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Impact of Beta-Adrenergic Agonist Supplementation and Heat Stress on the Microbiome and Gastrointestinal Transcriptome of Sheep

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IMPACT OF BETA-ADRENERGIC AGONIST SUPPLEMENTATION AND HEAT STRESS ON THE MICROBIOME AND GASTROINTESTINAL TRANSCRIPTOME OF SHEEP

By

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Improving animal growth and efficiency are critical points of research as the world's population and demand for agriculture products increase. Therefore, adaptions or changes in the gut are of interest to maximize growth efficiency and wellbeing of livestock. The gastrointestinal tract of the rumen plays many critical roles with the assistance of the associated microbial community. One way to improve animal performance is supplementation of β-adrenergic agonists (β-AA) which are commonly fed to cattle during the last 20-40 days of the finishing period, improving muscle growth by decreasing adipose deposition and increasing muscle accretion. Two β-AA, Ractopamine HCl (β1-AA) and Zilpaterol HCl (β2-AA) are currently approved for use in beef cattle in the United States. Alternatively to the beneficial effects of β-AA, heat stress in livestock decreases production efficiency and growth. Based on anecdotal reports it has been suggested that supplementation of β-AA could potentially exacerbate the stress response caused by hyperthermia leading to increased mortality. However, little research has been conducted to investigate the interaction between these two factors. Therefore, the purpose of these studies was to investigate the impact of heat stress, β-AA and the interaction between the two with respect to the ruminant gastrointestinal tract. Microbial

communities were isolated from rumen and cecum contents and RNA was isolated from rumen epithelium of lambs supplemented Zilpaterol HCl and Ractopamine HCl housed in either an ambient or heat stressed environment for 21 d. Additionally RNA was isolated from rumen epithelium of lambs supplemented Ractopamine HCl housed in either an ambient or heat stressed environment for 30 d. No interaction was found between β-AA and heat stress in either the microbial community or RNA studies. Heat stress and β-AA supplementation changed the composition of particular taxa in the rumen microbiome, while the addition of ammonium chloride to the second group significantly impacted the cecal microbial composition. Additionally, heat stress, but not β-AA supplementation, impacted the transcriptome profile of the rumen epithelium by upregulating the oxidative stress response. Based on these results, we conclude that β-AA do not induce an increased stress response within the ruminant gastrointestinal tract.

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CHAPTER I: A LITERATURE REVIEW

Introduction

Ruminant animals have served an important role in sustainable agriculture systems by converting resources, such as rangeland, pasture, and crop residues, into consumable products for humans. Ruminants' ability to utilize foodstuffs inedible by humans and turn them into high quality protein for human consumption is increasingly important as the world's population continues to increase. To keep up with increasing demands, livestock production systems must apply new advancements that feed the growing population in an efficient manner while considering animal wellbeing. The purpose of this work is to explore how the ruminant gastrointestinal environment responds to nutritional supplementation and environmental stress. Understanding how stressors impact ruminants and their ability to derive nutrients will contribute to the goal of improving and meeting demands for sustainable animal agriculture.

The Ruminant Animal and its Associated Microbiome

The rumen is a complex structure that, with the help of the associated microbial community, provides for the digestion of fibrous feed for utilization as a source of energy and nutrients. Ruminal microbiota play many roles in feed digestion and the microbial population can be affected by environmental and dietary changes. The rumen has several essential physiological functions, including nutrient uptake (Baldwin et al., 2004), metabolic activity (Baldwin and McCleod, 2000), host protection (Roh et al., 2007), and overall host health (Guarner, 2006). The microbial community of the rumen is altered by

diet (McCann et al., 2014), age (Jami et al., 2013), and physical environment (Tajima et al., 2007; Uyeno et al., 2010). Although the bacterial population of the rumen has been well described, there is a lack of information on how changes in the microbial population impact the wellbeing and overall function of the host. Being able to link the ruminal microbial structure to host phenotypic traits, such as metabolic function, will help us better understand the interactions between host and microbe, and the role of the microbiome in animal efficiency.

The Ruminant Diet

A majority of ruminant livestock eat a diet consisting primarily of polymers including carbohydrates, nitrogenous substances, lipids, and lignin. These components of the diet, except for lignin, are hydrolyzed to monomers which are then metabolized to fermentation products (Cummings and Macfarlane, 1997). The products of fermentation are mainly acids, such as volatile fatty acids, and gasses, such as methane, with many end products of fermentation depending on the bacteria that break it down (Baldwin, 1965; Tamminga and Van Vuuren, 1988). The abundance and type of bacteria in the rumen that break down the feed is dependent on the digestibility of the feed, which is why the abundance of bacterial communities in animals fed concentrate-based diets are 10- to 100-fold higher than animals fed forage-based diets (Nagaraja, 2016).

Forages have high concentrations of sugars and soluble proteins, but digestibility of these decreases as the cell wall of the forage thickens; a thicker cell wall decreases the ruminal fermentation rate, limiting feed intake (Jung et al., 1995). On the other hand, concentrate feeds increase the rate of ruminal fermentation, thus not restricting feed

intake (Chen et al., 2015). However, diets high in concentrate are reported to have an adverse effect on rumen metabolism (Ametaj et al., 2010, Hua et al., 2017) and are associated with metabolic disorders such as acidosis and liver abscesses (Castillo-González et al., 2014). Concentrate diets also cause a decrease in rumen pH (Fuentes et al., 2009; Hook et al., 2011), which in turn changes the composition of the microbial community and decreases nutrient availability (Hook et al., 2011).

Rumen Bacterial Community

Ruminants are able to utilize and derive nutrients from a wide range of feed due to their highly diverse microbial communities. Following the development of techniques for cultivating anaerobic organisms, a variety of bacteria were isolated and characterized from the rumen (Bryant, 1958). Although beneficial, these *in vitro* bacterial cultivation techniques were limited in their ability to identify distinct species of bacteria. The development of cultivation-independent techniques such as 16S ribosomal RNA gene sequencing and shotgun metagenomic studies have since revealed many bacterial species in the rumen unique from those that were cultivated (Kim et al., 2011; Fouts et al., 2012). Since then, studies using next-generation sequencing (NGS) have evaluated microbial community diversity (Fernando et al., 2010), function (Muegge et al., 2011), and impact on the host (Paz et al., 2017).

The ruminal microbiome is made up of a diverse symbiotic population of anaerobic bacteria, archaea, protozoa, and fungi (Hobson and Stewart, 1997). Rumen bacteria colonize the rumen shortly after birth and contribute to carbon and nitrogen metabolism through fermentation (Guan et al. 2008). The bacteria that make up the

rumen each have different functions in feedstuff degradation and utilization (Figure 1). Bacteria within each class, whether it be cellulolytic, fibrolytic, proteolytic, lipolytic or amylolytic, each possesses enzymes to degrade feedstuff (Table 1).

Although bacteria composition differs amongst species, the core members of the ruminant microbiome of livestock remain consistent (Jami et al., 2012; Kim and Yu, 2014; Henderson et al., 2015; Li and Guan, 2017). Firmicutes, Bacteroidetes, and Proteobacteria are the primary phyla in the rumen although their ratio and relative composition fluctuates with age (Jami et al., 2013). Proteobacteria are the most prominent in the first couple of days after birth, however, at around two months of age the abundance of Proteobacteria decreases as Bacteroidetes and Firmicutes increase to make up the majority of the community (Jami et al., 2013). Additionally, although ruminant bacterial communities can change due to environmental (Tajima et al., 2007; Uyeno et al., 2010), age (Jami et al., 2013), and dietary factors (Tajima et al., 2001; McCann et al., 2014), there appears to be a distinct "core" population of family level taxa amongst ruminants, which includes Prevotella, Butyrivibrio, Ruminococcus, unclassified Lachnospiraceae, Ruminococcaceae, Bacteroidales and Clostridiales (Hyder et al., 2017).

Cecal Importance and Function in Ruminant Species

The ruminant large intestine, consisting of the cecum, colon, and rectum, is the site of water absorption from passing material (Figure 2). Although the cecum of the ruminant serves little function compared to hind gut fermenters such as the horse, the large intestine is the second site of fermentation in ruminants and where water and end-products from passing digesta is absorbed (Tucker et al., 1968). Ulyatt et al. (1975)

reported that VFA production in the cecum ranged from 0.2 to 0.9 moles/day, which represents 8.6-16.8% of total VFA production. Similarly to ruminal fermentation, cecal digestion of feed is also performed by specialized microbiota, however, fermentable substrates arriving in the cecum are different from those fermented in the rumen, which may result in compositional or structural differences in the microbial communities of the two digestive compartments (Popova et al., 2017). The cecum microbial community ferments fiber to produce vitamins for the host (Van, 1994), as well as, poor-quality nutrients (Ulyatt et al., 1975) not utilized by the rumen. Additionally, Argenzio et al. (1975) speculated that VFA absorption in the cecum may serve as an energy source for the active transport of Na, meaning that VFA production in the cecum is critical aside from its' role in supplying energy. Moreover, surgical removal of the cecum in 7-month-old steers showed decreases in digestibility of dry matter, neutral-detergent fiber, acid-detergent fiber, and cellulose decreased (Maala et al., 1983), suggesting a significant role of the cecum in nutrient processing.

Ruminant Transcriptome

The mechanisms that alter growth and barrier function of the gastrointestinal epithelium have received particular attention over the past decade, especially with advancements in molecular-based techniques, such as microarrays and next-generation RNA sequencing. Understanding how host gene expression profiles are associated with environmental stressors or animal efficiency could lead to strategies that improve animal performance. Specifically, studies of the ruminal epithelium have increased with particular interest in the modulation of ruminal function in response to increasing rapidly

fermentable carbohydrates (Penner et al., 2011; Steele et al., 2011a) and supplemental butyrate (Górka et al., 2011; Baldwin et al., 2012; Kowalski et al., 2015). When characterizing genes expressed in the rumen epithelium it was observed the expression profile of rumen tissue clustered with that of skin and tonsil from non-ruminants, but not with other gastrointestinal transcriptomes (Xiang et al., 2016), pointing towards a specialized function of the rumen. Additionally, the expression profile of rumen had many genes involved in stratified epithelium keratins, enrichment of the cell cycle process and innate immunity proteins (Xiang et al., 2016).

Similar to the rumen microbiome, many factors impact the rumen epithelium transcriptome. Comparing high to low residual feed intake steers, 122 genes were differentially expressed, suggesting that the epithelium of efficient steers may have increased tissue morphogenesis resulting in increased absorption of nutrients and increased energy (Kong et al., 2016). The infusion of butyrate caused changes to the rumen epithelium by altering expression of transcripts involved in responses to bacteria and biotic stimuli (Baldwin et al., 2012). Understanding how expression profiles of the rumen epithelium change due to diet, environmental stressors, and supplementation will provide better insight into beneficial practices that can be adopted by the livestock industry to produce a more efficient animal.

Heat Stress

Environments with high temperature and humidity are detrimental to the productivity and economics of the livestock industry (St-Pierre et al., 2003). Homeothermic animals have a thermoneutral zone where normal body temperature is

maintained and energy loss is minimal (Abd-El-Samee and Marai, 1997). When the ambient temperature rises above the thermoneutral zone of livestock heat loss necessary to maintain thermoneutrality exceeds the animals' capacity for heat dissipation (Bernabucci et al., 2010). Livestock have zones of thermal comfort (ZTC) that are dependent on species, health and physiology of the animal, relative humidity, velocity of ambient air, and the degree of solar radiation (NRC, 1981). When livestock are housed in conditions outside of their ZTC, not only is animal wellbeing at risk, but economic losses also result. In the United States, an estimated annual economic loss between $1.69 and $2.36 billion to the livestock industry are attributed to heat stress;, $370 million of these losses occur in the beef industry (St-Pierre et al., 2003).

Temperature Humidity Index (THI)

The temperature-humidity index (THI) was developed as a tool to monitor heat stress in livestock and predict periods in which an animal's health may be in danger (Silanikove, 2000). The common calculation of THI is: THI = (Dry bulb temperature °C) + (0.36 x dew point temperature °C) + 41.2 with dry bulb temperature being the ambient temperature and dew point temperature the relative humidity (St-Pierre et al., 2003). It is important to note that the interpretation of THI is dependent on the species and their specific thermo-tolerance. For example, sheep tend to have a higher tolerance to heat stress and can sustain thermoregulatory functions at a higher THI value (Srikandakumar et al., 2003). On the other hand, for cattle values above 78 are considered dangerous (Silanikove, 2000). Figure 2 shows a THI chart used in the beef cattle industry.

Molecular Effects of Heat

Heat stress has been suggested to be responsible in inducing oxidative stress in livestock (Ganaie et al., 2013; Nizar et al., 2013), leading to cytotoxicity (Bernabucci et al 2002; Lord-Fontaine and Averill-Bates, 2002). Oxidative stress occurs when there is a disturbance in the concentrations of pro-oxidants and antioxidants, leading to the overproduction of free radicals and reactive oxygen species (ROS), and a decrease in antioxidant defense (Ganaie et al., 2013). Gene expression profiles show that the response to heat and oxidative stress is similar (Salo et al. 1991). In accordance with these findings, oxidative stress has been linked to increased cell death and increased reactive oxygen species in heat exposed cells (Burdon et al. 1987; Skibba et al 1991; McAnulty et al. 2005). Additionally, Adachi et al. (2009) reported that oxidative stress influences the heat shock response and unfolded protein recovery, reducing protective functions against heat stress.

As mentioned, heat stress can be cytotoxic by disturbing cell functions and inducing oxidative cell damage. Mitochondria are one of the primary cellular organelles responsible for ROS production; when the concentration of ROS is increased, mitochondria are damaged (Silmen et al 2015). Heat stressed rodents showed abnormalities and altered morphology of mitochondria in skeletal muscle (Hsu et al 1995). Furthermore, mitochondria under heat stress became swollen, with broken cristae and low matrix density (Song et al. 2000). This may suggest that mitochondrial damage due to heat stress renders the cell unable to meet the increased energy requirements of the animal.

Heat shock proteins (HSP) are chaperone proteins activated by heat and other stressors. HSP act as molecular chaperons that provide the cell with the ability to survive injury and oxidative stress (Collier et al., 2008). HSP carry out crucial intracellular housekeeping functions such as folding, unfolding and refolding of stress-denatured proteins (Morimoto et al. 1990). In heat stressed sheep (Romero et al. 2013; Salces-Ortiz et al. 2013) and cattle (Deb et al. 2014; Kishore et al. 2014), HSP-70 and HSP-90 were increased, likely due to an increase in damaged proteins which induced HSP as a mechanism for cellular repair (Slimen et al., 2015)). Furthermore, pretreatment with HSP70 significantly protected intestinal epithelial barrier function impaired by chronic stress (Yang et al., 2009). The increased expression of these proteins during heat stress suggests their importance in cell survival to injury and stress.

