIC ADDITIVE FROM *ASPERGILLUS ORYZAE* INCREASED MILK PRODUCTION AND DECREASED INFLAMMATION OF LACTATING DAIRY COWS EXPOSED TO HEAT STRESS

A version of this chapter is being prepared for publication in the Journal of Dairy Science by: Jeffrey D. Kaufman, Hannah R. Bailey, Fernando Bargo, Ignacio R. Ipharraguerre, Gina M. Pighetti, and Agustín G. Ríus.

J. D. Kaufman conducted and organized the experiment, analyzed the data, and wrote the manuscript. H. R. Bailey collected samples and edited the manuscript. F. Bargo and I. R. Ipharraguerre provided the funding, helped design the experiment, and edited the manuscript. G. M. Pighetti directed the ex-vivo challenge and PCR work and edited the manuscript. A. G. Ríus directed the project, acquired the funding, and edited and approved the manuscript as the corresponding author.

ABSTRACT

Warm climate decreases productivity and triggers inflammation in dairy cattle. The objective of this study was to determine the effect of supplementing a novel postbiotic from *Aspergillus oryzae* (AO) fermentation on physiological and milk production responses in dairy cows exposed to natural heat stress. Forty-eight mid-lactation, Holstein cows were enrolled in a completely randomized design and randomly assigned to 1 of 4 treatments for 36 d. Treatments were increasing levels of the AO postbiotic (Biozyme Inc., St. Joseph, MO) at 1) 0 g/d (control), 2) 3 g/d, 3) 6 g/d, and 4) 18 g/d. The basal diet was formulated with 41% forage and 59% concentrate and fed individually twice daily. The postbiotic was top-dressed at each feeding. Cows experienced warm climate during June and July 2018. Heat abatement (i.e., fans and sprinklers) maintained mild heat stress (average temperature-humidity index, THI = 74.6 ± 2.4) from d 1 to 10 (period 1). Heat stress intensity was increased (average THI = 77.3 ± 4.2) by removing heat abatement during the day on d 11 to 36 (period 2). Body temperatures and

respiration rates were measured throughout the study. Milk samples were collected and analyzed for milk components. Blood samples were collected to analyze metabolites and acute phase proteins. On d 36, an ex-vivo challenge using lipopolysaccharide (LPS) was conducted on whole blood samples to analyze pro-inflammatory cytokine expression. In period 1, AO cubically decreased afternoon vaginal temperature, and 6 g/d of AO reduced temperature by 0.3°C. The 6 g/d of AO increased yields of milk and energy-corrected milk (ECM) and feed-use efficiency from 1.54 to 1.70 (Milk/DMI in kg/d) and 1.66 to 1.74 (ECM/DMI in kg/d) in period 1. In period 2, the 6 g/d of AO decreased morning vaginal temperature from 39.0 to 38.7 and 38.8°C, respectively. The 3 and 6 g/d of AO increased ECM yield by 3.8 kg/d, and yields of milk protein, fat, and lactose. The 3 g/d of AO decreased serum amyloid A and LPS binding protein concentrations in plasma. For the ex-vivo LPS challenge, the postbiotic linearly decreased the *IL-6* expression when expressed as a ratio of LPS to no LPS stimulation. In summary, 6 g/d of AO increased synthesis milk and milk components and feed-use efficiency, and 3 g/d of AO reduced concentrations of inflammatory markers in heat-stressed cows.

INTRODUCTION

Heat stress negatively impacts the productive activity and thermoregulatory mechanisms of lactating dairy cows (Kadzere et al., 2002). High environment temperatures and humidity that combine as a THI greater than 68 result in heat stress (Cook et al., 2007). Dairy cows exposed to increasing THI (82 and 76) showed a decrease in DMI and milk yield (Rhoads et al., 2009; Kassube et al., 2017). In addition, heat stress decreased yields of milk protein and milk fat by 41 and 24% compared with cows in thermoneutral conditions (Gao et al., 2017). Substrate availability to the cow limits the milk productivity during warm climates possibly from thermoregulatory mechanisms and inflammation. Heat stress decreases plasma concentrations of

glucose and AA (Gao et al., 2017) and increases the disposal rate of glucose from circulation (Wheelock et al., 2010). Also, heat stress increased plasma circulation of pro-inflammatory cytokines (i.e., IL-1β, IL-6, and TNF-α) compared with cows under thermoneutral conditions (Chen et al., 2018).

Aspergillus oryzae (**AO**) is a fungal mold that secretes relatively large amounts of enzymes (e.g., cellulase and α-amylase), cellular components, and VFA from fermentation of highly fibrous plant material (Machida, 2002; Tricarico et al., 2005). A postbiotic is biologically active molecules released from microorganism fermentation on a fiber source, which provides health benefits to the host animal (Maguire and Maguire, 2019). Similar products from AO provided at 3 g/d have been previously shown to improve feed-use efficiency (i.e., the amount of DMI used to produce milk and milk components), milk production, and body temperature in heat-stressed cows (Huber et al., 1994). The inclusion of 3 to 3.5 g/d of AO increased milk yield by 5 to 7% from dairy cows (Gomez-Alarcon et al., 1991; Sallam et al., 2019). Extracted cellular components from a fungal probiotic reduced plasma concentrations of SAA and endotoxin LPS in Holstein steers (Garcia Diaz et al., 2018). We hypothesized that supplementing a novel AO postbiotic will increase milk production and decrease pro-inflammatory markers from lactating dairy cows exposed to warm climate. The objective of this study was to determine the effect of feeding various levels of a novel AO postbiotic on milk production, body temperature, metabolism, and inflammation from lactating dairy cows experiencing warm climate conditions.

MATERIALS AND METHODS

Animals, Housing, and Treatments

Procedures for the experiment were accepted by the University of Tennessee Institutional Animal Care and Use Committee. Forty-eight Holstein cows (43 multiparous and 5 primiparous) were used from the ETREC-LRD herd (Walland, TN) and housed in two adjacent pens in a freestall barn during June and July of 2018. Each pen was equipped with fans and bunk-line sprinklers. Before assigning treatments, cows were stratified into 1 of 4 groups based on DIM (105 ± 27 SD), milk yield (44.0 ± 1.9 kg/d), and parity (2.5 ± 0.13), and then randomly assigned to 1 of 2 pens and then to 1 of 4 treatments (n = 12 cows/treatment) for 36 d in a completely randomized design. There were 2 experimental periods of heat stress consisting of 1) 10 d with heat abatement provided throughout the day (**P1**) and 2) 26 d with no heat abatement for 13 h during the day (**P2**). All treatments were equally represented in each pen. Treatments consisted of a basal diet with no additive (**CTL**; 0 g/d), and the same diet top-dressed twice daily with an AO postbiotic (Biozyme Inc., St. Joseph, MO) at 3, 6, or 18 g/d. Previous research has tested various levels of AO at 3 g/d (Gomez-Alarcon et al., 1991), 3.5 g/d (Sallam et al., 2019), and 5 g/d (Sun et al., 2017); therefore, multiple levels of AO were tested in the present study. The additive was mixed with the top portion of the TMR at feeding, and consumption of the additive was assessed using visual observation. The diet was individually fed at 0700 and 1700 h using an electronic feeding system (American Calan, Inc., Northwood, NH) to provide 10% daily refusal. The basal diet was formulated for a mid-lactation cow with 26.0 kg/d of DMI, 706 kg of BW, 3.0 BCS, and 40.2 kg/d of milk yield with 3.9% fat and 2.9% protein (NRC, 2001). The diet was formulated with 41% forage and 59% concentrate on a DM basis at 17.8% CP, 1.71 Mcal/kg NEL, 32.0% NDF, 19.9% ADF, 28.5% starch, 5.4% fat, and 43.6% DM. The forages consisted

of corn silage, ryegrass silage, and orchardgrass hay and the grain mix mostly consisted of corn grain, hominy, soybean meal, soybean hulls, and mineral/vitamin premix. Cows were acclimated to the basal diet for 14 d prior to start of the study. Cows were milked at 0700 and 1800 h in a double-eight herringbone parlor. At the beginning and end of the study, BW and BCS were assessed and recorded after the 0700 h milking by one experienced researcher using the 1 to 5 scale explained by Edmonson et al. (1989).