Metabolic and Physiologic Impacts of Heat Stress

Physiological responses to heat stress include increased respiratory rate (Marai et al., 2002), increased water consumption (Marai et al., 2007), decreased feed intake (Marai et al., 2007; Guo et al., 2018; Johnson, 2018), ADG, and final weights (Mitlöhner et al., 2002; Blaine and Nsahlai, 2011). These negative responses to heat stress have detrimental effects on the animals' health and wellbeing. Productivity of sheep in heat stress inducing environments causes reduced voluntary feed intake leading to reduced metabolizable energy (Dixon et al. 1999). In a study performed Mitlöhner et al. (2001), heifers in heat stress had lower DMI than those provided shade. Additionally, control heifers provided with shade reached their target BW 20 days earlier than the unshaded, heat stressed heifers. Heat stress upregulates the secretion and expression of the receptors

of two adipokines: leptin and adiponectin (Bernabucci et al. 2009). Leptin stimulates the hypothalamic axis resulting in reduced feed intake (Rabe et al. 2008) while adiponectin regulates feeding behavior through a 'starvation signal' (Hoyda et al 2011). Therefore, heat stress stimulates the hypothalamic axis by increasing the levels of these adipokines resulting in reduced feed intake, partially impacting changes seen in ADG and DMI.

Exposure to heat results in redistribution of blood to the periphery and compensatory reduction in the blood supply to the gut, with blood flow to the abdominal organs, stomach and ileum is decreasing by 55%, 58%, and 32% respectively (Sakurada and Hales 1998). The lack of blood flow to the gut damages the cell lining, allowing for endotoxins to enter the body. The endotoxins released into the body can then cause tissue damage and an acute phase immune response (Cronjé 2015). When exposure to heat ceases and normal blood flow resumes, reactive oxygen species and cytokines are released, which cause multiple organ injury (Cronjé, 2005). It is therefore possible that the integrity of the gut lining is critical for heat stress abatement. This is especially important in livestock species as energy–dense production diets damage the gut lining, even in the absence of heat stress (Conlon et al., 2015), permitting endotoxins to enter the body (Cronjé, 2005).

Beta-Adrenergic Agonists

Beta-adrenergic agonists (β-AA) are similar to endogenous catecholamines that increase rate of gain, decrease carcass fat, and improve feed efficiency when fed before harvest by stimulating the adrenoreceptors, which are similarly stimulated during stress (Nelson, 1980; Vasconcelos et al., 2008; Elam et al., 2009; Montgomery et al., 2009).

These compounds have been an area of interest for researchers and producers for the past two decades due to their ability to improve efficiency in livestock production (Montgomery et al., 2009). They are commonly fed to livestock to redirect nutrients from fat deposition towards muscle formation. However, their mechanism of action is not fully understood and several factors such as diet, dose and duration of treatment, age, genetics, and weight impact the efficiency of β-AA supplementation. Therefore, due to β-AA signaling through adrenoreceptors, understanding if the mechanism by which β-AA act will allow for a better understanding of whether their use in animal agriculture increases stress to the animal or alters an animal's ability to respond properly to other stressors.

Proposed mechanism of action

Beta-adrenergic receptors (β-AR), on the surface of muscle, fat, and other cells and are part of a large family of G protein-coupled receptors (Mills and Mersmann, 1995). Subtypes of β-AR include β1-AR, β2-AR and β3-AR. The β1-AR and β2-AR are the most well characterized and most abundant in mammalian cells (Mersmann, 1998). The function of these receptors is similar, only differing by the ligands they bind. The type of β-AR varies by tissue type and across species although one β-AR type generally predominates within a tissue (Barnes, 1995). For example, bovine and ovine skeletal muscle and adipocytes contain primarily β2-AR (Johnson et al., 2014) while rat heart contains over 90% β1-AR and rat adipocytes contains over 90% β3-AR (Mersmann, 1998; Mersmann, 2002).

Substrates bind the ligand binding site of β-AR located at the center of the seven transmembrane domains (Mills and Mersmann, 1995). Activation of β-AR is coupled

with G_s proteins and the activation of adenylate cyclase. When adenylate cyclase is stimulated this leads to the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Liggett and Raymond, 1993). Cyclic AMP is an intracellular signaling molecule that binds to protein kinase A (PKA) to release its catalytic subunit. PKA then phosphorylates intracellular enzymes such as serine residues of metabolic hormones and regulates their use. The phosphorylation results in the partial hydrolysis of triacylglycerol and inhibits de novo biosynthesis of fatty acids (Ricks et al., 1984; Mersmann, 1998). Based on the proposed mechanism of action for β-AA, it has been hypothesized that β-AA reduce adipose tissue accretion by inhibiting de novo biosynthesis of fatty acids and by stimulating triacylglycerol hydrolysis (Mersmann, 2002). Furthermore, in muscle cells the activation of the β-AA signaling pathway results in increased muscles mass by inhibiting protein turnover and promoting myofibrillar protein synthesis (Johnson et al., 2014). Overall it is hypothesized that the binding of β-AA induces signals in fat cells to decrease fat synthesis and increase fat degradation, resulting in a slower rate of fat accretion while in muscle cells, binding of a β-AA results in increased muscle protein synthesis (Anderson et al., 2005).

B-Adrenergic Agonist Types

The β2-AA cimaterol and clenbuterol were the first β-AA investigated in livestock production as a way to increase production efficiency in both cattle and sheep (Warriss et al., 1989). Although they were effective in increasing overall weight gain and gain to feed ratio (Kim et al. 1987), their overall benefit was not significant enough to account for their cost (Warriss et al., 1989; Koohmaraie et al., 1991; Pringle et al., 1993).

During the 1990s and early 2000s, two β-AA were approved for use in cattle in the United States: zilpaterol HCl (ZH) a β2 agonist, and ractopamine HCl (RAC) a β1 agonist (Delmore et al., 2010; Boler et al., 2012).

Zilpaterol HCl (ZH) is produced by Merck Animal Health and sold under the trade name Zilmax. Zilpaterol HCl is on average fed to cattle in the last 26.6 ± 9 days on the feedlot, having a substantial impact on live and carcass traits (Lean et al. 2014). A meta-analysis by Lean et al (2014) reported that animals fed ZH had a BW 8kg and ADG of 0.15kg more than non-supplemented controls, while also having reduced DMI (0.1kg/hd/day). Additionally, supplementation with ZH improves hot carcass weight, cool carcass weight, dressing percentage, and carcass meat percentage in heifers (Montgomery et al. 2009). While ZH benefits livestock production by increasing body weight, the β-AA acts as a repartitioning agent to redirect nutrient from fat to protein deposition reducing marbling score and fat depth (Lean et al. 2014), which is often accompanied by a decrease in tenderness (Knight, 2014)

Ractopamine HCl (RAC) is produced by Elanco Animal Health and sold under the trade name Optaflexx for cattle, Paylean for swine and Topmax for turkeys and is fed during the last 20 to 40 days. Animals supplemented with RAC show an increase in both BW and ADG of 8kg and 0.19 kg/day, respectively, compared to non-supplemented controls (Lean et al. 2014). Moreover, supplementation with RAC increases G:F ratios by 20.5% (Abney et al., 2007) and final live weight (Vogel et al., 2005; Lopez-Carlos et al., 2011).

Contrary to the previously stated results, administration of either ZH or RH did not improve carcass traits of Pelibuey x Katahdin male lambs (Estrada-Angulo et al., 2008; Robles-Estrada et al., 2009). Additionally, control and supplemented ewe lambs, fed 10 mg of ZH/day had similar *(P* > 0.10) feedlot performance and body fat deposition (Macías-Cruz et al., 2010). These studies demonstrate a variation in response to ZH or RH across animals that could be due genetics, species, and other variables.

Due to rapid elimination of β-AA residue from the animal, β-AA have gained popularity in livestock systems. RAC has not been shown to leave residues after feeding so a withdrawal period is not necessary (Elanco, 2017). Conversely, ZH has a minimum three-day withdrawal period before slaughter to ensure residues in the muscle tissue are eliminated before consumption (Merck, 2017). Additionally, ZH and RAC have no antibiotic activity and therefore do not act as antibiotic growth promoters (Anderson et al. 2005). However, neither β-AA is approved for use in sheep or chickens (AMSA, 2015). Additionally, while both are approved for use by the FDA, animal welfare concerns related to the use of ZH in feedlot cattle resulted in the removal of ZH from production systems, leading to RAC as the primary β-AA used in cattle production (AVMA, 2014)

Factors that influence response to β-AA

Although β-AA improve animal performance and efficiency, their effect varies across studies (Fiems, 1987). It has thus been hypothesized, and some data have supported that the age of the animal, weight, accessibility of β-AR, type of β-AA, species, and genetics may impact the overall effect of the β-AA. For example, the use of two different β-AA, metaproterenol and terbutaline, had different impact on feedlot

parameters and carcass characteristics of lambs fed the same diet (Nourozi et al., 2008). Additionally, β-AA may differ in effect due to varying abundance of β-AR across cell and tissue types (Moody et al. 2000). The β-AR available on the cell surface also may become desensitized over time with β2-AR being more readily desensitized than β1-AR (Lafontan 1993, Johnson et al 2014). Due to the desensitization of β-AR reports have suggested that at high doses, RAC can bind β2-AR and elicit a biological response, but both the cost of that level of supplementation and possible side effects keep this from being a viable option (Vogel et al., 2005).

Potential Adverse Effects of Beta-Adrenergic Agonists

While the use of β-AA is beneficial in improving growth and animal performance, reports suggest that these supplements have adverse effects as well. RAC improves both growth performance through increased protein synthesis (Moody et al. 2000) and decreased protein degradation (Mershmann 1998; Moody et al. 2000; Dunshea et al. 2005). Given that an effect attributed to β-AA supplementation is decreased protein degradation, it is possible its use may lead to tougher meat. In a literature review, Koohmaraie et al. (2002) concluded that improvements in animal growth could be due to decreased protein degradation, which reduces the rate of post-mortem proteolysis in muscle resulting in tougher meat. However, Merck Animal Health reported that although tenderness is decreased in animals fed ZH, flavor is unaffected (Merck, 2017) and sensory panel tasting scores showed that there were no differences between the products of control and ZH fed cattle (Weber et al., 2013).

In 2013, instances of increased mobility issues and mortality were observed in feedlots using ZH and as a result, concerns arose about the wellbeing of cattle being fed ZH (Loneragan et al., 2014). Although no scientific data were available to support the connection of ZH and lameness, the possible association of ZH with these issues caused Tyson and other packing plants to stop accepting cattle fed the supplement (Sorensen, 2016). The claims against ZH led to Merck Animal Health removing ZH from the market in the US, leading to a decrease of its use in the United States and a negative public perception of ZH (Sorensen, 2016).

To investigate the claims that ZH diminishes animal wellbeing, studies have compared mobility of animals supplemented ZH to controls. As time on feed increased, mobility decreased for all animals, regardless of ZH supplementation (Boyd et al., 2015). This work suggested that ZH was not the cause of mobility issues but instead the increase in overall mass as cattle aged may have resulted in the issues reported. Further, transport and flooring at the packing plants were hypothesized to contribute to mobility problems (Boyd et al., 2015). Overall, research conducted to investigate adverse effects due to the feeding of ZH has shown no measurable negative effect of ZH supplementation on animal wellbeing (Hales et al., 2014; Boyd et al., 2015; Buntyn et al., 2016; Sorensen, 2016).

β-AA Impact on the Microbiome

Although β-AA improve ADG, feed efficiency and carcass weight, the potential effect β-AA have on the ruminant ecosystem in feedlot animals is not yet understood. Natural catecholamines, norepinephrine, epinephrine, and dopamine, stimulate bacterial

growth (Roberts et al. 2002), affect gut motility and the secretory response (Ruckebush, 1983; McIntyre and Thompson 1992), and also increase the amount of gram-negative bacteria species in vitro (Lyte and Ernst 1992). The latter impact of β-AA on the microbiome is important as many gram-negative bacteria of the rumen are vital for fermentation (Walker and Drouillard 2012).

Walker et al. (2006) showed a reduced response to RAC when finishing heifers were fed ruminally degraded forms of nitrogen. The reported interaction between RAC and the nitrogen supplied suggested a direct effect of RAC on microbial populations of the rumen. Therefore, it is possible that RAC alters proteolysis in the rumen, resulting in less degradable protein available to the rumen microbes. Additionally, Walker et al. (2006) demonstrated that the ratio of degradable to undegradable protein provided in diet to the ruminal microflora is important for maximizing response to RAC.

The effect of natural catecholamines on bacteria is not fully understood; however, it is possible that catecholamines increase the ability of bacteria to uptake iron, which is essential for bacterial growth (Kinney et al. 2010). Catecholamines can also function to chelate iron and thus allow bacteria to recognize and use these siderophores (Kinney et al. 2000). RAC may therefore be providing a competitive advantage by increasing iron availability for bacteria to utilize; this may explain the increased bacterial population growth seen by Walker and Drouillard (2010) in an *in vitro* assay using RAC. Walker and Drouillard (2010) reported that RAC increased gas production in vitro providing evidence that the impact of RAC on an animal is not limited to the tissue response. Because β-AR are present all along the digestive tract, the binding of β-AA directly

affects its motility and secretory functions (Ruckebush, 1983; McIntyre and Thompson, 1992).

Interaction between heat stress and beta-adrenergic agonist supplementation

While a lot of attention has been given to the impacts of heat stress and β-AA separately, little attention has been paid to the interaction between the two. Heat stress and β-AA both stimulate the β- adrenergic system, however it is unknown how their simultaneous stimulation impacts the animal. Based upon anecdotal observations, it has been hypothesized that the supplementation of β-AA could potentially exacerbate the stress response caused by hyperthermia (Grandin, 2013; Loneragan et al., 2014). Additionally, Allen et al. (2017) found an association, but no supported causation, between cattle fed during the summer months and a decrease in feed intake compared to animals fed ZH during cooler months. Little to no research has examined the interaction between β-AA usage, heat stress, and their combined impact on the ruminant GI tract ecosystem and physiology.

Marcías-Cruz et al. (2010) represents one of the few studies evaluating how heat stress β-AA supplementation impact growth traits and carcass characteristics. In the study, ewe lambs were fed 10 mg of ZH/ewe/day and were housed in an environment with an average ambient temperature of 34.1°C with 50.4% humidity. No difference was observed in final live weight, ADG, feed intake, feed conversion, and G:F between ZH and control lambs even though previous studies have shown all these traits excepting feed intake increase with ZH supplementation (Vasconcelos et al., 2008; Montgomery et al., 2009; Hales et al., 2014). Therefore, Macías-Cruz et al. (2010) suggests that under heat

stress conditions the effect of ZH on performance traits is diminished. Additionally, ZH-supplemented lambs showed an increase in hot and cold carcass weight, dressing percentage, conformation and rib-eye area when compared to control (Macías-Cruz et al., 2010). These data indicate that there is no consequential interaction between heat stress and β-AA. However, this is a single study and additional research must be conducted to fully understand what is occurring when β-AA are fed during heat stress conditions.