Environmental Management and Assessment

Environmental temperature (°C) and relative humidity (%) were measured every 10 min using a HOBO External Temp/RH Data Logger (Onset Computer Corporation, Bourne, MA). Temperature-humidity index was assessed using the equation from Dikmen and Hansen (2009), where T = environmental temperature (°C) and RH = relative humidity (%):

$$THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)].$$

Period 1 was imposed to determine treatment effects on productivity in cows exposed to warm climate and receiving heat abatement commonly used in the U.S. Throughout P1, fans and sprinklers were set to come on at > 20°C, and sprinklers cycled at time intervals of 40 s on and 10 min off. Throughout P2, fans and sprinklers were activated from 2200 to 0900 h at the previously mentioned thresholds and were inactivated from 0900 to 2200 h with the aim of increasing heat stress intensity.

The cow's thermal load was assessed using rectal, vaginal, and udder surface temperatures and respiration rates. Excluding vaginal temperatures, measurements were taken at 0900 and 1600 h on d -1, 10, 31, 32, 33, 34, and 35 of the study as previously described (Kaufman et al., 2018b). Rectal temperatures were measured using a GLA M500 digital

thermometer (GLA Agricultural Electronics, San Luis Obispo, CA; accuracy ± 0.1°C). Udder surface temperatures were measured at 15 cm in distance on a cleanly shaven patch of the right, rear udder using a FLIR imaging gun (TG165 Imaging IR Thermometer; Wilsonville, OR; accuracy ± 1.5°C). Respiration rates were counted for 15 s at the flank and reported as breaths/min. Vaginal temperatures were monitored continuously over 24 h at 10 min intervals on d 10, 31, 32, 33, 34, and 35 of the study using fixed, intravaginal temperature loggers (DS1922L Thermochron iButton Device, Maxim Integrated, San Jose, CA; accuracy ± 0.01°C) as previously described (Kaufman et al., 2017).

Samples and Analyses

Corn and ryegrass silage, orchardgrass hay, and grain mix were sampled three times weekly and dried at 55°C (Table 3-1). A composite pooled sample of forages were chemically analyzed (Cumberland Valley Analytical, Waynesboro, PA) for CP, starch, lignin, crude fat, and minerals using near-infrared spectroscopy (Foss 500; Foss North America; Table 3-2). A composite pooled sample for the grain mix was chemically analyzed for total N (AOAC International, 1999; method 990.03) using a CN628 Carbon/Nitrogen Determinator (LECO; Saint Joseph, MI); for starch (Hall, 2008); for ether extract (AOAC International, 1999; method 2003.05); and for minerals (AOAC International, 1999) using inductively coupled plasma spectrometry (Thermo iCAP 6300; Waltham, MA). All feed ingredients were analyzed for NDF (Van Soest et al., 1991) and for ADF (AOAC International, 1999; method 973.18). The nutrient composition of the diet is reported in Table 3-1, and the nutrient composition of forages and the grain mix are reported in Table 3-2.

Milk samples were collected from morning and afternoon milkings on d -1, 10, 31, 32, 33, 34, and 35 of the study. Individual milk samples were analyzed in the Tennessee DHIA Lab (Knoxville, TN) for fat, protein, lactose, solids, and SCC by mid-infrared (Bentley 2000; Bentley Instruments, Chaska, MN).

Blood samples were collected from the coccygeal vessel after morning milking on d 10, 12, 34, and 35 of the study in 140 IU sodium heparin (Benton Dickinson and Co., Franklin Lakes, NJ). Plasma was harvested from blood through centrifugation at 1,500 x *g* for 20 min at 4°C. Samples collected on d 10, 34, and 35 were used to assess concentrations of plasma metabolites (total free fatty acids and urea-N). Total free fatty acid concentrations were analyzed using a non-esterified fatty acid commercial kit (Wako Diagnostics, Mountain View, CA). Urea-N concentrations were analyzed using the Infinity Urea Liquid Stable Reagent commercial kit (Thermo Fisher Scientific, Waltham, MA). The intra- and inter-assay CV were 12% and 9.9% for total free fatty acid and 30% and 13% for plasma urea-N, respectively. Samples collected on d 12 of the study were ~36 h after the acute increase in heat stress intensity, whereas d 34 represented the cumulative effect of exposure to higher intensity heat stress. Using d 12 and 34 samples, ELISA was used to analyze HPT (bovine HPT: Immunology Consultants Laboratory, Inc., Lake Oswego, OR), SAA (multispecies SAA: Tridelta Development, Maynooth, County Kildare, Ireland), and LPS-binding protein (bovine **LBP**; ABclonal Technology, Woburn, MA) according to manufacturers' protocols. The intra- and inter-assay coefficient of variation were 41% and 2.2% for HPT, 7.0% and 20% for SAA, and 19% and 13% for LBP, respectively.

Ex-Vivo LPS Challenge and Leukocyte Analysis

Two whole blood samples were taken from the coccygeal vessel of cows supplied 0, 3, and 6 g/d of AO on d 36 at 0900 h to conduct an ex-vivo LPS challenge. The ex-vivo challenge provided the opportunity to understand how whole blood responded to treatments with a known stimulation of endotoxin or LPS that initiates an immune response (Pearce et al., 2014). One blood sample was stimulated with LPS from *Escherichia coli* O111:B4 (MilliporeSigma, Burlington, MA) at a final concentration of 5 μg/μL. The second blood sample received Dulbecco's Modified Eagle Medium (MilliporeSigma) to act as a control. Both samples were incubated in a water bath for 3.5 h at 38.4°C (Røntved et al., 2005). Hematocrit, hemoglobin, and counts of RBC and total leukocytes were determined for the LPS-stimulated and non-LPS-stimulated whole blood samples. Samples were made into microscope slide smears to analyze differential leukocyte (basophil, eosinophil, lymphocyte, monocyte, and neutrophil) counts previously described (Levkut et al., 2002; Kull et al., 2018) using HEMA 3 Fixative solutions kit (ThermoFisher Scientific, Waltham, MA). In addition, RNA in the blood samples was isolated and stabilized using a Tempus Spin RNA Isolation kit (Thermo Fisher Scientific). Briefly following manufacturer's instructions, 3.5 mL of blood was injected into Tempus tube, vortexed for 10 s, and stored at 4°C until analysis. To isolate RNA, the sample was transferred into a culture tube, diluted with 1X phosphate-buffered saline, vortexed for 30 s, and centrifuged at 3,000 × *g* for 20 min in 4°C. The pellet of RNA was resuspended, purified, filtered, and stored in nucleic acid purification elution solution at -20°C until analysis.