Conclusion

Much is known about the ruminant gastrointestinal tract and its importance in nutrient absorption, utilization, and energy production. Changes in diet as well as environmental stressors can affect rumen function impacting animal performance. Heat stress and β-AA have been studied individually, but little is known about their interaction. Studying the impact of heat stress and β-AA supplementation on the rumen is important because many feedlot cattle are fed β-AA during summer month. By gaining a better understanding of the response to each factor individually as well as in concert, we can improve growth and efficiency along with animal wellbeing. Therefore, this research aimed to identify how β-AA supplementation and heat stress impacts the rumen gastrointestinal ecosystem and physiology to further improve the sustainability of animal production.

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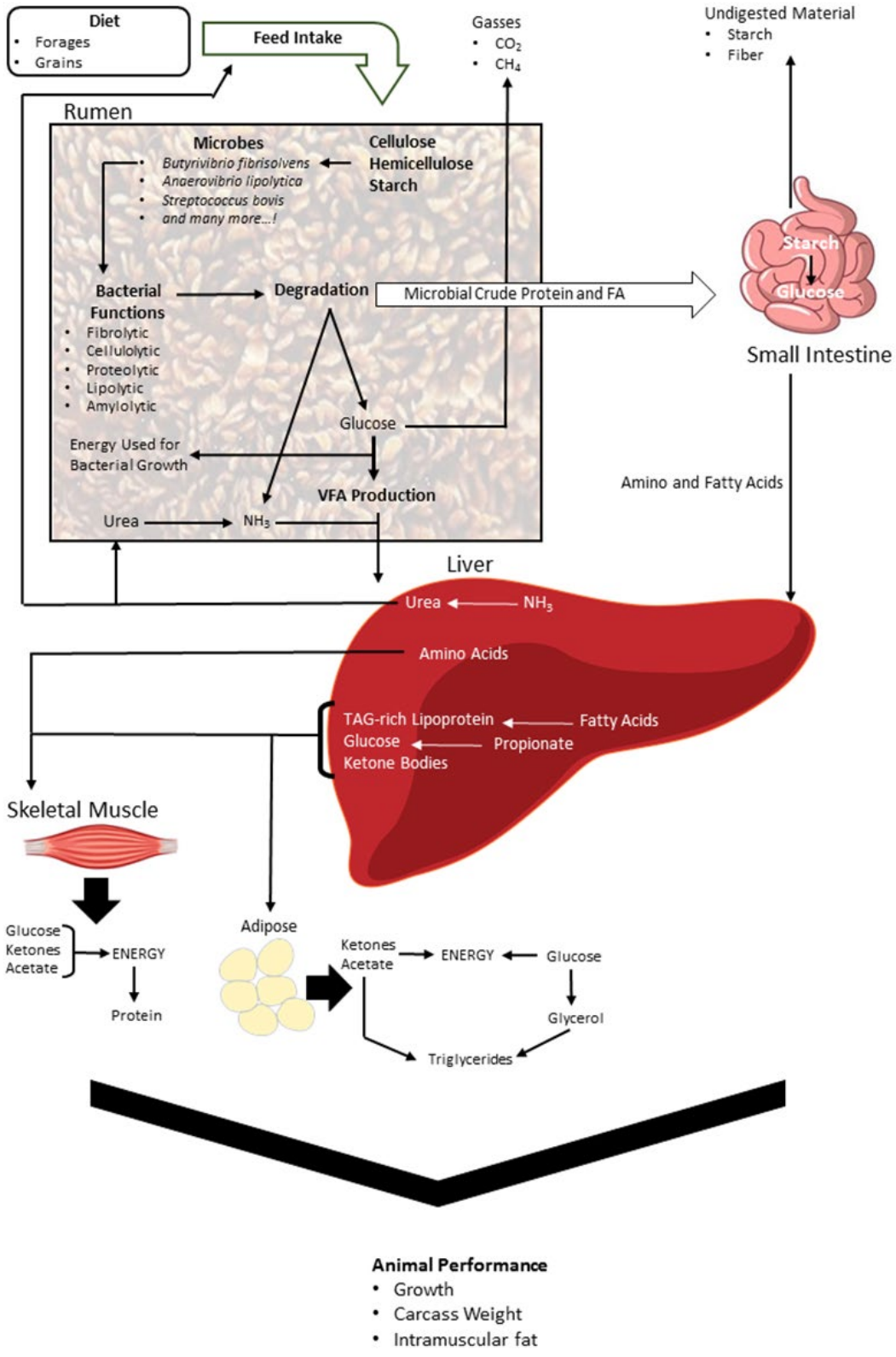

Diet
Forages
Grains
Feed Intake
Gasses
CO_2
CH_4
Undigested Material
Starch
Fiber
Rumen
Microbes
Butyrivibrio fibrisolvens
Anaerovibrio lipolytica
Streptococcus bovis
and many more...!
Cellulose
Hemicellulose
Starch
Bacterial Functions
Fibrolytic
Cellulolytic
Proteolytic
Lipolytic
Amylolytic
Degradation
Microbial Crude Protein and FA
Starch
Glucose
Small Intestine
Glucose
Energy Used for Bacterial Growth
VFA Production
Urea
NH_3
Amino and Fatty Acids
Liver
Urea
NH_3
Amino Acids
TAG-rich Lipoprotein
Fatty Acids
Glucose
Propionate
Ketone Bodies
Skeletal Muscle
Glucose
Ketones
Acetate
ENERGY
Protein
Adipose
Ketones
Acetate
ENERGY
Glucose
Glycerol
Triglycerides
Animal Performance
Growth
Carcass Weight
Intramuscular fat

Figure 1. The link between nutrition, metabolism, and the rumen microbiome (Adapted from McCann et al. (2014) and Dairy Essentials: Babcock Institute for International Dairy Research and Development http://bizplan-uz.com/learning/course/): Changes in microbiome composition affect bacteria within functional niches responsible for feedstuff degradation. Fermentation of feedstuffs results in the production of volatile fatty acids (VFA), methane (CH_4), carbon dioxide (CO_2), and ammonia (NH_3). CO_2 and CH_4 are released as gasses into the environment while other fermentation products, VFAs and NH_3 are absorbed by the rumen to be transported to the liver. Fatty acids (FA) and microbial crude protein are taken up by the small intestine and also taken to the liver. NH_3 is converted to urea to be recycled to the GI tract. Additionally, propionate is converted to glucose and distributed with amino acids and lipoproteins to skeletal muscle and adipose. These metabolic reactions all work with one another to alter animal performance.

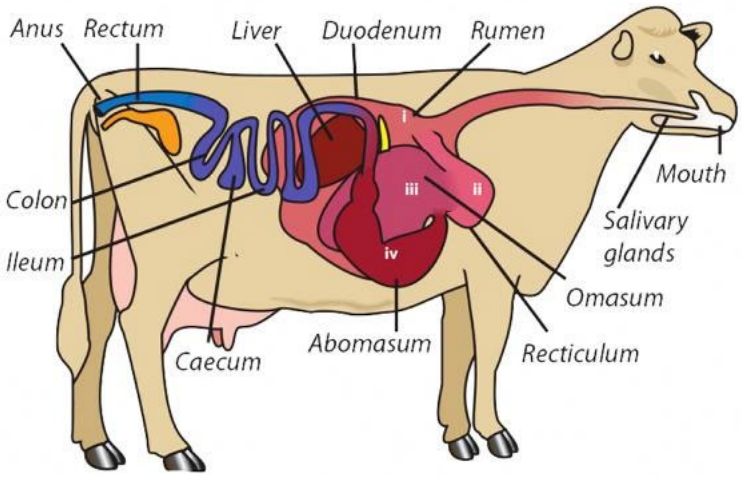
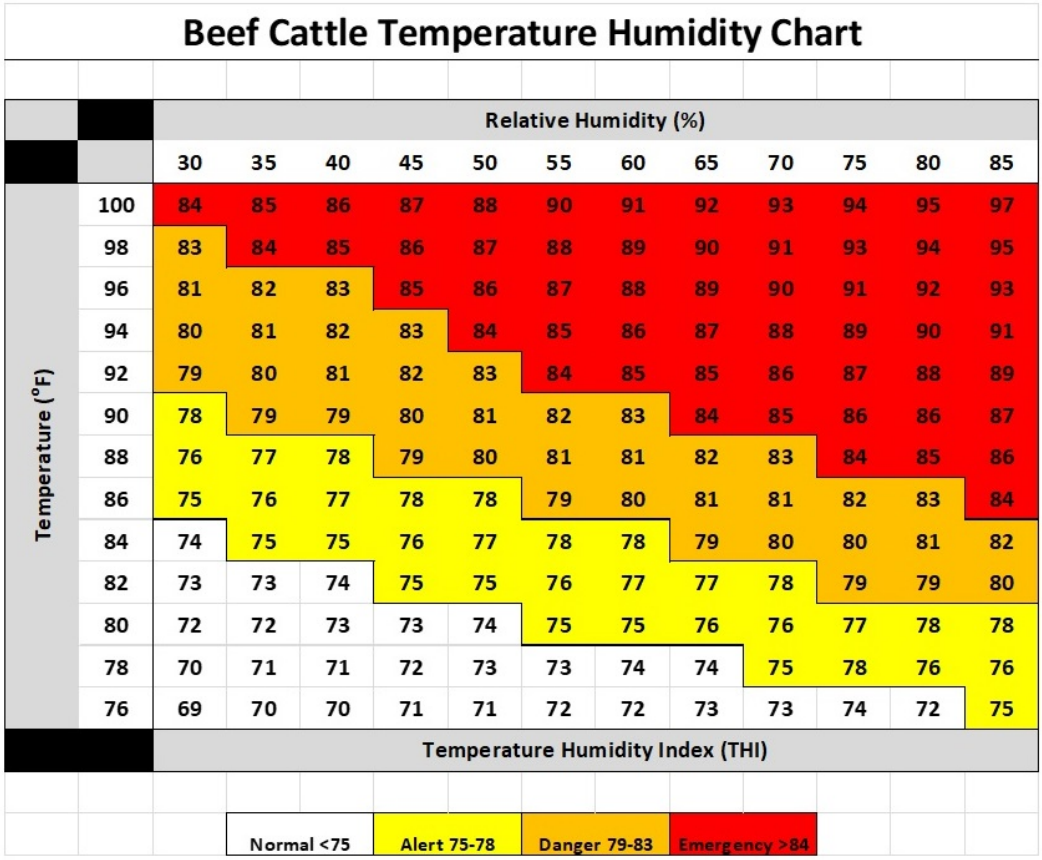


Figure 2. A diagram of the ruminant digestive system of cattle (Source: Irish Farmers Journal www.farmersjournal.ie/)



Beef Cattle Temperature Humidity Chart

Temperature (°F) \ Relative Humidity (%)	30	35	40	45	50	55	60	65	70	75	80	85
100	84	85	86	87	88	90	91	92	93	94	95	97
98	83	84	85	86	87	88	89	90	91	93	94	95
96	81	82	83	85	86	87	88	89	90	91	92	93
94	80	81	82	83	84	85	86	87	88	89	90	91
92	79	80	81	82	83	84	85	85	86	87	88	89
90	78	79	79	80	81	82	83	84	85	86	86	87
88	76	77	78	79	80	81	81	82	83	84	85	86
86	75	76	77	78	78	79	80	81	81	82	83	84
84	74	75	75	76	77	78	78	79	80	80	81	82
82	73	73	74	75	75	76	77	77	78	79	79	80
80	72	72	73	73	74	75	75	76	76	77	78	78
78	70	71	71	72	73	73	74	74	75	78	76	76
76	69	70	70	71	71	72	72	73	73	74	72	75

Temperature Humidity Index (THI)

Normal <75 | Alert 75-78 | Danger 79-83 | Emergency >84

Figure 2. Standard THI diagram for beef cattle. (Source: University of Nebraska - Lincoln https://beef.unl.edu/beefwatch/heat-stress-handling-cattle-through-high-heat-humidity-indexes)

Table 1. Bacterial classes and their functions

Bacterial Class	**Function**
Cellulolytic	Break down cellulose; changes in activity may be caused by a shift in ruminal pH (Nozière et al. 2010) as well as temperature (Castillo-González et al. 2014).
Fibrolytic	Conduct microbial fermentation by hydrolyzing the β 1,4 bonds in plant polysaccharides which breaks down these plant fibers for nutrient utilization for both the bacteria and the host animal. Fermentation leads to the production of volatile fatty acids (VFAs). Once absorbed the VFAs are transported by the blood to the liver where they are converted to other forms of energy.
Amylolytic	Starch digesters that produce enzymes such as alpha-amylase and pullulanase, a debranching enzyme (Nagaraja 2016). Higher levels of amylolytic organisms, caused by increased starch availability, induces an increase in the propionate (Nozière et al. 2010) which is processed to create glucose to be used by adipose and skeletal muscle.
Proteolytic	Secrete proteases to hydrolyze peptide bonds in proteins from feedstuffs. The breakdown of these proteins results in amino acids and short peptides that can be used as potential nutrients for the growth of rumen microorganisms, but they may also be degraded to ammonia and lost from the rumen (Wallace 1996).
Lipolytic	Utilize lipase enzymes for decomposing plant and animal fats. The decomposition of these fats results in the formation of glycerol, fatty acids, and water. The bacteria perform the degradation of lipids to provide themselves with carbon as an energy source, as well as for the host.

CHAPTER II: INVESTIGATION OF THE RUMEN MICROBIOME AND RUMINAL VOLATILE FATTY ACID CONCENTRATIONS OF LAMBS SUPPLEMENTED WITH BETA-ADRENERGIC AGONISTS AND SUBJECTED TO HEAT STRESS FOR 21 DAYS

Introduction

The ruminant gastrointestinal tract and the associated microbial community is a complex system that is vital for regulation of nutrient uptake (Baldwin et al., 2004), metabolic activity (Baldwin and McCleod, 2000), host protection (Roh et al., 2007), and overall host health (Guarner, 2006). Within the rumen, cellulolytic microbes break down cellulose and fibrous compounds whereas amylolytic bacteria ferment starches to produce volatile fatty acids (VFA). Volatile fatty acids are produced primarily from dietary carbohydrates and provide a significant amount of energy through direct oxidation or as precursors to hepatic gluconeogenesis or lipid biosynthesis (Dijkstra, 1994). The different classes of bacteria play distinct roles and therefore understanding the bacterial population of the rumen may allow for more efficient nutritional management to improve overall health and optimize production efficiency. The development of next-generation sequencing (NGS) technology has allowed for studies of the microbiome to be performed independent of culture-based methodology. Studies using NGS have evaluated microbial community diversity (Fernando et al., 2010), function (Muegge et al., 2011) and impact on the host (Paz et al., 2017). By better understanding changes in the microbiome, management practices can be altered to increase production efficiency and animal wellbeing.