Real-Time Quantitative PCR

The gene expression of pro-inflammatory cytokines (*IL-1β*, *IL-6*, and *TNF-α*) from the RNA of the ex-vivo challenge were quantified using real-time quantitative PCR. The RNA quality and concentration was assessed using electrophoresis (Experion System; Bio-Rad, Hercules, CA). Complementary DNA was synthesized using reverse transcriptase (GoScript; Promega Co., Madison, WI) following manufacturer's protocol. Reactions were performed using real-time PCR (QuantStudio6; Applied Biosystems, Foster City, CA), Power SYBR Green Master Mix (Applied Biosystems), and primer sequences (5'-3') for target genes designed using Primer3 (Untergasser et al., 2012), ordered from Integrated DNA Technologies (Coralville, IA), and shown in Table 3-3. Reactions occurred under the following conditions: 2 min at 50°C, 10 min at 95°C, and then 50 cycles for 15 s at 95°C and for 1 min at 60°C. All standards and samples were analyzed in triplicate and normalized with the geometric mean (Vandesompele et al., 2002) of two reference genes (*YWHAZ* and *RPS24*) as the change in threshold cycle [**ΔCt** = Ct target – Ct reference; (Livak and Schmittgen, 2001)] with primer sequences that are stable with LPS stimulation shown in Table 3-3. Technical variation between plates was removed by normalizing the samples with an inter-run calibrator created from pooling an equal volume from each sample (**ΔΔCt** = ΔCt sample – ΔCt calibrator). Cytokine expression was calculated using the formula of $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Statistical Analyses

Energy-corrected milk was calculated with the equation provided by Tyrrell and Reid (1965). Feed-use efficiency was calculated as the ratio of milk yield or ECM yield with DMI. To determine changes from treatments between P1 and P2, percent change of DMI, milk production,

feed-use efficiency, BW, and BCS was calculated as the difference of P2 to P1 and divided by the LSM of P1. Data were analyzed using the Mixed model procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) for the main effect and the dose response to feeding the postbiotic additive levels. The experimental unit was the dairy cow. For body temperatures, respiration rate, milk production, acute phase protein, and cytokine gene expression data, least square differences and a contrast between the CTL and AO postbiotic (CTL vs. AO) were reported, and polynomial contrasts were performed for evaluating the curvilinear response to the increasing doses of the AO postbiotic. Data from two cows (0 and 18 g/d treatments) were removed due to death unrelated to treatments and prevented them from finishing the study. Cytokine gene expression data were normalized using total leukocyte counts before analysis. Three analyses were conducted on normalized, cytokine gene expression: 1) without LPS stimulation, 2) with LPS stimulation, and 3) ratio of LPS to no LPS stimulation. Cytokine expression data was log transformed when necessary, and non-transformed LSM are reported. Data collected immediately prior to P1 were included as a covariate adjustment in the model for P1 for body temperature, DMI, and milk production parameters. In addition, maximum afternoon respiration rate from d 31 to 35 were included as a covariate adjustment in the model for P2. The covariate was excluded in the analysis of rectal, vaginal, and udder temperature and respiration rate. Measurements taken in non-random, consecutive order were analyzed as repeated measures with an autoregressive variance-covariance structure. The P1 model included:

$$Y_{ijkl} = \mu + T_i + P_j + A \times (T \times P)_{ijk} + \beta(\chi)_{ijk} + e_{ijk},$$

where μ = the overall mean, T_i = the fixed effect of ith treatment (i = 0, 3, 6, and 18 g/d), P_j = the fixed effect of jth pen, $A \times (T \times P)_{ijk}$ = the random effect of kth animal (A) in the ith and j^{th} treatment and pen, $\beta(\chi)_{ijk}$ = the covariate effects, and e_{ijk} = the random error. The P1 model was

used to determine the effect of treatments during mild heat stress with cooling. The P2 model had the same terms described for P1 but included the fixed effect of lth day (D) as repeated measures including interactions with T and P. Significant differences were declared at $P \leq 0.05$ and trends were declared at $0.05 < P \leq 0.10$. All results are reported as LSM (± SEM).

RESULTS

Dietary Composition

The CP observed from the diet during the study differed slightly from the initial formulation shown in Table 3-1 (18.1 vs. 17.8% CP). The observed NEL content decreased compared with the formulated diet. Actual NEL decreased by 0.10 Mcal/kg compared with the formulated diet (1.61 vs. 1.71 Mcal/kg).

Environment and Heat Stress Assessment

The THI ranged from 71.6 to 75.2 at 2200 to 0900 h and from 73.3 to 78.7 at 0900 to 2200 h throughout P1 (Figure 3-1). Throughout P2, the THI ranged from 71.6 to 77.7 at 2200 to 0900 h and from 75.4 to 83.3 at 0900 to 2200 h (Figure 3-1). In P2, the increase in THI from morning to afternoon was associated with increased ($P < 0.001$) rectal, vaginal, and udder surface temperatures and respiration rate (Table 3-4). In P1, AO increased morning rectal temperature (CTL vs. AO; $P = 0.017$). There was a cubic effect ($P < 0.001$) of AO on vaginal temperature such that 3 and 18 g/d of AO increased or did not change morning and afternoon vaginal temperatures, whereas 6 g/d of AO decreased afternoon vaginal temperatures during P1. In P2, there was a cubic effect ($P < 0.001$) of AO indicating that 3 g/d did not affect morning vaginal temperature, whereas 6 and 18 g/d doses decreased vaginal temperature. There was a cubic effect ($P < 0.001$) indicating that supplementing 3, 6, or 18 g/d of AO increased afternoon

vaginal temperature during P2. There was a quadratic trend (P = 0.084) indicating that AO decreased afternoon respiration rate during P2.

Milk Production and Feed-Use Efficiency

In P1, the supplementation of AO increased milk yield compared with the 0 g/d dose (CTL vs. AO; P = 0.019; Table 3-5). In P1, there was a quadratic trend (P = 0.061) for AO indicating that 3 g/d did not affect milk yield, whereas 6 and 18 g/d doses increased milk yield. There was a quadratic trend (P = 0.056) for AO such that 3 and 18 g/d increased or did not change ECM yield, whereas 6 g/d of AO increased ECM yield during P1. There was a cubic effect (P = 0.003) for AO indicating that 3 g/d did not affect efficiency of feed conversion into milk (i.e., milk/DMI in kg/d), whereas 6 and 18 g/d doses increased feed-use efficiency. There was a cubic effect (P = 0.021) such that AO at 6 g/d increased efficiency of feed conversion into ECM (i.e., ECM/DMI in kg/d) compared with 3 g/d but did not affect feed-use efficiency compared with 0 and 18 g/d. Treatments did not affect DMI, milk protein, milk fat, and SCC in P1.