The rumen microbial community is altered by many host factors including diet (Tajima et al., 2001; McCann et al., 2014), age (Jami et al., 2013), and physical environment (Tajima et al., 2007; Uyeno et al., 2010). An environmental variable of concern in livestock production is heat stress, which reduces feed intake and feed efficiency (Hagenmaier et al., 2016; Barnes et al., 2004). In the United States beef industry alone, the impact of heat stress results in an estimated loss of $370 million per year (St-Pierre et al., 2003). With such substantial losses and other impacts on animal wellbeing, it is important to understand how changes in the rumen bacterial community impact livestock performance and health.

Heat stress may indirectly change the microbial community of the rumen by decreasing feed intake (Marai et al., 2007). Additionally, significant reductions in VFA levels have been reported during increased environmental temperature in both ad libitum and limit-fed animals (Weldy et al. 1964; Kelley et al. 1967; Freestone and Lyte 2010), which may be attributed to changes in feeding behavior and the bacterial community. Ruminants also respond to environmental heat by redistributing blood flow from gastrointestinal organs to their periphery (Sakurada and Hales, 1998). The gut microbiome is important in maintaining intestinal epithelial health. Therefore, the host-microbe interaction is critical during times of stress (Pearce et al., 2018). The reduction in blood supply to the gut can damage its epithelial lining, permitting endotoxins to enter the body (Cronjé, 2005). Changes in the epithelium tissue that impact this interaction may also alter the associated microbial community.

Alternatively to the negative effects of heat stress, β-adrenergic agonists (β-AA), which are fed as supplements to finishing cattle, act via specific adrenergic pathways to increase the efficiency of muscle growth (Buntyn et al., 2016). However, these same adrenergic pathways are associated with stress responses when stimulated by endogenous ligands, catecholamines (Nelson, 1980). The catecholamines, norepinephrine, epinephrine, and dopamine, stimulate bacterial growth (Roberts et al., 2002), affect gut motility and secretory response (Ruckebush, 1983; McIntyre and Thompson 1992), and increase gram-negative bacteria species in vitro (Lyte and Ernst 1992); the latter potentially impact feed conversion because many gram-negative bacteria of the rumen are starch fermenters (Walker and Drouillard 2012).

Two β-AA are approved for use in cattle in the United States: zilpaterol HCl (ZH) a β2 agonist, and ractopamine HCl (RAC) a β1 agonist (Delmore et al., 2010; Boler et al., 2012). These are named for the isoform of the β-adrenergic receptor (β-AR) to which they preferentially bind. Average daily gain (ADG) was increased by 17.9% in cattle supplemented RH (Vogel et al., 2005) and up to 74% in cattle supplemented ZH (Montgomery et al., 2009; Hales et al., 2014). Additionally, live weights were improved by 5% when supplementing ZH (Montgomery et al., 2009; Robles-Estrada et al., 2009). Particular operational taxonomic units (OTUs) of the rumen have been attributed to improved ADG and average daily feed intake (ADFI) (Paz et al., 2017), suggesting that β-AA supplementation may change the microbial community in a means that improves animal performance.

Researchers also have observed that β-AA reduce the frequency and intensity of ruminal contractions (Ruckebusch et al. 1983; Brikas et al. 1989; Leek 2001), which are important to digestion, and others have shown that β-AA increase absorption in the digestive tract (McIntyre and Thompson, 1992). These changes in ruminant digestion attributed to β-AA may lead to changes in VFA production. Additionally, β-AA may influence the production of VFA directly to increase the efficiency of digestion and provide more energy to the animal. Considering the impact of β-AA on growth efficiency (Scramlin et al., 2010; Romero-Maya et al., 2013) and the noted effects of the adrenergic system on gut activity (Ruckebush, 1983; McIntyre and Thompson 1992) and bacterial abundance (Lyte and Ernst 1992), the role of β-AA in determining the rumen microbiome of livestock remains of interest.

While attention has been given to the impacts of heat stress (Hagenmaier et al., 2016) and β-AA (Romero-Maya et al., 2013) separately, fewer studies have evaluated the interaction between the two. Heat stress and β-AA both stimulate the β- adrenergic system, however it is unknown how their simultaneous stimulation impacts the animal. Based upon anecdotal observations, it has been hypothesized that the supplementation of β-AA could potentially exacerbate the stress response caused by hyperthermia (Grandin, 2013; Lonergan et al., 2014). Additionally, supplementing β-AA during warmer conditions could potentially increase the symptoms of heat stress (Boyd et al., 2015). Marcias-Cruz et al (2010), however, reported that lambs supplemented ZH and housed in heat stress environments showed no difference from non-supplemented lambs in final

live weight, ADG, feed intake, feed conversion, and feed efficiency, although the positive effect of ZH on performance traits was diminished by heat stress.

Because β-AA supplementation in heat-stressed animals may negatively exacerbate stimulation of the adrenergic system, we hypothesized that the interaction between environment and supplementation with β-AA will have a negative impact on the rumen microbiome; resulting in decreased animal performance by altering VFA production. Therefore, the objective of this study was to determine the impact that supplementation with β-AA (ractopamine HCl or zilpaterol HCl) and heat stress on the rumen bacterial community and concentration of VFAs in the rumen using lambs as a model for ruminant livestock. With the profound role of the rumen microbiome in nutrient utilization and animal health, it is possible that heat stress can impact the microbiota decreasing its metabolic activity and fermentation, thus decreasing heat produced and energy provided to the animal. Additionally, β-AA may alter the microbiome to increase nutrient digestion providing more energy to the animal in the form of VFAs. However, to our knowledge, the influence of heat stress and β-AA supplementation together on the rumen microbiome and VFA concentrations has not been investigated.

Materials and Methods

Animals and Experimental Design

The following experimental procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and approved by the Institutional Animal and Care and Use Committee at the University of Nebraska -

Lincoln (IACUC protocol 1300). Animal studies were performed at the University of Nebraska-Lincoln Animal Science Complex (Lincoln, NE). Forty-nine Suffolk x Rambouillet crossbred lambs (53.26 ± 3.68 kg) were stratified by body weight and divided into two groups (group one = 39.99 ±1.92 kg; group two = 37.35 ± 1.92 kg) to accommodate for the capacity of the environmental chambers (12 stalls).

The lambs were housed in one of two environmental conditions. The thermoneutral (TN) condition was approximately 21 °C and 25% humidity over the 21 d study period. Heat stress (HS) was achieved by maintaining a temperature of approximately 34 °C and 35% humidity from 0800 to 2000 h, and 29 °C between 2000 and 0800 with temperature changing over a period of 2 h at the beginning and end of each heat cycle. In both environmental conditions, the light was from 0630 to 2045 h.

Lambs assigned to group one were transitioned to a 90% concentrate finishing diet (49% SweetBran®; 37.8% dry-rolled corn; 8.3% chopped alfalfa hay; 4% mineral supplementation; dosage of coccidiostat (20 g/ton)) over a period of 21 days. Lambs assigned to group two were held on a maintenance diet (54.8% SweetBran®; 41.1% chopped alfalfa hay; 4% mineral supplementation; dosage of coccidiostat (20 g/ton)) at 2% of body weight for 41 days prior to a 21 day transition to the 90% concentrate finishing diet previously stated with the addition of ammonium chloride (10 mg/hd/d).

On trial, lambs were fed at 2.5% body weight and administered one of three supplemental treatments: zilpaterol hydrochloride (ZH) supplemented at 5.44 g/ton (2.5 mg/kg), ractopamine hydrochloride (RH) supplemented at 18.14 g/ton (40 mg/kg), or no supplement (control; C). The supplement was mixed into 200 g of fine ground corn with

the control receiving only the 200g of fine ground corn. β-AA supplementation began on study day 1 and was mixed into 907.19 g of feed offered at 0800h to ensure consumption of the supplement. The remainder of the diet was fed at 1200h.

Within group, lambs were stratified by body weight and randomly assigned to one of six experimental groups for a 2x3 (diet/environment) factorial design: Ractopamine HCl/thermoneutral (RHTN), Zilpaterol HCl/thermoneutral (ZHTN), control/heat stress (CHS), Ractopamine HCl/heat stress (RHHS), and Zilpaterol HCl/heat stress (ZHHS). All lambs were harvested on day 21 of the study via captive bolt followed by exsanguination. Rumen contents were flash frozen in liquid nitrogen and stored at −80 °C until use.

DNA extraction, 16S rRNA library preparation, and sequencing of the V4 Bacteria region

Total DNA was extracted from the rumen samples (~0.25-1 g) using the PowerMag Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol, with the exception of the following modification. During cell lysis, two bead-beating steps were performed in a TissueLyser (Qiagen Inc., Valencia, CA, USA) for 10 minutes (min) at 30 Hz and samples were incubated in a 95 °C water bath for 5 min between the two bead-beading steps to ensure cell lysis.

The V4 region of the 16s rRNA gene was amplified from the extracted DNA using universal 16S primers as described previously by Kozich et al. (2013). The 25 μL PCR reaction consisted of 0.5 μL Terra PCR Direct Polymerase Mix (0.625 Units), 10 μL

2× Terra PCR Direct Buffer, 1 µL of primers (10 µM), 2 µL of extracted DNA (20 to 70 ng DNA), and 7.5 µL of molecular grade water. PCR conditions for amplification of the 16S rRNA gene included: an initial denaturation of 98 °C for 3 min, followed by 25 cycles of 98 °C for 30 s, 55 °C for 30 s, and 68 °C for 45 s, and a final extension of 68 °C for 4 min. Following amplification, 5 µL of amplicon product was run of a 1.2% agarose gel at 120 V for 55 minutes for size and amplification verification.

Following amplification, PCR products from each sample were normalized (1 to 2 ng/µL) using the SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer, then pooled (10 µL/sample) using the Eppendorf epMotion (M5073, Germany). The pooled libraries were purified using the MinElute PCR Purification Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. The resulting concentrated samples were size selected using the Pippin Prep (Sage Science, Inc., Beverly, MA, USA) automated size selection instrument with 1.5% agarose gel cassettes. The resulting libraries were quantified using a DeNovix Spectrophotometer/Fluorometer (Wilmington, DE, USA) and PCR product size and quantity was verified using the Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The libraries were sequenced on an Illumina Miseq (Illumina, San Diego, CA, USA) using the V2 500 cycle kit according to the manufacturer's protocol.

Volatile fatty acid extraction

Rumen fluid and contents collected from each lamb were analyzed for VFA concentrations according to Erwin et al. (1961), with the following modifications. Five g of each sample was weighed into a flask and 15 ml of 0.5N H_2SO_4 was added. The flasks

were incubated overnight at 4°C. After overnight incubation, the protocol was followed as suggested. A standard was prepared containing known amounts of VFA, and 2.0 ml of this solution was also combined with 0.5 ml of 25% metaphosphoric acid and 25 mM 2-ethyl butyrate solution. Samples and standards were refrigerated for 30 min and then centrifuged at 10,000 × g for 15 min. The supernatant was filtered through a 25-mm Whatman (GE Healthcare Life Sciences, Pittsburgh, PA) syringe filter using a 3-ml BD (Becton, Dickinson and Company, Franklin Lakes, NJ) tuberculin syringe into a 2 ml vial. The samples were analyzed for VFA using a Thermo Scientific Trace 1300 (Thermo Fisher Scientific, Waltham, MA) gas chromatographer.

Microbial Community Analysis

Contigs were assembled and quality filtered to remove sequences with ambiguous bases, incorrect length (min=245, max=275), or those improperly assembled (MOTHUR v.1.38.1; Schloss et al., 2009). Utilizing the UPARSE pipeline (USEARCH v7.0.1090; Edgar, 2013), sequences were then clustered into operational taxonomic units (OTUs). Clustering steps included dereplication, sorting by cluster size (descending and not retaining singletons), mapping sequences to OTUs at 97% identity, and filtering chimeric sequences using UCHIME (Edgar et al., 2011) with ChimeraSlayer gold.fa as the reference database.

Taxonomy assignments were performed as described in MOTHUR (Schloss et al., 2009) using a Naive Bayes classifier similar to the RDP Classifier (Wang et al., 2007), with the Greengenes database (gg_13_8_otus; McDonald et al., 2012) reference sequences. Taxonomy was then assigned to the OTU table previously generated and the

resulting table was converted to a BIOM (McDonald et al., 2012) file for further analysis. The Silva database (Quast et al., 2013) was used to generate a customized reference database to check for sequences outside the V4 region. Outliers were removed, and sequences aligned to the customized reference alignment. Overall OTUs were removed if there was poor alignment, singletons, or classified within the Archaea kingdom of Cyanobacteria phylum. The resulting alignment was used to construct a phylogenetic tree using Clearcut (Sheneman et al., 2006).

Alpha metrics were used to describe bacterial richness (observed OTUs), diversity (Shannon–Weiner index [logarithm base 2]; Shannon and Weaver, 1949), and dominance (1-Simpson index). To reduce the effect of animal-to-animal variation in rumen bacterial community composition, a core measurable microbiome (CMM) was defined as OTUs that were present in all animals split by environment (HS, TN), supplementation (ZH, RH, C), and group (G1, G2). After filtering out OTUs that were not found in all animals in each group, the filtered OTU tables were then merged back together. Once the CMM was defined, OTUs were filtered from the original OTU table that were not to be included. Once unwanted OTUs were filtered from the original OUT table, the CMM OTU table was formed.

Statistical Analysis of the Microbial Community

The OTU table was rarefied to lowest sequencing depth of 10,943 reads using QIIME v.1.9.1 (Caporaso et al., 2010) implementing the Mersenne Twister pseudo-random number generator. Alpha and beta diversity indices and statistical comparisons of the total microbial community and of the core microbial community were also conducted

with QIIME v.1.9.1. Alpha diversity metrics were compared using a nonparametric two-sample t-test with multiple comparisons that were corrected for false discovery rate (Benjamini and Hochberg, 1995). Good's coverage (Good, 1953) was calculated to evaluate adequate sampling depth after rarefaction. Differences between total and core microbial composition were evaluated in R (R Core Team, 2018) with the adonis function of the vegan package (Oksanen et al., 2017). The weighted UniFrac distance matrix was used as an input for PERMANOVA using environment, supplement, group as the main effects and testing for an interaction between environment and supplement. Significance was declared for alpha and beta diversity metrics at $P \leq 0.10$.