In P2, administration of AO tended to increase milk yield compared with the 0 g/d dose (CTL vs. AO; P = 0.065; Table 3-5). There was a quadratic effect for AO indicating that 18 g/d did not affect ECM, milk protein, and milk fat yields (P < 0.001) and milk lactose yield (P = 0.005), whereas 3 and 6 g/d doses increased ECM, milk protein, milk fat, and milk lactose yields. Additionally, AO linearly increased milk protein content (P < 0.001) and milk fat content (P = 0.033) by 0.03 and 0.18 percentage units and tended to linearly increase (P = 0.056) feed-use efficiency of milk yield by 0.04 when compared with the 0 g/d dose in P2. Administration of AO linearly decreased SCC (P = 0.021) by 318×10^3 cells/mL compared with the 0 g/d dose in P2;

however, SCC differences between cows prior to start of the study were not balanced between treatments, which may be responsible for variations in SCC results.

For percent change from P1 to P2, supplementation of AO at 6 and 18 g/d tended to decrease linearly (P = 0.055) feed-use efficiency for milk yield compared with the 0 g/d dose, whereas 3 g/d of AO did not affect feed-use efficiency (Table 3-6). Whereas, supplementation of AO at 18 g/d decreased linearly (P = 0.049) percent change from P1 to P2 for feed-use efficiency for ECM yield compared with the 0 g/d dose, whereas 3 and 6 g/d of AO did not affect feed-use efficiency from ECM yield.

Plasma Metabolites and Acute Phase Proteins

Treatments did not affect total free fatty acid and urea-N concentrations in plasma (Table 3-7). At d 12 of the study (~36 h after removal of heat abatement), supplementation with 3 g/d of AO decreased (quadratic; P = 0.030) plasma concentrations of LBP by 1.31 μg/mL, whereas 6 g/d did not affect LBP concentrations (Table 3-8). Supplementation of AO tended to decrease plasma concentrations of SAA compared with the 0 g/d dose at d 12 (CTL vs. AO; P = 0.066). At d 34 of the study, supplementing with 3 g/d of AO decreased (quadratic; P = 0.011) plasma concentrations of SAA by 45.4 μg/mL, whereas 6 g/d did not affect SAA concentrations. There was a quadratic trend (P = 0.052) such that supplementation with 3 g/d of AO decreased plasma concentrations of LBP by 1.31 μg/mL, whereas 6 g/d did not affect LBP concentrations at d 34 of the study. Supplementation with AO did not affect plasma concentrations of HPT during P1 and P2 compared with no supplementation.

Ex-Vivo LPS Challenge and Leukocyte Analysis

The no LPS stimulation results showed relative expression of cytokines from whole blood of cows exposed to heat stress on the last day of P2 (Table 3-9). These values were used in the ratio calculation to adjust for gene expression of cytokines already present from heat stress in comparison to expression from the LPS stimulation. There was a linear effect of supplementation with AO indicating that 3 g/d decreased (P = 0.022) the *IL-6* relative expression ratio (LPS to no LPS stimulation), whereas the 6 g/d dose did not further affect the *IL-6* relative expression ratio. Supplementation of AO tended to decrease the relative expression ratio of *IL-6* compared with the 0 g/d dose (CTL vs. AO; P = 0.070). The supplementation with AO did not affect the relative expression ratio of *IL-1β* and *TNF-α*. There were no differences in hematocrit, hemoglobin, and counts of RBC, total leukocytes, and leukocyte differentials between treatments. The mean percentages for differential leukocyte counts were 72.6 ± 1.87% for lymphocytes, 21.2 ± 1.79% for neutrophils, 5.3 ± 0.81% for eosinophils, 0.80 ± 0.18% for monocytes, and 0.10 ± 0.07% for basophils.

DISCUSSION

Environment and Heat Stress Assessment

During P1, fans and sprinklers provided heat abatement, which allowed for morning and afternoon body temperatures and respiration rate to remain relatively constant, which is in agreement with previous research performed at the dairy unit (Kaufman et al., 2018a). As expected, the removal of heat abatement from 0900 to 2200 h in P2 increased THI and caused a marked increase in body temperature and respiration rate in the afternoon measurements. In a previous study in this location, afternoon removal of heat abatement increased body temperature

and respiration rate of heat-stressed cows compared with morning measurements (Kaufman et al., 2017). Thus, we successfully attained the objective of increasing the intensity of heat stress during P2.

During P1, the cubic response to AO showed that 6 g/d reduced afternoon vaginal temperature compared with 0 g/d (38.6 vs. 38.9°C). During P2, 6 g/d of AO reduced morning vaginal temperatures the greatest compared with 0 g/d (38.7 vs. 39.0°C). Vaginal temperature provides a continual measurement of the body's thermal load over multiple times (i.e., days), which makes this measurement a suitable parameter to assess cow's capacity to regulate body temperature under heat stress conditions (Kaufman et al., 2018b). In agreement with our results, previous studies reported that providing AO to cows decreased body temperature compared with no supplementation (Huber et al., 1985; Higginbotham et al., 1994). Therefore, 6 g/d of AO was effective at attenuating the increase in body temperature of lactating dairy cows exposed to mild and moderate heat stress conditions.

Milk Production and Feed-Use Efficiency

Results showed that 3 and 6 g/d of AO improved milk and ECM yield in both P1 and P2. In P1, the 6 g/d dose caused an increase in ECM yield of 3.6 kg/d compared with 0 g/d. In P2, 3 and 6 g/d of AO caused increases in ECM yield of 3.9 and 3.8 kg/d compared with 0 g/d. The 6 g/d dose of AO provided the lowest percent change between ECM yield of P1 and P2. Collectively, the 6 g/d dose provided the best milk production response in cows with or without heat abatement under warm climates. In agreement with our results, feeding 3 g/d of an AO product increased milk yield in cows exposed to warm climate compared with no supplementation (Chiou et al., 2002). The increase in milk production may derive from

biologically active molecules coming from AO. In summary, AO was effective at increasing milk in P1 and ECM yield in both periods in heat-stressed cows with the greatest response at 6 g/d.

Supplementation with 3 and 6 g/d of AO increased milk component yields in P2. Compared with 0 g/d, 3 and 6 g/d of AO increased milk protein by 0.09 and 0.08 kg/d, milk fat by 0.17 and 0.16 kg/d, and milk lactose by 0.10 and 0.12 kg/d, respectively. Additionally, the 6 g/d dose of AO provided the lowest percent change between milk fat and lactose yield of P1 and P2. In agreement with the present results, 3 g/d of AO increased milk protein yield by 0.03 kg/d in cows exposed to heat stress compared with no supplementation (Chiou et al., 2002). Fermentation from AO is known to promote protein flow post-ruminally (Machida, 2002), which may result from greater ruminal microbial CP and outflow because of improved rumen fermentation. In addition, the increases in yields of milk protein, fat, and lactose are directly associated with numerically greater milk yield from AO doses and similar or small changes in milk component contents compared with 0 g/d. In summary, supplying 3 and 6 g/d of AO increased the yields of milk protein, fat, and lactose from lactating cows experiencing moderate heat stress conditions.

In P1, the cubic response showed that 6 g/d increased DMI conversion to milk and ECM yield the greatest compared with 0, 3, and 18 g/d doses. The feed-use efficiency (milk/DMI) was increased by 10.4% as a result of 6 g/d of AO compared with 0 g/d in P1. The feed-use efficiency expressed as ECM/DMI was increased by 8.8% as a result of 6 g/d of AO compared with the 3 g/d dose in P1. In P2, supplementation of AO increased feed-use efficiency (milk/DMI) in a linear trend. The increase in feed-use efficiency may derive an improvement in nutrient utilization at the mammary gland of heat-stressed cows supplemented with AO. The AO

provides biological active molecules to the cow, which may result in greater nutrient utilization. In comparison with the present study, heat-stressed cows supplemented with 3 g/d of a yeast culture-based product to a basal diet increased ECM/DMI efficiency by 6.7% compared with cows with no supplementation (Schingoethe et al., 2004). Likewise, supplementation with 3 g/d of AO increased milk/DMI efficiency by 5.4% compared with no supplementation in the diet for heat-stressed cows (Gomez-Alarcon et al., 1991). In summary, AO increased feed-use efficiency, but this response was greater in cows receiving heat abatement.

Pro-Inflammatory Response

The analysis of acute phase proteins was conducted under acute (d 12 of the study) and chronic (d 34 of the study) heat stress. Comparing data under no supplementation of AO demonstrates the pro-inflammatory state the cows experienced under these conditions (Lomborg et al., 2008). Observations at 0 g/d of AO for plasma concentrations of HPT and SAA showed an ~1.5 to 3-fold increase from d 12 to d 34. Therefore, exposure to chronic heat stress conditions increased the concentrations of HPT and SAA, which can be synthesized in hepatocytes (Lomborg et al., 2008). However, concentrations of LBP in plasma did not numerically differ between acute and chronic heat stress. The increase in HPT and SAA concentrations from heat stress conditions were found additionally from supplementing with 6 g/d of AO. The relative expression of cytokines in blood without LPS stimulation from 6 g/d of AO is in agreement with the acute phase protein concentrations from cows exposed to the chronic, moderate heat stress. The 6 g/d of AO numerically increased expression of *IL-1β* and *IL-6* in blood with no LPS stimulation compared with 3 g/d and expression of *TNF-α* compared with 0 g/d. The analysis without LPS stimulation represents the expression of cytokines reflecting the impacts of heat stress on the cow. In agreement, a previous study showed that chronic, moderate heat stress

increased plasma concentrations of IL-6 (Min et al., 2016). In addition, research has shown that cytokines are associated with synthesis of acute phase proteins in hepatocytes (Zhang et al., 2014). In summary, increased concentrations of acute phase proteins and increased expression of cytokines in blood without LPS stimulation demonstrate the cows were in a pro-inflammatory state under chronic, moderate heat stress conditions.

The ex-vivo LPS challenge provided an insight into how AO influenced the immune system and the ability of leukocytes to respond to LPS. The 6 g/d dose of AO resulted in a linear decrease in the LPS to no LPS stimulation ratio for *IL-6* expression. The change in responsiveness resulted from the numerically higher expression from 6 g/d of AO during no LPS stimulation compared with 0 and 3 g/d. Interleukin-6 can be synthesized by various cells in the body (e.g., macrophages, lymphocytes, and endothelial cells) as a result of multiple stimulants (Bradford et al., 2015), which makes it difficult to understand the direct relationship with AO. Based on the present analysis, the AO product did not influence the leukocytes ability in blood to respond to LPS. These results demonstrate an increased pro-inflammatory response going from supplementation of 3 to 6 g/d of AO, as acute phase protein concentrations increased. In agreement, supplementation of extracted cellular components from a fungal probiotic showed reduced SAA concentrations in plasma from Holstein steers (Garcia Diaz et al., 2018). These combined data indicate the 6 g/d of AO may be less capable of decreasing a pro-inflammatory state compared with the 3 g/d of AO, which demonstrated lower plasma concentrations of LBP and SAA – indicators of inflammation.

The shift in the pro-inflammatory state is interesting when compared with milk production responses. Administration of AO increased milk production during P2 with the greatest response from 6 g/d, but the lowest pro-inflammatory response was from 3 g/d. This

demonstrates a potential separation between milk production and the pro-inflammatory state of lactating cows exposed to heat stress. Although, this is unexpected as increased inflammation (i.e., HPT concentrations) was associated with a greater decrease in milk production from early-lactation cows (Bertoni et al., 2008). Alternatively, the return to an increase in inflammation with the 6 g/d dose may reflect the start of an inflammatory effect on milk production, as milk yield was lower when 18 g/d of AO was administered under more intensive heat stress conditions. Research has summarized that bioactive products from yeast fermentation supplements may cause release of pro-inflammatory cytokines from macrophages (Broadway et al., 2015). Unfortunately, due to physical labor restrictions, samples were not analyzed for acute phase proteins and cytokine gene expression with cows supplemented with 18 g/d of AO, and the cytokine analysis was added after initial design of the study. The supplementation with AO changes the inflammatory response and may be associated with the milk production responses. Further research on the mechanistic aspect of AO may provide insight into the relationships found in this study.

CONCLUSIONS

Supplying AO to cows exposed to increasing levels of heat stress reduced body temperature and respiration rate and increased yields of ECM, milk, milk protein, milk fat, and milk lactose. As a result, AO increased estimates of feed-use efficiency from milk and ECM yield. The 6 g/d dose of AO provided the overall best response for milk production and feed-use efficiency in P1 and P2. In addition, AO at 3 g/d decreased markers for inflammation during a chronic, moderate heat stress; whereas, 6 g/d sustained the pro-inflammatory state as compared with 0 g/d. The 6 g/d dose of AO is associated with greater inflammation than 3 g/d but with greater milk production. How the mechanism of AO influences the nutrient utilization and

relationship with the immune system is of importance to understand fully the improvement on milk production.

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APPENDIX

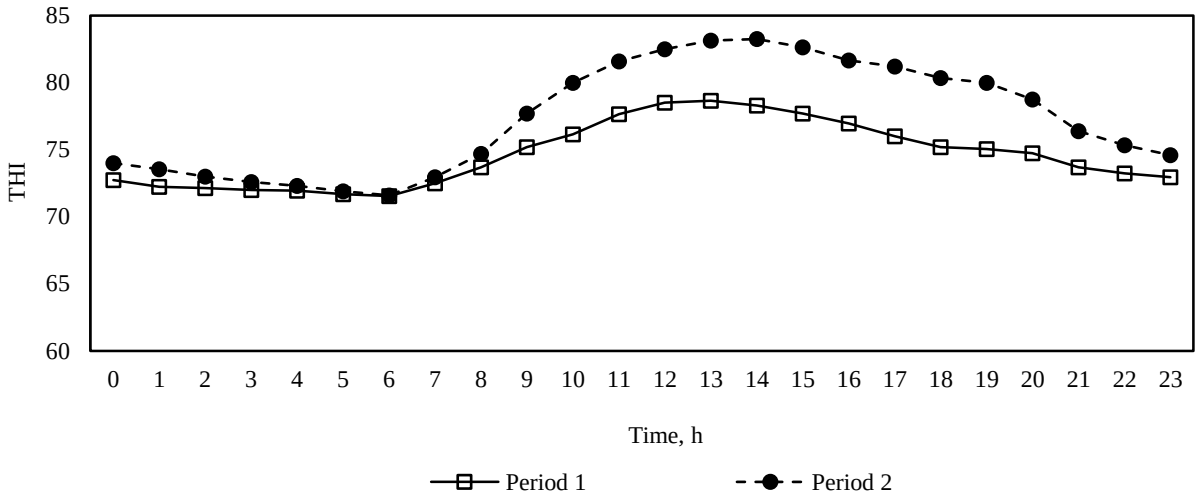

Figure 3-1. Temperature-humidity index (THI) over 24 h between periods 1 and 2. Ten-minute data points were averaged over the hour for the entirety of each period. Period 1 consisted of 10 d of normal temperature of June in East Tennessee, and period 2 consisted of 26 d of temperatures with no heat mitigation (i.e., fans and sprinklers) of July in East Tennessee.