Pairwise comparisons of total and core microbial communities across main effects were tested with the linear discriminatory analysis (LDA) effect size (LefSe; Segata et al., 2011) to identify differentially abundant OTUs/bacterial features. LefSe was implemented using default parameters with a significance threshold of $P < 0.05$ for the factorial Kruskal-Wallis test among classes and a threshold of 2.0 for the LDA score for discriminative features.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict metagenome functional content based of 16S rRNA gene data (Langille et al 2013). To utilize the PICRUSt software a biom-formatted OTU table was generated by close reference picking against the Greengenes database (gg_13_8) followed by normalization by copy number and metagenome predictions using the KEGG Orthologs option. After identification of metagenomic functional profiles, statistical analysis of taxonomic and functional profiles (STAMP; Parks et al., 2014) was

used to analyze and determine biological significance of taxonomic and metabolic profiles determined by PICRUSt. Differences were evaluated in STAMP using Welch's t-test with a cut off $P < 0.10$.

Statistical Analysis of Volatile Fatty Acids

Data were analyzed using the GLM procedure of SAS (SAS Institute INC., Cary, NC). The model for measurement of VFA included the effects of supplement, environment, group, and the interaction between diet and environment. Differences were declared significant at $P<0.05$, and tendencies noted at $P<0.10$. If significant differences were determined, Tukey's Honest Significant Difference test was used to evaluate pairwise comparisons for significance. Data are presented as means ± SEM.

Results

Total Microbial Community

Approximately 1.3 million quality filtered sequences were generated from the 48 samples. Before rarefaction, 1,630 OTUs were identified; after rarefaction to 10,943 sequences per sample, 1,447 OTUs remained. The Good's coverage index demonstrated that the sampling depth obtained represented on average 99.3% of the total bacterial community.

The Shannon (diversity) and Simpson (dominance) metrics indicated no difference ($P > 0.10$) among any groups evaluated (the interaction between environment and supplement, environment, supplement, or group). Species richness measured by observed OTUs was decreased ($P = 0.08$) in HS compared to TN lambs. Beta diversity, calculated using the weighted unifrac distance matrix as input for a permutational

multivariate analysis of variance (PERMANOVA), suggested that environment had an impact ($P = 0.06$) on the overall composition of the bacterial community. However, group ($P = 0.25$), supplement ($P = 0.60$), and the interaction between environment and supplement ($P = 0.33$), did not affect the overall rumen microbial composition.

Core Measurable Microbiome

The core OTU table had 194 OTUs in total. PERMANOVA analysis of only the core OTUs showed no effect of environment ($P = 0.14$), rep ($P = 0.27$), supplement ($P = 0.64$), or environment x supplement (P = 0.24) on the core rumen microbial composition. Alpha diversity metrics revealed that ZH ($P = 0.10$) and RH-supplemented ($P = 0.10$) lambs had greater OTUs from the control. All other comparisons for the alpha diversity metrics were not affected.

A majority (95%) of the CMM were represented by 5 phyla (Figure 1), primarily composed of Firmicutes (56%) and Bacteroidetes (26%). The major family members in the CMM included Lachnospiraceae (15.01%), Prevotellaceae (14.84%), Erysipelotrichaceae (13.47%), Veillonellaceae (8.85%), Ruminococcaceae (8.07%), Paraprevotellaceae (4.33%), Coriobacteriaceae (3.46%), and S24-7 (Figure 2).

LEfSe analyses of differentially abundant OTUs found 16 OTUs that differed ($P < 0.05$) in abundance between HS and TN lambs (Figure 3), equating to 8.25% of the core microbiome. Four OTUs in RH and 12 OTUs in ZH-supplemented lambs were differentially abundant ($P < 0.05$; Figure 4a & 4b) when compared to control lambs and 27 OTUs were differentially abundant ($P < 0.05$; Figure 5) between group one and two (Table 2). When evaluating differentially abundant families of the core microbial

community, Bacillaceae and Mycoplasmataceae were more abundant ($P < 0.05$) in HS animals. Lachnospiraceae and Pirellulaceae were more abundant ($P < 0.05$) in ZH supplemented lambs compared to the controls, while RH supplemented lambs were not different from the controls.

LEfSe identified that the bacterial OTU 52, belonging to the family Veillonellaceae, was more abundant ($P < 0.05$) in ZH supplemented lambs compared to control supplemented lambs. PICRUSt predicted Veillonellaceae to have functional categories related to biosynthesis of secondary metabolites, carbon metabolism, glycolysis and gluconeogenesis, the TCA cycle, and propionate and butyrate metabolism. Predicted functionality was not available for any other differentially abundant OTUs. STAMP identified 14 features that differed ($P < 0.10$) between the CMM of the HS and TN lamb from the PICRUSt output (Figure 6a). RH supplemented lambs had 0 and ZH supplemented lambs had 19 different features ($P < 0.10$) from the control (Figure 6b). No features differed between group one and group two.

Volatile Fatty Acids

Due to low detection of VFA, data from 7 animals were removed leaving that of 6-8 lambs per treatment for analysis. Group had no impact of VFA concentrations ($P > 0.05$) and was therefore removed from analyses. The ratio of acetate, propionate, and butyrate remained constant among experimental groups, with an average of 75:15:10. Total VFA concentrations did not differ ($P = 0.65$) between TN and HS lambs (11.88±1.12 mM and 12.6±1.12 mM, respectively). Total VFA concentrations were decreased ($P < 0.05$) in RH- (10.0±1.37 mM) and ZH-supplemented lambs (11.6±1.42

mM) compared to non-supplemented lambs (15.12±1.32 mM). RH-supplemented lambs had reduced ($P = 0.05$) concentrations of isobutyrate compared to non-supplemented lambs.

A significant supplement-environment interaction ($P < 0.05$) was found for acetate, propionate, and total VFA concentration. RH-supplemented HS lambs produced less ($P < 0.05$) acetate than non-supplemented HS lambs (Figure 7). The concentration of propionate was less ($P < 0.05$) in HS lambs supplemented with RH or ZH and in non-supplemented TN lambs compared to non-supplemented HS lambs (Figure 7). Additionally, non-supplemented TN lambs produced less propionate than non-supplemented HS lambs ($P = 0.02$). Finally, HS lambs supplemented with RH or ZH produced less ($P < 0.05$) total VFA than non-supplemented HS lambs. There was no difference in total VFA concentrations between non-supplemented TN and non-supplemented HS lambs.

Discussion

In this study, we have reported that β-AA supplementation and heat stress each affect specific bacterial OTUs and family members, which may explain changes in overall lamb performance of feedlot animals. Notably, there were no interactions between environment and supplementation on the microbial community, suggesting that β-AA in heat-stressed animals does not change their rumen microbial population differently than in thermoneutral animals. However, there were interactions between environment and supplementation on the concentration of acetate, propionate, and total VFA in the rumen. Although heat stress and β-AA supplementation each altered specific members of the

rumen bacterial composition, the overall microbial population was not altered by experimental group. Similar to previous rumen bacterial studies (Petri et al., 2013; Henderson et al., 2015), most bacteria of the ruminant communities in our lambs in the current study were classified in the phyla Bacteroidetes and Firmicutes. However, the compositions of these phyla in our study were represented by different OTUs, which varied by among our experimental group. Overall, these results indicate that the changing physiology of the lambs under heat stress or supplemented a β-AA only minimally selected for specific groups of bacteria.

Effects of Heat Stress

The core measurable microbiome was analyzed rather than the total microbial community to reduce noise and to focus on differences that were consistent within our experimental groups. In the core microbial community, heat stress altered the relative abundance of 16 OTUs and 2 familial taxa. Although Tajima et al (2007) and Uyeno et al (2010) previously reported that heat and humidity had notable effects on the rumen microbial community, our results did not reveal changes in the same taxa reported by these studies. The discrepancy between the present and previous observations could be attributed to the differences in housing, diet, and species.

Differentially Abundant Bacterial Taxa in Heat Stress

The present study identified the family Mycoplasmataceae as increased by heat stress. Mycoplasmataceae are obligate parasites that need to obtain nutrients from the environment to survive and multiply (Jarquin, 2011). The prevalence of Mycoplasma, a genus of the family of Mycoplasmataceae, was also increased in response to heats stress.

Mycoplasma attach to the surface of epithelial cells, causing damage that can interfere with membrane receptors and alter transport mechanisms of the host cells (Jarquin, 2011). No known toxins are released by Mycoplasma, but STAMP results from our study indicate that bacterial features involved in bacterial toxins were increased in HS lambs. Although the abundance of Mycoplasmataceae was relatively low (0.085% in HS lambs), the increase in members of this family may have compromised the epithelial lining of the HS lambs and thus nutrient uptake and fermentation capacities. The greater abundance of the Bacillaceae family in response to heat stress us understandable, as most form endospores that are resistant to heat allowing for prolonged survival in adverse conditions (Setlow, 2006; Baril et al., 2012). Therefore, the increase in relative abundance of Bacillaceae, as indicated by LEfSe, may have resulted from the ability to protect themselves against heat stress.

Conversely, abundance of OTU87, *Ruminococcus albus*, a fiber fermenting, cellulolytic bacteria was reduced by heat stress. Ruiping et al. (2013) reported that in mid- and late-lactation heifers, *R. albus* was similarly decreased by heat stress. Cellulolytic bacteria often rely on other microbes to meet their nutrient requirements (Walker and Drouillard 2012). Therefore, their reduction indicates that shifts in the bacterial community during heat stress resulted not providing nutrients for *R. albus* to survive.

β-AA Supplementation

Although studies have reported that natural catecholamines impact bacterial growth, to our knowledge there is limited literature available discussing the impact of ZH and RH

on bacterial communities. Similar to Walker and Drouillard (2010), who reported that RH added directly to buffered ruminal fluid had no impact microbial growth in culture, no significant effect of RH was observed on the overall bacterial community. Therefore, these data conclude that RH is not changing or interacting with the microbial community, and thus is not impacting microbial fermentation.

Differential Taxa Due to β-AA

Although there were no changes in family abundances in RH-supplemented animals, Lachnospiraceae were more abundant in ZH-supplemented lambs compared to those on the control diet. Lachnospiraceae are important members of the GI tract because of their ability to produce butyrate (Bryant 1986) which promotes microbial growth (Liu et al., 1999), host epithelial cell growth (Roediger 1980; McIntyre et al., 1993; Hague et al., 1996; Pryde et al., 2002), and is utilized as an energy source (Bugaut,1987). An increase in the production of butyrate due in lambs supplemented with ZH due to Lachnospiraceae may help to explain increased ADG in animals supplemented with ZH (Montgomery et al., 2009; Hales et al., 2014).

OTU 52, which was of greater abundance in ZH supplemented lambs by LEfSe was identified to belong to the family Veillonellaceae, which produce propionate as a major fermentation product (Kishimoto et al., 2006). Additionally, PICRUSt identified OTU 52 to be involved in in butyrate and propionate metabolism. Propionate is the only major VFA that contributes to gluconeogenesis (Young, 1977). Increased production of propionate may lead to changes in glucose metabolism potentially increasing feed efficiency and promoting growth (Nafikov and Beitz, 2007). Myer et al., (2015) reported

that Veillonellaceae were decreased in abundance in cattle that had decreased ADG and increased average daily feed intake. The decreased abundance of Veillonellaceae observed by Myer et al. (2015) may have impacted cattle performance resulting in low ADG. Furthermore, STAMP results identified a feature increased in ZH supplemented lambs to be pentose and glucuronate interconvention, which is involved in carbohydrate metabolism and thus may also contribute to a mechanisms by which ZH increases animal efficiency. The differences in relative abundance in particular OTUs may play a part in the increase in carbohydrate metabolism in ZH supplemented lambs, suggested by STAMP.

Ruminal VFA Concentrations

Previous reports (Weldy et al. 1964; Kelley et al. 1967; Freestone and Lyte 2010) have shown heat stress reduces VFA levels; while our study revealed a significant interaction between environment and supplement on acetate, propionate, and total VFA in the rumen. Control lambs in HS tended to have a higher level of VFAs compared to the others. HS events may impact rumen functionality by altering blood flow to the rumen (Crandall et al., 2008), which may consequently affect rumen epithelium and its ability to absorb nutrients (Storm et al., 2012). Therefore, we hypothesize that non-supplemented lambs in the HS environment were not producing more totals VFAs but rather the environment altered the ability of the rumen to uptake VFAs produced, leaving them unabsorbed. Reduced absorption of VFAs leads to reduced energy uptake by the animal which can reduce overall efficiency impacting livestock production systems (Bergman 1990; Doreau et al., 1997).

Changes in VFA attributed to heat stress have been hypothesized to be caused by changes in the bacterial composition in response to heat stress itself or due to decreased feed intake and rumination, in turn decreasing buffering agent entering the rumen (Hyder et al., 2017). Additionally, temperature and humidity have a notable effect on the rumen microbial composition which may lead to changes in VFA production (Tajima et al., 2007). There was a difference in microbial taxa present in our lambs comparing HS to TN conditions, therefore we expected to see a clear difference in VFA as well. However, these results suggest that the response due to the interaction between environment and supplementation is more complex. Our results could be attributed to a change in the metabolic properties of microbial fermentation. By the microbes changing their metabolic function they were able to combat heat stress and maintain adequate ruminal fermentation.

A study observing the effect of ZH on ruminal fermentation in finishing steers showed no impact of the supplement on VFA production (Romero et al., 2008). Zilpaterol hydrochloride, however, increases muscle glucose oxidation (Barnes et al. 2017; Cadaret et al. 2017), suggesting its mode of action is elsewhere rather than in the GI tract. We did see a significant interaction in the production of propionate and total VFA in ZH supplemented lambs in the HS environment, suggesting that ZH together with HS cause a negative impact on propionate and total VFA. Additionally, the interaction between environment and RH supplement for production of acetate and propionate and the effect of RH on total VFA production was results were similar to Walker et al. (2007) who attributed decreased VFA production to RH. These results

suggest that the interaction between supplementation with β-AA and the environment fails to support efficient ruminal fermentation, which can decrease animal performance. Changes in ruminal fermentation may be caused by poor ruminal contractions which can cause feed to pass through the rumen quickly, not allowing for microbes to come into contact with feedstuffs. Conversely, the decreased amount of VFA in ruminal contents attributed to the interaction of environment and supplement in this study may cause VFAs to be metabolized and absorbed more rapidly, leaving less unabsorbed in the contents collected.

The observed change in total VFA is contrary to Walker and Drouillard (2010) in which RH added directly to buffered ruminal fluid had no impact on VFA levels, showing that RH does not impact microbial growth or fermentation end products when grown in pure cultures. We have found that RH supplementation did not change bacterial composition, however RH and the interaction of RH with environment impacted production of VFAs. This implicates another mechanism, besides the change in the bacterial community itself in altered VFA production. The relative reduction in total VFAs when supplemented with β-AA may therefore be suggestive of decreased microbial activity. Additionally, the reduction in VFA production in this study suggests that production depends on the overall anatomy and function of the rumen *in vivo* which would explain why *in vitro* studies saw no reduction in the production of VFA.