Table 3-1. Ingredient and nutrient composition of the experimental diet

Item	% of DM
Ingredient	
Corn silage	34.1
Ryegrass silage	4.90
Orchardgrass hay	1.59
Corn grain, ground, dry	16.9
Soybean meal, solvent (48% CP)	8.89
Soybean hulls, ground	11.5
Soybeans, roasted	5.43
Protected soybean meal[1]	2.71
Corn hominy, high fat	6.99
Molasses, sugarcane	0.63
Urea	0.60
Palmit 80[2]	0.43
Nurisol[2]	0.42
Monensin[3]	0.005
Alimet[4]	0.07
Salt	0.26
Sodium bicarbonate	1.92
Calcium carbonate	1.59
Potassium chloride	0.39
Magnesium oxide	0.19
Trace mineral and vitamin mix[5]	0.49
Nutrient Composition[6]	
DM[7], %	45.2
CP	18.1
RDP	11.4
RUP	6.7
NDF	33.0
ADF	20.9
NFC	37.0
Starch[8]	22.0
Crude fat	4.90
NE_L, Mcal/kg	1.61
Ca	0.90
P	0.30

[1]SoyPLUS, West Central Cooperative (Ralston, IA).
[2]Natu'oil Services Inc. (Port Coquitlam, BC Canada).
[3]Rumensin 90, Elanco (Greenfield, IN).
[4]Novus Int. (Saint Charles, MO).
[5]AgCentral Cooperative (Athens, TN).
[6]Actual values from chemistry analysis of ingredients, DMI, milk yield, and milk components from basal diet. 12.2×10^3 IU/kg of vitamin A, 2.0×10^3 IU/kg of vitamin D, 4.9 IU/kg of vitamin E, 0.01 mg/kg of Co, 7.5 mg/kg of Cu, 0.07 mg/kg of I, 178 mg/kg of Fe, 39.4 mg/kg of Mn, 0.1 mg/kg of Se, and 31.2 mg/kg of Zn.
[7]Actual DM of TMR.
[8]Rate of inclusion of each feed ingredient and nutrient content was used to estimate starch.

Table 3-2. Observed nutrient and chemical composition of the feed ingredients used in the experimental diet (% of DM, unless otherwise stated)

	Ingredient			
Item	Corn Silage	Ryegrass Silage	Orchardgrass Hay	Grain Mix
DM, % of feed	29.8	26.3	82.6	92.3
NDF	48.7	55.0	69.7	32.2
ADF	31.0	37.9	41.6	18.7
CP	9.00	15.2	9.30	22.2
Lignin	4.36	4.49	5.10	1.26
Starch	25.1	-	1.10	22.6
Crude Fat	3.36	4.06	2.42	2.16
NE_L, Mcal/kg	1.54	1.37	1.26	1.61
Calcium	0.19	0.53	0.40	1.27
Phosphorus	0.22	0.34	0.29	0.38
Magnesium	0.21	0.23	0.23	0.35
Potassium	1.46	2.87	2.16	1.89
Sodium	0.00	0.19	0.03	1.03

Table 3-3. GenBank gene name, accession number, and sequence of primers used to analyze relative gene expression by real-time quantitative PCR

Gene	Primers[1]	Primers (5'-3')	References
RPS24	F	TTTGCCAGCACCAACGTTG	Bevilacqua et al., 2006
	R	AAGGAACGCAAGAACAGAATGAA	
YWHAZ	F	GCATCCCACAGACTATTTCC	Goossens et al., 2005
	R	GCAAAGACAATGACAGACCA	
IL-1β	F	CAAGGAGAGGAAAGAGACA	Konnai et al., 2003
	R	TGAGAAGTGCTGATGTACCA	
IL-6	F	TCATTAAGCGCATGGTCGACAAA	Leutenegger et al., 2010
	R	TCAGCTTATTTTCTGCCAGTGTCT	
TNF-α	F	CGGTGGTGGGACTCGTATG	Witchell et al., 2010
	R	GCTGGTTGTCTTCCAGCTTCA	

[1]Direction of primer (F = forward; R = reverse).

Table 3-4. Morning and afternoon body temperature and respiration rate (RR) of lactating Holstein cows fed different doses of an *Aspergillus oryzae* postbiotic (AO) in two periods of heat stress (LSM ± SEM)

		AO Dose, g/d					*P*-Value	Polynomial Contrasts (*P*-Value)		
Item	Period[1]	0	3	6	18	SEM	CTL vs. AO[2]	Linear	Quadratic	Cubic
Rectal, °C										
a.m.	1	38.7[b]	39.1[a]	39.0[ab]	39.1[a]	0.104	0.017	0.137	0.254	0.106
	2	38.7	38.7	38.8	38.7	0.056	0.196	0.989	0.083	0.931
p.m.	1	38.7	38.8	38.7[y]	38.8[y]	0.105	0.479	0.880	0.939	0.231
	2	39.3[z]	39.4[z]	39.6[z]	39.4[z]	0.095	0.225	0.607	0.110	0.579
Vaginal, °C										
a.m.	1	38.8[b]	39.1[a]	38.8[b]	39.0[a]	0.051	0.030	0.498	0.409	<0.001
	2	39.0[a]	39.0[a]	38.7[c]	38.8[b]	0.022	<0.001	<0.001	<0.001	<0.001
p.m.	1	38.9[b]	39.1[a]	38.6[c]	38.9[b]	0.053	0.652	0.190	0.006	<0.001
	2	39.2[c,z]	39.7[a,z]	39.4[b,z]	39.4[b,z]	0.039	<0.001	0.314	0.002	<0.001
Udder Surface, °C										
a.m.	1	35.3	35.6	35.6	35.6	0.243	0.284	0.567	0.501	0.546
	2	34.7	34.9	34.8	34.7	0.170	0.641	0.827	0.650	0.838
p.m.	1	34.5	34.8[y]	34.8[y]	34.7	0.327	0.579	0.892	0.678	0.601
	2	36.7[z]	36.5[z]	36.4[z]	36.6[z]	0.195	0.504	0.872	0.293	0.968
RR, breaths/min										
a.m.	1	61.7	68.2	63.7	64.0	3.24	0.372	0.844	0.618	0.178
	2	60.3[ab]	64.0[a]	59.3[b]	62.0[ab]	1.89	0.522	0.911	0.700	0.056
p.m.	1	60.5	55.0[y]	60.7	54.5[y]	3.56	0.398	0.349	0.770	0.181
	2	91.9[a,z]	88.4[ab,z]	86.6[b,z]	89.8[ab,z]	2.20	0.178	0.925	0.084	0.913

[a-c]Values within a row with differing superscripts denote AO dose differences between means ($P < 0.05$) and trend between means ($0.05 < P \leq 0.10$).

yValues on p.m. denote a difference between a.m. means in period 1 ($P < 0.05$).

zValues on p.m. denote a difference between a.m. means in period 2 ($P < 0.05$).