Conclusion

Overall, within a heat stress environment and supplementation of a β-AA did not have a strong effect on the overall core microbial community. However, changes in the

abundance of particular OTUs did occur. The underlying causes for shifts in bacterial communities is mostly unknown, however it is possible the changes in environmental temperature may be an indirect cause, via changes in the physiology of animals in response to stress. Additionally, the present study found the environment-supplementation interaction had a significant impact on the concentration of acetate, propionate, and total VFA found in the rumen. Heat stress decreases feed intake (Lu et al., 2007; Padua et al., 1997; Marai et al., 1997a), which could impact the microbial composition. As the HS and TN lambs were not pair fed, the effect of reduced feed intake on the microbiome cannot be elucidated from these data.

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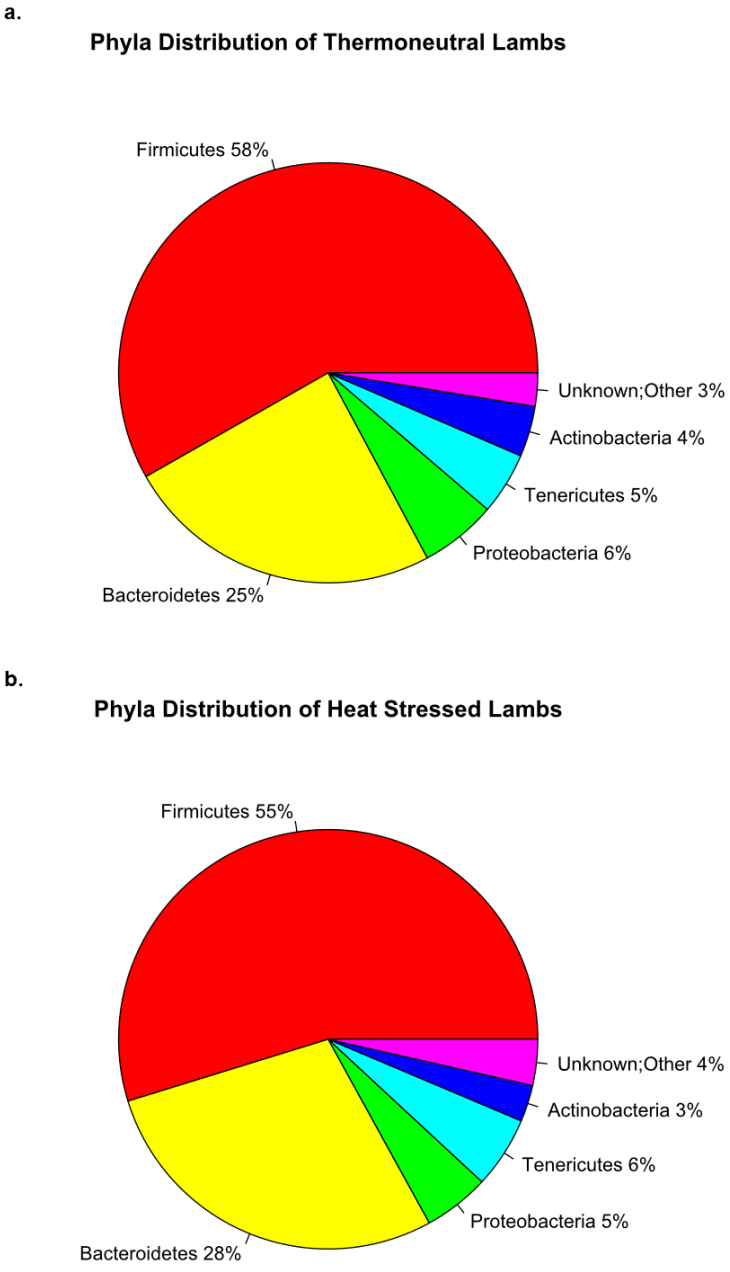

Figure 1. Ruminal bacterial community taxonomy at the phylum level for either a). thermoneutral environment or b). heat stress environment

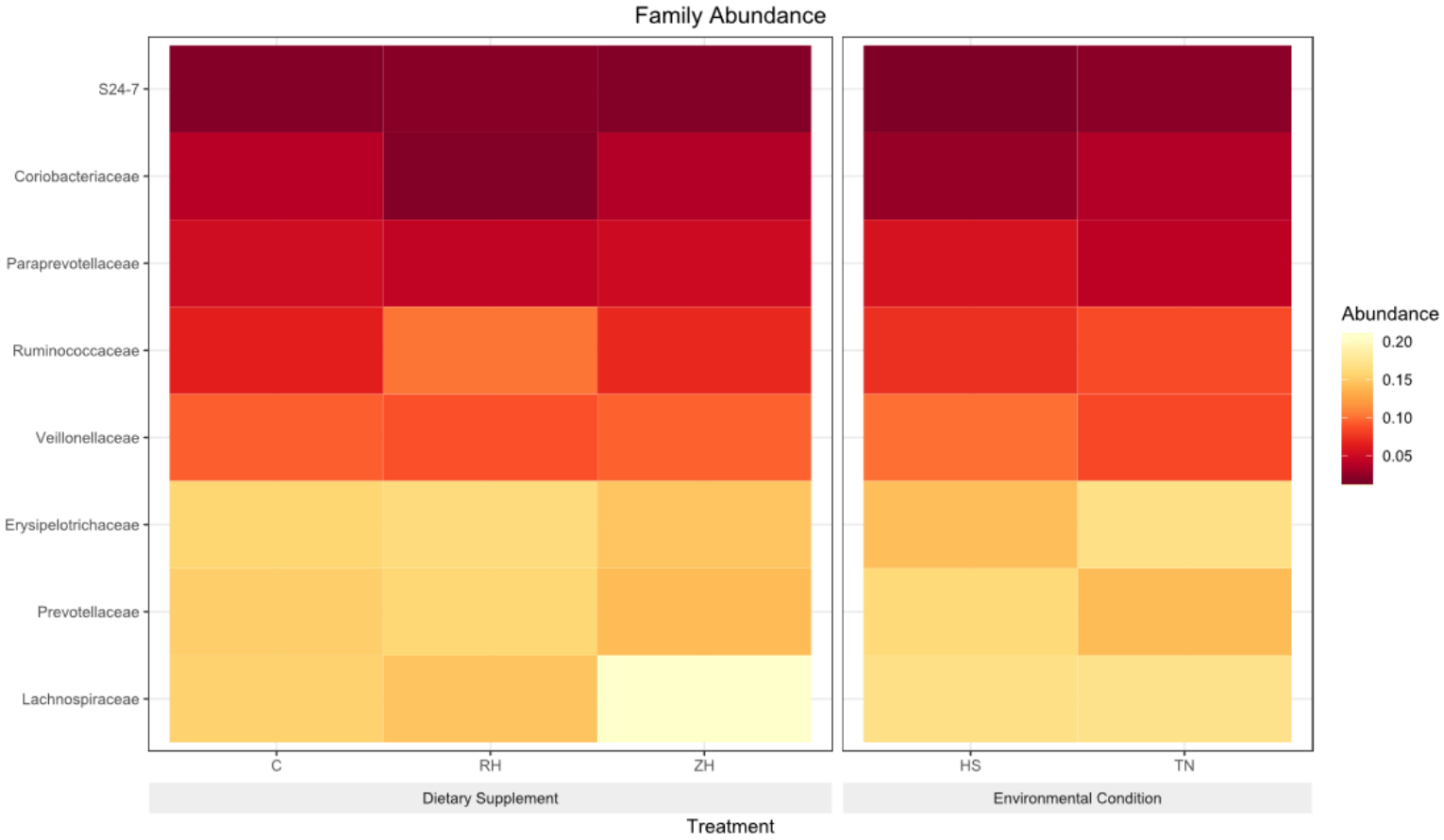

Figure 2. Heatmap showing differing family abundances for dietary supplement (control = C, zilpaterol HCl = ZH, and ractopamine HCl = RH) and environmental condition (thermoneutral = TN and heat stress = HS). The gradient from red to yellow represents low to high relative abundance of the evaluate family taxa.

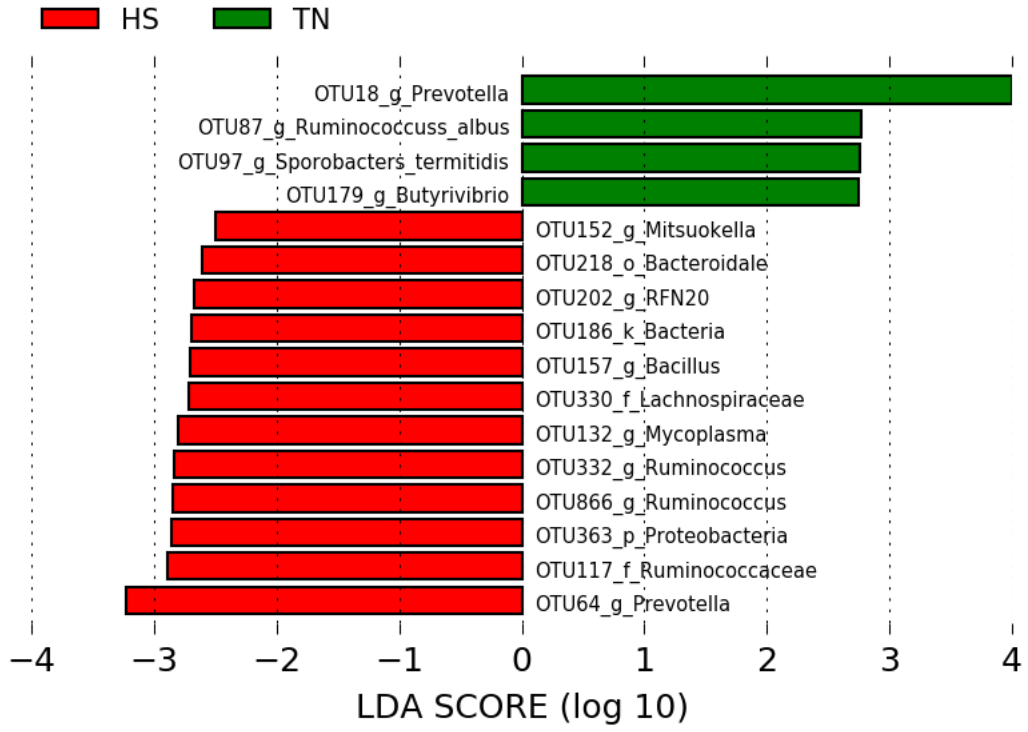

Figure 3. Differential analysis of microbiota community among environmental conditions. The Linear discrimination analysis (LDA) coupled with effect size (LEfSe) was used to identify the most differentially abundant taxa among heat stressed (HS) and thermoneutral (TN) lambs. Only the results meeting an LDA significant threshold of >2 are shown.

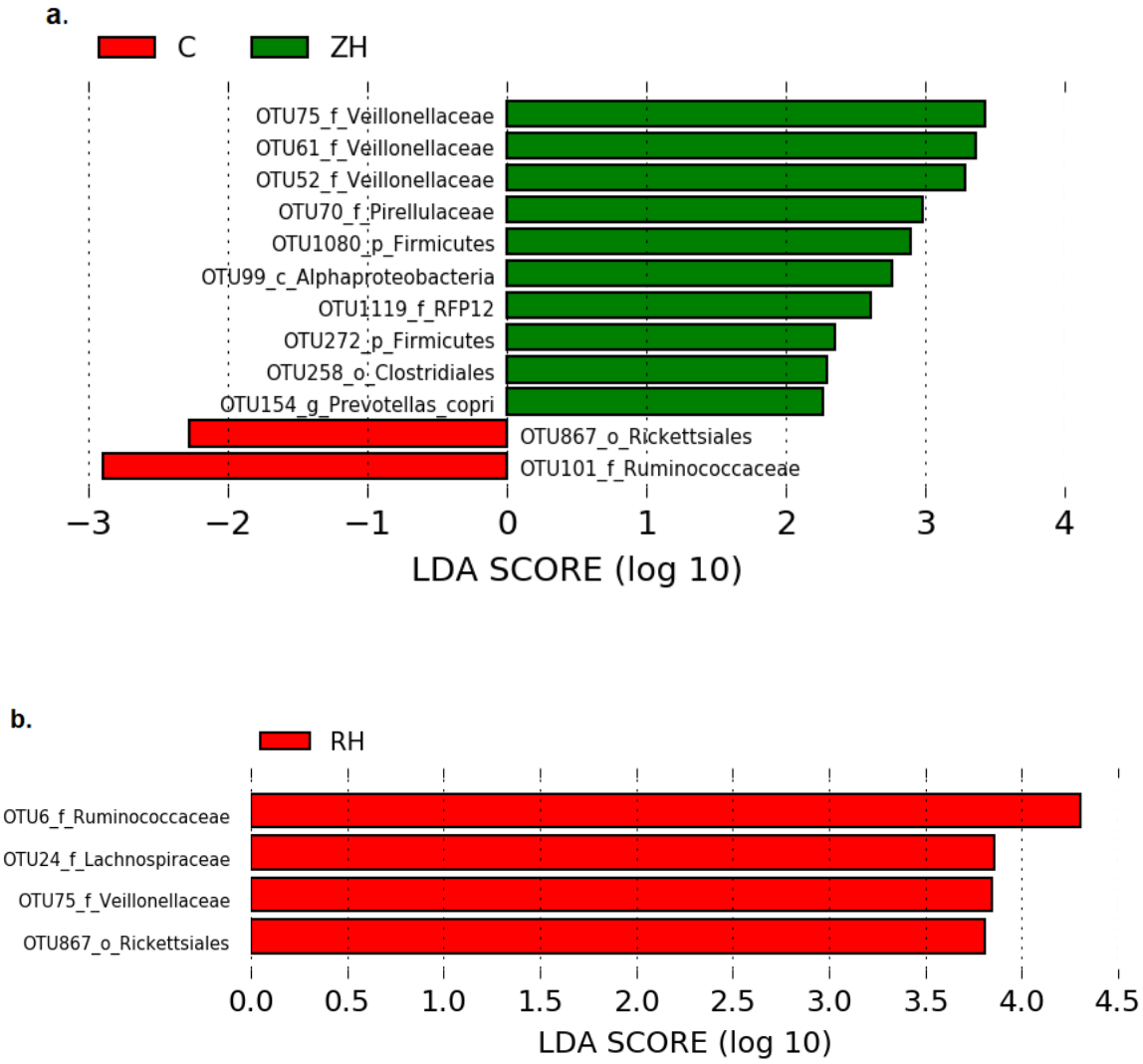

Figure 4. Differential analysis of microbiota community among dietary supplement. The Linear discrimination analysis (LDA) coupled with effect size (LEfSe) was used to identify the most differentially abundant taxa among RH-supplemented and non-supplemented controls (a), ZH-Supplemented and non-supplemented controls (b).