[1]Period 1 consisted of normal environmental conditions of June in Tennessee with heat abatement. Period 2 consisted of more intense environmental conditions of July in Tennessee by removal of heat abatement.

[2]Contrast between CTL and AO postbiotic.

Table 3-5. Dry matter intake, milk and milk components, and feed efficiency of lactating Holstein cows fed different doses of an *Aspergillus oryzae* postbiotic (AO) in two periods of heat stress (LSM ± SEM)

		AO Dose, g/d					*P*-Value	Polynomial Contrasts (*P*-Value)		
Item	Period[1]	0	3	6	18	SEM	CTL vs. AO[2]	Linear	Quadratic	Cubic
DMI, kg/d	1	26.6	27.7	26.4	26.9	0.670	0.593	0.849	0.978	0.136
	2	24.2	24.8	24.7	24.0	0.304	0.500	0.387	0.318	0.637
Milk, kg/d	1	40.9[b]	42.8[ab]	44.1[a]	44.1[a]	0.960	0.019	0.065	0.061	0.979
	2	34.5	37.1	37.6	36.8	1.19	0.065	0.560	0.095	0.438
ECM, kg/d	1	37.7[b]	37.9[b]	41.3[a]	39.6[ab]	1.11	0.162	0.285	0.056	0.170
	2	36.4[b]	40.3[a]	40.2[a]	37.6[b]	0.600	<0.001	0.403	<0.001	0.062
True Protein, kg/d	1	1.02	1.03	1.08	1.03	0.029	0.545	0.882	0.143	0.498
	2	0.98[b]	1.07[a]	1.06[a]	0.97[b]	0.017	0.013	0.010	<0.001	0.071
True Protein, %	1	2.86	2.86	2.84	2.81	0.039	0.695	0.281	0.997	0.697
	2	2.85[a]	2.88[a]	2.84[a]	2.66[b]	0.027	0.114	<0.001	0.056	0.465
Fat, kg/d	1	1.40	1.43	1.59	1.51	0.074	0.238	0.376	0.130	0.383
	2	1.37[b]	1.54[a]	1.53[a]	1.41[b]	0.036	0.004	0.366	<0.001	0.090
Fat, %	1	3.97	4.02	4.15	4.06	0.210	0.686	0.843	0.562	0.818
	2	3.99[ab]	4.17[a]	4.11[a]	3.85[b]	0.086	0.601	0.033	0.094	0.315
Lactose, kg/d	1	1.68	1.69	1.80	1.74	0.042	0.255	0.451	0.085	0.217
	2	1.69[b]	1.79[a]	1.81[a]	1.75[ab]	0.031	0.015	0.839	0.005	0.521
Lactose, %	1	4.69	4.74	4.73	4.66	0.033	0.635	0.208	0.201	0.481
	2	4.91[a]	4.80[b]	4.82[b]	4.79[b]	0.025	0.001	0.044	0.099	0.061
SNF, kg/d	1	3.01	3.02	3.19	3.07	0.079	0.387	0.673	0.128	0.312
	2	2.96[b]	3.16[a]	3.18[a]	3.02[b]	0.051	0.010	0.495	<0.001	0.279
SNF, %	1	8.39	8.45	8.39	8.24	0.072	0.719	0.047	0.463	0.575
	2	8.60[a]	8.51[a]	8.49[a]	8.29[b]	0.040	<0.001	<0.001	0.961	0.422
SCC, $\times 10^3$ cells/mL	1	445	183	620	681	193	0.872	0.269	0.999	0.331
	2	605[a]	287[ab]	320[ab]	231[b]	78.3	0.016	0.021	0.307	0.318
Milk/DMI	1	1.54[c]	1.54[c]	1.70[a]	1.61[b]	0.026	0.011	0.075	<0.001	0.003
	2	1.47	1.51	1.53	1.56	0.031	0.116	0.056	0.485	0.830
ECM/DMI	1	1.66[ab]	1.60[b]	1.74[a]	1.70[ab]	0.038	0.703	0.234	0.344	0.021
	2	1.58	1.60	1.59	1.61	0.035	0.628	0.515	0.994	0.714
Initial BW, kg	Cov	686	700	716	673	15.2	0.588	0.223	0.073	0.771
Final BW, kg	2	696	712	735	672	22.6	0.705	0.203	0.097	0.729
Initial BCS	Cov	3.04	2.98	3.10	3.01	0.112	0.968	0.978	0.646	0.461
Final BCS	2	3.17	3.02	3.07	3.04	0.092	0.259	0.671	0.835	0.328

[a-c]Values within a row with differing superscripts denote AO dose differences between means ($P < 0.05$) and trend between means ($0.05 < P \leq 0.10$).

[1]Period 1 consisted of normal environmental conditions of June in Tennessee with heat abatement. Period 2 consisted of more intense environmental conditions of July in Tennessee by removal of heat abatement.

[2]Contrast between CTL and AO postbiotic.

Table 3-6. Percent change in average DMI, milk and milk components, and feed efficiency from period 1 to period 2 for lactating Holstein cows fed different doses of an *Aspergillus oryzae* postbiotic (AO) challenged with heat stress (LSM ± SEM)[1,2]

	AO Dose, g/d					*P*-Value	Polynomial Contrasts (*P*-Value)		
Item, % change	0	3	6	18	SEM	CTL vs. AO[3]	Linear	Quadratic	Cubic
DMI	-4.37	-8.87	-9.28	-7.83	2.62	0.206	0.725	0.220	0.621
Milk Yield	-14.9	-18.4	-14.2	-13.2	2.43	0.906	0.300	0.855	0.193
ECM Yield	-20.8[b]	-16.2[ab]	-11.1[a]	-13.4[ab]	3.27	0.073	0.221	0.082	0.789
True Protein Yield	-21.6	-20.4	-16.5	-14.9	3.04	0.244	0.120	0.491	0.647
True Protein Percent	-0.381	-2.35	-2.68	-2.44	1.32	0.193	0.457	0.279	0.705
Fat Yield	-19.4[b]	-11.0[ab]	-6.20[a]	-11.6[ab]	5.01	0.111	0.543	0.080	0.941
Fat Percent	-0.165	10.3	9.51	2.98	5.66	0.261	0.834	0.168	0.518
Lactose Yield	-19.4[b]	-15.7[ab]	-12.3[a]	-10.7[a]	3.19	0.101	0.089	0.291	0.919
Lactose Percent	1.89	3.35	2.09	2.54	0.777	0.416	0.945	0.801	0.160
SNF Yield	-19.9	-17.2	-14.0	-12.3	3.24	0.176	0.121	0.418	0.860
SNF Percent	1.43	1.40	0.199	0.627	1.12	0.611	0.589	0.539	0.611
SCC	5.77	1.11	-2.28	19.0	7.99	0.985	0.122	0.210	0.926
Milk/DMI	-12.7[b]	-10.1[ab]	-4.44[a]	-4.75[a]	2.60	0.058	0.055	0.106	0.493
ECM/DMI	-10.1[b]	-10.3[b]	-7.61[ab]	-0.992[a]	3.80	0.420	0.049	0.874	0.767
BW[4]	1.42[ab]	1.38[ab]	3.42[a]	-0.418[b]	0.984	0.970	0.122	0.061	0.261
BCS[4]	5.23	-0.776	0.983	-0.308	2.98	0.159	0.388	0.402	0.339

[a-b]Values within a row with differing superscripts denote AO dose differences between means ($P < 0.05$) and trend between means ($0.05 < P \leq 0.10$).