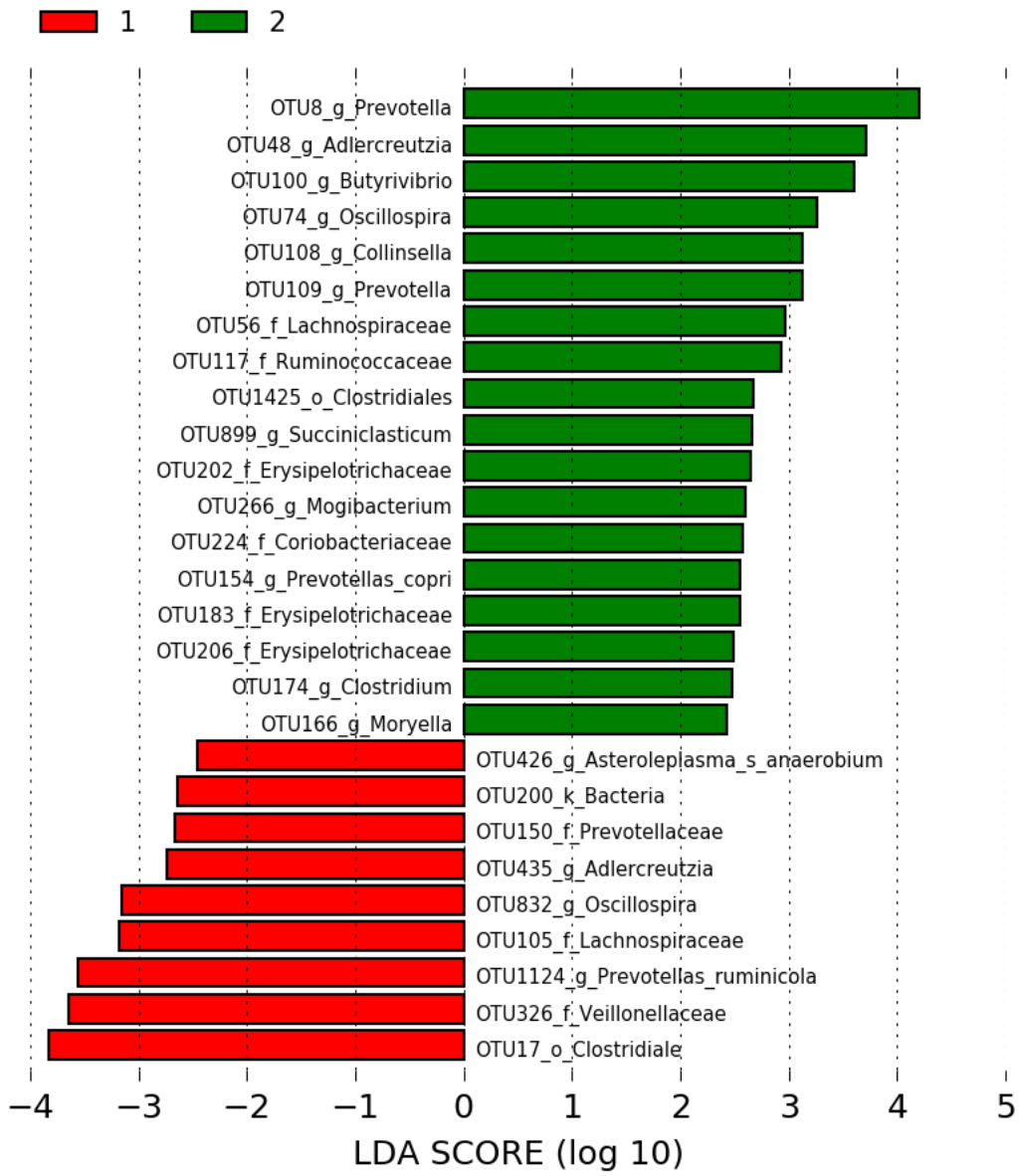

Figure 5. Differential analysis of microbiota community among group. The Linear discrimination analysis (LDA) coupled with effect size (LEfSe) was used to identify the most differentially abundant taxa among group one (1, red) and group (2, green). Only the results meeting an LDA significant threshold of >2 are shown

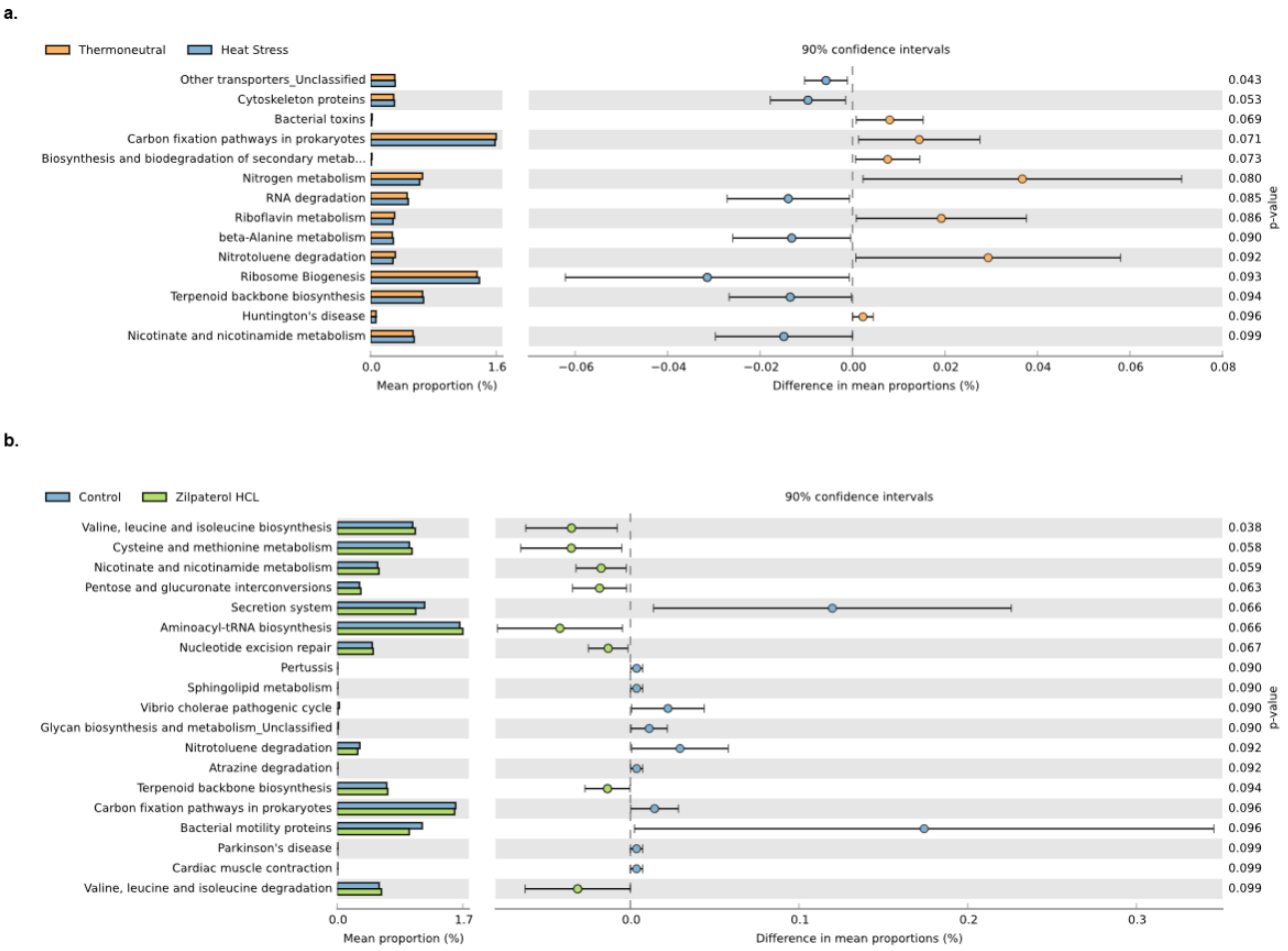

Figure 6. STAMP profiles showing differing features for a). environmental condition and b). dietary supplement (control vs. ZH)

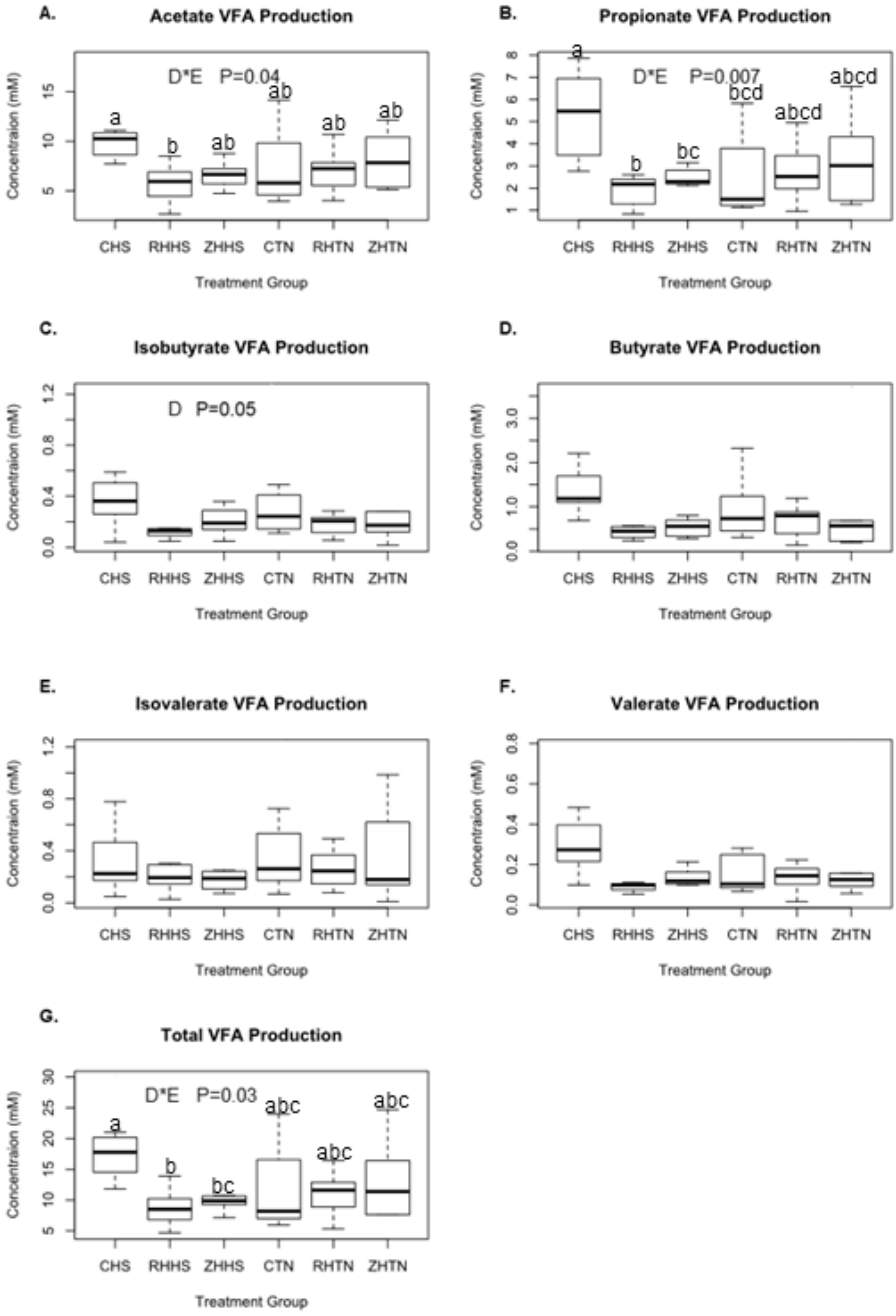

Figure 7. Production of (A.) acetate, (B.) propionate, (C.) isobutyrate, (D.) butyrate, (E.) isovalerate, (F.) valerate, and (G.) total VFA in lambs in one of six treatments in a 3 x 2 factorial of diet: no supplement (C), ractopamine HCl (RH), zilpaterol HCl (ZH), and environment: heat stress (HS), thermoneutral (TN). P-values are shown for the effects of diet (D), environment (E) or the interaction (D*E) when significant.

CHAPTER III: CHARACTERIZATION OF THE CECAL MICROBIAL COMMUNITY IN LAMBS SUPPLEMENTED WITH BETA AGONISTS AND SUBJECTED TO HEAT STRESS FOR 21 DAYS

Introduction

An animal's ability to utilize feedstuffs for energy to promote increases in growth and efficiency are critical aspects of the livestock industry. While it is known that the rumen of the ruminant gastrointestinal tract and the associated microbial community participate in nutrient uptake (Baldwin et al., 2004), metabolic activity (Baldwin and McCleod, 2000), and overall host health (Guarner, 2006), little attention has been given to the cecal microbiome and associated functions. Similar to ruminal fermentation, cecal digestion of feed is also performed by specialized microbiota. However, fermentable substrates arriving in the cecum are different from those fermented in the rumen, which may result in compositional or structural differences in the microbial communities of the two digestive compartments.

Although the cecum of the ruminant serves little function compared to hind-gut fermenters such as the horse, the large intestine is the second site of fermentation and where water and end-products from passing digesta are absorbed (Tucker et al., 1968). In ruminants, the cecum provides an extra energy source, up to 8.6% of metabolizable energy, in addition to energy that is produced from the rumen which is the main fermentative component (Siciliano-Jones and Murphy, 1989). Furthermore, surgical removal of the cecum in 7-month-old steers showed decreased digestibility of dry matter,

neutral-detergent fiber, acid-detergent fiber, and cellulose (Maala et al. 1983), suggesting the importance of the cecum in the digestion of feed that has passed through the rumen.

In dairy cattle, the cecum had the lowest number of observed phyla (12 phyla) compared to other compartments of the gastrointestinal tract (Popova et al., 2017). Similar to the rumen, Firmicutes were the dominant phyla with proteobacteria being the second predominant phyla. This differs from rumen bacterial communities where Bacteroidetes are the second most prevalent phyla. Additionally, diversity indices indicate that microbial diversity is lower in the cecum relative to other portions of the GI tract (Popova et al., 2017). Nutrient availability has been shown to have selective pressure on the biodiversity of microbiota (Mello et al., 2016), supporting studies observing lower microbial diversity in the cecum than in the rumen. Furthermore, microbial genes involved in carbohydrate metabolism in the microbiota were significantly higher in forestomach than cecum samples (Popova et al., 2017). Other factors such as host genetics, diet, physical environment or immune status may also have a stronger influence on shaping cecal microbial community than for the rumen (Popova et al. 2017).

An environmental stressor that may impact the cecal microbial community is heat stress. Little information is available on the effects of heat stress on the cecum microbial community in ruminants A previous study evaluating heat stress induced changes in the cecum of ducks reported that the order Rickettsiales, which contains species pathogenic to humans and animals (Darby et al., 2007), was significantly increased in heat stressed animals' cecum (He et al., 2019). However, it is important to note that ducks are nor

ruminant species, therefore their cecum has different function in digestion. Because the cecum of the ruminant is important for additionally digestion of feed that passed through the rumen it is critical to understand how heat stress may allow for pathogenic organisms to invade healthy animals' gastrointestinal tract.