[1]Percent change from period 1 to period 2, where period 1 data were adjusted using data from day -1 of the study as a covariate.

[2]Period 1 consisted of normal environmental conditions of June in Tennessee with heat abatement. Period 2 consisted of more intense environmental conditions of July in Tennessee by removal of heat abatement.

[3]Contrast between CTL and AO postbiotic.

[4]Percent change for BW and BCS is difference from d -1 to 35 of the study.

Table 3-7. Plasma free fatty acid and urea-N concentrations (μmol/L) in lactating Holstein cows fed different doses of an *Aspergillus oryzae* postbiotic (AO) in two periods of heat stress (LSM ± SEM)[1]

		AO Dose, g/d					*P*-Value	Polynomial Contrasts (*P*-Value)		
Item[2]	Period[3]	0	3	6	18	SEM	CTL vs. AO[4]	Linear	Quadratic	Cubic
Fatty Acid	1	85.8	101	98.7	92.3	6.66	0.158	0.994	0.137	0.389
	2	87.1	78.8	93.8	82.7	8.96	0.853	0.851	0.602	0.249
PUN	1	2,808	3,071	2,789	3,397	369	0.532	0.270	0.733	0.516
	2	1,631	1,745	1,920	1,941	200	0.330	0.327	0.461	0.827

[1]Intra- and inter-assay CV for fatty acids are 12 and 9.9% and for PUN are 30 and 13%.
[2]PUN = plasma urea-N.
[3]Period 1 consisted of normal environmental conditions of June in Tennessee with heat abatement. Period 2 consisted of more intense environmental conditions of July in Tennessee by removal of heat abatement.
[4]Contrast between CTL and AO postbiotic.

Table 3-8. Acute phase protein concentrations (μg/mL) in plasma of lactating Holstein cows fed different doses of an *Aspergillus oryzae* postbiotic (AO) on two different days during heat stress (LSM ± SEM)[1]

		AO Dose, g/d				*P*-Value	Polynomial Contrasts (*P*-Value)	
Item	Study Day[2]	0	3	6	SEM	CTL vs. AO[3]	Linear	Quadratic
HPT	12	3.46	1.66	2.75	1.56	0.529	0.764	0.457
	34	11.5	7.43	14.0	2.60	0.812	0.487	0.101
LBP	12	5.10[a]	3.79[b]	4.67[ab]	0.39	0.089	0.524	0.030
	34	5.63[a]	4.32[b]	6.00[a]	0.60	0.543	0.589	0.052
SAA	12	46.2	27.4	34.3	6.36	0.066	0.230	0.110
	34	69.2[a]	23.8[b]	51.2[ab]	10.7	0.026	0.275	0.011

[a-b]Values within a row with differing superscripts denote AO dose differences between means ($P < 0.05$) and trend between means ($0.05 < P \leq 0.10$).

[1]Intra- and inter-assay CV for haptoglobin (HPT) are 41 and 2.2%, for lipopolysaccharide binding protein (LBP) are 19 and 13%, and for serum amyloid A (SAA) are 7.0 and 20%.

[2]Day 12 consisted of 36 h after start of period 2 that consisted of more intense environmental conditions of July in Tennessee by removal of heat abatement.

[3]Contrast between CTL and AO postbiotic.

Table 3-9. Relative gene expression for cytokines from ex-vivo LPS stimulation of whole blood taken from lactating Holstein cows fed different doses of an *Aspergillus oryzae* postbiotic (AO; LSM ± SEM)

	AO Dose, g/d				*P*-Value		Polynomial Contrasts (*P*-Value)	
Item[1]	0	3	6	SEM	Pen	CTL vs. AO[2]	Linear	Quadratic
No LPS								
IL-1β[3]	0.156	0.198	0.315	0.103	0.687	0.851	0.273	0.775
IL-6[3]	0.134	0.136	0.267	0.089	0.627	0.859	0.191	0.480
TNF-α	0.535	0.790	0.773	0.165	0.393	0.235	0.309	0.513
LPS								
IL-1β[3]	0.536	0.717	0.414	0.307	0.072	0.938	0.568	0.693
IL-6[3]	2.66	2.08	2.60	0.684	0.229	0.511	0.963	0.524
TNF-α[3]	1.87	2.76	2.47	0.749	0.382	0.584	0.565	0.530
Ratio[4]								
IL-1β[3]	2.77	3.15	2.50	0.685	0.041	0.997	0.789	0.546
IL-6	8.17[a]	5.92[ab]	2.81[b]	1.64	0.912	0.070	0.022	0.833
TNF-α[3]	0.519	0.509	0.503	0.153	0.389	0.838	0.938	0.990

[a-b]Values within a row with differing superscripts denote AO dose differences between means ($P < 0.05$) and trend between means ($0.05 < P \leq 0.10$).

[1]*IL-1β* = interleukin 1β; *IL-6* = interleukin 6; *TNF-α* = tumor necrosis factor-α.

[2]Contrast between CTL and AO postbiotic.

[3]Data were log transformed for statistical analysis, and non-transformed LSM are reported.

[4]Ratio of LPS and No LPS stimulation.

CHAPTER 4.

CONCLUSIONS

Cows exposed to high environmental temperatures and humidity experience heat stress, which impacts milk production, metabolism, and the immune system. Nutritional strategies can provide metabolic relief to improve milk production in heat-stressed cows. Providing dietary CP at 16.1% of DM increased ECM and milk fat yield in cooled cows but not heat-stressed cows. Subsequently, a lower dietary CP level (12.5%) sustained milk production responses in cows experiencing unabated heat stress. Higher CP increased glucose and insulin concentrations and decreased free fatty acid concentrations. The increased glucose may derive from improved rumen fermentation and liver function, which better supported milk production of cooled cows. Therefore, heat-stressed cows sustain milk production with low dietary CP, but higher dietary CP increases milk production in cooled cows. Heat-stressed cows also benefit from supplementation of a postbiotic, fungal additive developed from AO fermentation of a polysaccharide. The 6 g/d of AO increased yields of ECM, milk, protein, and fat and efficiency of feed-use for milk. The increase in milk production may derive from greater nutrient utilization with a reduction in body temperature or thermoregulation and a reduction of an inflammatory response at 3 g/d (i.e. reduced acute phase proteins and cytokine expression). Thus, these studies verify that manipulation of dietary CP proportions and a postbiotic additive can be used as nutritional strategies to improve the metabolic and immune adjustments of heat-stressed cows to support milk production.

VITA

Jeffrey Daniel Kaufman was born and raised in Fremont, IN on January 23, 1989. He graduated in May 2007 from Fremont High School. He earned his Bachelor of Science degree in Biology at the Purdue University of Fort Wayne in Fort Wayne, IN in 2012. He earned his Master of Science degree in Animal Science with a focus on Nutritional Physiology and a Minor in Statistics at the University of Tennessee in Knoxville, TN in 2016. He then pursued a Doctor of Philosophy degree in Animal Science on Nutritional Physiology and Immunology at the University of Tennessee under the mentorships of Dr. Agustín Ríus and Dr. Gina Pighetti. Jeff defended his Ph.D. in August 2019, and he will be working as a Dairy Cow Nutritionist at Kalmbach Feeds in Upper Sandusky, OH.