Contrary to the negative effects of heat stress, the supplementation of beta-adrenergic agonists is common in livestock production systems to promote growth efficiency. While there are no known studies evaluating the impact of β-AA on the cecal microbiota of ruminants, previous studies using a porcine model observed that norepinephrine altered the adherence of pathogenic *E.coli* O157:H7 (EHEC) to porcine cecal-colonic mucosa (Chen et al., 2006). Norepinephrine, an endogenous catecholamine similar to the supplemented β-AA, promotes EHEC adherence, which appears to be mediated by alpha2-adrenergic receptors that are linked to decreased cyclic AMP and protein kinase A in colonic epithelial cells (Green et al. 2004; Chen et al. 2006). Given the impact of β-AA on growth efficiency (Scramlin et al., 2010; Romero-Maya et al., 2013), and with noted effects of the adrenergic system on gut activity (Ruckebush, 1983; McIntyre and Thompson 1992) and bacterial abundance (Lyte and Ernst 1992), the role of β-AA in post ruminal fermentation is of interest in livestock production.

Given that there has been anecdotal suggestions that heat stress in conjunction with β-AA supplementation may negatively exacerbate the adrenergic system (Grandin, 2013; Lonergan et al., 2014), it is hypothesized that the interaction between environment and supplementation with β-AA may have a negative impact on the cecal microbiome resulting in changes in post-ruminal fermentation. Therefore, the objective of this study

was to determine the impact that supplementation with β-AA (ractopamine HCl or zilpaterol HCl) and heat stress on the cecal bacterial community of lambs fed a high concentrate diet for 21 days.

Material and Methods

Animals and Experimental Design

The following experimental procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and approved by the Institutional Animal and Care and Use Committee at the University of Nebraska - Lincoln (IACUC protocol 1300). Animal studies were performed at the University of Nebraska-Lincoln Animal Science Complex (Lincoln, NE). Forty-nine Suffolk x Rambouillet crossbred lambs (53.26 ± 3.68 kg) were utilized in a 2x3 factorial design. Lambs were stratified by body weight and divided into two groups (group one = 39.99 ±1.92 kg; group two = 37.35 ± 1.92 kg) to accommodate for the capacity of the environmental chambers (12 stalls).

Environmental Conditions

Lambs were housed in one of two environmental conditions. The thermo neutral (TN) condition was approximately 21 ºC and 25% humidity during both study periods. Heat stress (HS) was achieved by maintaining a temperature of approximately 34 °C and 35% humidity from 0800 to 2000 h, and 29 °C between 2000 and 0800 with temperature changing over a period of 2 h at the beginning and end of each heat cycle. In both environmental conditions, the light was on from 0630 to 2045 h.

Supplementation and Feed

Lambs assigned to group one were transitioned to a 90% concentrate finishing diet (49% SweetBran®; 37.8% dry-rolled corn; 8.3% chopped alfalfa hay; 4% mineral supplementation; dosage of coccidiostat (20 g/ton)) over a period of 21 d. Lambs assigned to group two were held on a maintenance diet (54.8% SweetBran®; 41.1% chopped alfalfa hay; 4% mineral supplementation; dosage of coccidiostat (20 g/ton)) at 2% of BW for 41 d prior to 21 d transition to the 90% concentrate finishing diet previously stated with the addition of ammonium chloride (10 mg/hd/d).

On trial, lambs were fed at 2.5% body weight and administered one of three supplemental treatments: zilpaterol hydrochloride (ZH) supplemented at 5.44 g/ton (2.5 mg/kg), ractopamine hydrochloride (RH) supplemented at 18.14 g/ton (40 mg/kg), or no supplement (control; C). The supplement was mixed into 200 g of fine ground corn with the control receiving only the 200g of fine ground corn. β-AA supplementation began on study day 1 and was mixed into 907.19 g of feed offered at 0800h to ensure consumption of the supplement. The remainder of the diet was fed at 1200h.

Within group, lambs were stratified by body weight and randomly assigned to one of six treatment groups for a 2x3 (diet/environment) factorial design: control/thermoneutral (CTN), Ractopamine HCl/thermoneutral (RHTN), Zilpaterol HCl/thermoneutral (ZHTN), control/heat stress (CHS), Ractopamine HCl/heat stress (RHHS), and Zilpaterol HCl/heat stress (ZHHS). Orts, feed left over from the day before, were collected daily and recorded to evaluate average daily gain (ADG), feed to gain ratio (G:F), and feed intake. β-adrenergic agonists were supplemented into the diet daily

beginning on study d1 of each group, mixed into 907.19 g of feed. The remaining daily allotment of feed was given at approximately 1400 hours.

Necropsy and Sample Collection

Lambs were harvested on d 21 of the trial. The harvest order was randomly assigned and 12 were harvested each d via captive bolt followed by exsanguination. Cecum and rumen contents were collected, and flash frozen for microbial community analysis.

DNA extraction, 16S rRNA library preparation, and sequencing of the V4 Bacteria region

Total DNA was extracted from the cecum samples (~0.25-1 g) using the PowerMag Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol, with the exception of the following modification. During cell lysis, two bead-beating steps were performed in a TissueLyser (Qiagen Inc., Valencia, CA, USA) for 10 min at 30 Hz and samples were incubated in a 95 °C water bath for 5 min between the two bead-beading steps to ensure cell lysis.

The V4 region of the 16s rRNA gene was amplified from the extracted DNA using universal 16S primers (Kozich et al., 2013). The 25 µL PCR reaction consisted of 0.5 µL Terra PCR Direct Polymerase Mix (0.625 Units), 10 µL 2× Terra PCR Direct Buffer, 1 µL of primers (10 µM), 2 µL of extracted DNA (20 to 70 ng DNA), and 7.5 µL of molecular grade water. PCR conditions for amplification of the 16S rRNA gene included: an initial denaturation of 98 °C for 3 min, followed by 25 cycles of 98 °C for 30 s, 55 °C for 30 s, and 68 °C for 45 s, and a final extension of 68 °C for 4 min. Following

amplification, 5 µL of amplicon product was run of a 1.2% agarose gel at 120 V for 55 minutes for size and amplification verification.

Following amplification, PCR products from each sample were normalized (1 to 2 ng/µL) using the SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer, then pooled (10 µL/sample) using the Eppendorf epMotion (M5073, Germany). The pooled libraries were purified using the MinElute PCR Purification Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. The resulting concentrated samples were size selected using the Pippin Prep (Sage Science, Inc., Beverly, MA, USA) automated size selection instrument with 1.5% agarose gel cassettes. The resulting libraries were quantified using a DeNovix Spectrophotometer/Fluorometer (Wilmington, DE, USA) and PCR product size and quantity was verified using the Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The libraries were sequenced on an Illumina Miseq (Illumina, San Diego, CA, USA) using the V2 500 cycle kit according to the manufacturer's protocol.

Microbial Community Analysis

Contigs were assembled and quality filtered to remove sequences with ambiguous bases, incorrect length (min=245, max=275), or those improperly assembled (MOTHUR v.1.38.1; Schloss et al., 2009). Utilizing the UPARSE pipeline (USEARCH v7.0.1090; Edgar, 2013), sequences were then clustered into operational taxonomic units (OTUs). Clustering steps included dereplicate, sorting by cluster size (descending and not retaining singletons), mapping sequences to OTUs at 97% identity, and filtering chimeric

sequences using UCHIME (Edgar et al., 2011) with ChimeraSlayer gold.fa as the reference database.

Taxonomy assignments were performed as described in MOTHUR (Schloss et al., 2009) using a Naive Bayes classifier similar to the RDP Classifier (Wang et al., 2007), with the Greengenes database (gg_13_8_otus; McDonald et al., 2012) reference sequences. Taxonomy was then assigned to the OTU table previously generated and the resulting table was converted to a BIOM (McDonald et al., 2012) file for further analysis. The Silva database (Quast et al., 2013) was used to generate a customized reference database to check for sequences outside the V4 region. Outliers were removed, and sequences aligned to the customized reference alignment. Overall OTUs were removed if they had poor alignment, singletons, or classified within the Archaea kingdom of Cyanobacteria phylum. The resulting alignment was used to construct a phylogenetic tree using Clearcut (Sheneman et al., 2006).

Alpha metrics were used to describe bacterial richness (observed OTUs), diversity (Shannon–Weiner index [logarithm base 2]; Shannon and Weaver, 1949), and dominance (1-Simpson index). Asa measure of β-diversity, PCoA plots were generated by utilizing unweighted unifrac matrices (Lozupone et al., 2011). To reduce the effect of animal-to-animal variation in cecum bacterial community composition, a core measurable microbiome (CMM) was defined as OTUs that were present in all animals split by environment (HS, TN), supplementation (ZH, RH, C), and group (G1, G2). After filtering out OTUs that were not found in all animals in each group, the filtered OTU tables were then merged back together. Once the CMM was defined, OTUs were filtered from the

original OTU table that were not to be included. Once unwanted OTUs were filtered from the original OTU table, the CMM OTU table was formed.

Statistical Analysis

The OTU table was rarefied to lowest sequencing depth of 8,148 reads using QIIME v.1.9.1 (Caporaso et al., 2010) implementing the Mersenne Twister pseudo-random number generator. Alpha and beta diversity indices and statistical comparisons of the total microbial community and of the core microbial community were also conducted with QIIME v.1.9.1. Alpha diversity metrics were compared using a nonparametric two-sample t-test with multiple comparisons that were corrected for false discovery rate (Benjamini and Hochberg, 1995). Good's coverage (Good, 1953) was calculated to evaluate adequate sampling depth after rarefaction. Differences between total and core microbial composition were evaluated in R (R Core Team, 2018) with the adonis function of the vegan package (Oksanen et al., 2017). The weighted UniFrac distance matrix was used as an input for PERMANOVA using environment, supplement, group as the main effects and testing for an interaction between environment and supplement. Significance was declared for alpha and beta diversity metrics at $P \leq 0.10$.

Pairwise comparisons of total and core microbial communities across main effects were tested with the linear discriminatory analysis (LDA) effect size (LefSe; Segata et al., 2011) to identify differentially abundant OTUs/bacterial features. LefSe was implemented using default parameters with a significance threshold of $P < 0.05$ for the factorial Kruskal-Wallis test among classes and a threshold of 2.0 or greater for the LDA score for discriminative features.

Multivariate Association with Linear Models (MaAsLin), a statistical tool developed by the The Huttenhower Lab (Department of Biostatistics, Harvard School of Public Health, Boston, MA), was used to evaluate associations between metadata and the microbial community abundancy, utilizing OUT distribution in samples. The metadata used in the analysis included the environment, supplement, group, ADG, and average daily feed intake (ADFI). For any association with a q-value ≤ 0.05 (the minimum false discovery rate), the MaAsLin tool generated knotched box plots for factor data found in the metadata file. At the top of the plots, the coefficient effect size (r) is listed, followed by the standard deviation, P-value, and q-value. In addition to the knotched box plots, a complementary partial residual plot was generated. In the partial residual plot, the residuals are plotted against the independent variable, which is useful in order to detect outliers or assess the presence or absence of inhomogeneity of variance (Larsen and Mccleary, 1972).

DNA sequences associated with OTUs associated with the metadata from the MaAsLin results were then submitted to the NCBI Standard Nucleotide BLAST tool to identify the specific bacterial microorganisms represented. The resulting list of potential matches to microorganisms in the database were output, along with query cover percentage, E-value, and maximum identity percentage. The query cover percentage represents the proportion of the input sequence that overlaps the potentially matching microorganism's sequence. The E-value represents the number of expected hits that occur by chance when searching the database; the smaller the E-value, the more significant the

match. The identity percentage is that between the query and the hit in a nucleotide-to-nucleotide alignment (Agostino, 2013a).

Results

Total Microbial Composition

1.3 million quality filtered sequences were generated from 47 samples. Before rarefaction 2,109 OTUs were identified; after rarefaction to 8,148 sequences per sample, 1,866 OTUs remained representing 88% of the original OTU table. Good's coverage demonstrated that the sampling depth obtained represented on average 98.6% of the total bacterial community.

The Shannon (diversity) and observed OTUs (species richness) metrics identified no difference ($P > 0.10$) between all categories evaluated (supplement, environment, group, and the supplement-environment interaction). There was a significant difference ($P < 0.10$) for environment and supplement as determined by the Simpson (dominance) metric. Beta diversity, calculated using calculated using the weighted unifrac distance matrix as input for a permutational multivariate analysis of variance (PERMANOVA), differed ($P = 0.005$) based upon groip. However, environment ($P = 0.25$), supplement ($P = 0.33$), and the environment-supplement interaction ($P = 0.34$) , did not have an effect on the overall cecum microbial community. To better establish a visual model of the community structure and its influence by group, Principle Coordinate Analyses was performed using rarefied OTU tables to account for unequal read depth (Figure 1).

Core Microbial Composition

The core OTU table had 302 OTUs in total. PERMANOVA analysis of only the core OTUs showed no effect of environment ($P = 0.20$), supplement ($P = 0.33$), or environment x supplement ($P = 0.31$), on the core cecal microbial composition. However, group had a significant ($P = 0.002$) effect on the core microbial composition.

The CMM was primarily composed of Firmicutes (70%), Bacteroidetes (9%), and Proteobacteria (4%), with 5% of the phyla classified as unknown. Proteobacteria were more abundant in group one (6.48%) compared to group two (2.13%). The major family members in the CMM included Ruminococcaceae (19.10%), and Veillonellaceae (10.18%), Lachnospiraceae (8.86%).

LEfSe analyses of differentially abundant OTUs found 27 differed in abundance ($P < 0.05$, LDA scores ≥ 2) between HS and TN lambs (Figure 2). Fourteen OTUs in RH (Figure 3) and 17 OTUs in ZH-supplemented (Figure 4) lambs were differentially abundant ($P < 0.05$, LDA scores ≥ 2) when compared to control lambs and 80 OTUs were differentially abundant ($P < 0.05$, LDA scores ≥ 2) between group one and two (Figure 5a, Table5), equating to 24.10% of the core microbiome. When evaluating differentially abundant families of the core microbial community, Verrucomicrobiaceae and Veillonellaceae were more abundant ($P < 0.05$, LDA scores ≥ 2) in HS animals, while Ruminococcaceae were more abundant ($P < 0.05$, LDA scores ≥ 2) in TN animals. Spirochaetaceae were more abundant ($P < 0.05$, LDA scores ≥ 2) in ZH-supplemented lambs compared to the controls, while family taxa found in RH-supplemented lambs were not different from the controls. When evaluating the effect of group on family abundance it was observed that Desulfovibrionaceae,

Peptostreptococcaceae, Pasteurellaceae, and WCB1_25 were more abundant ($P < 0.05$, LDA scores ≥ 2) in group one.

Results generated by the MaAsLin analysis identified significant ($P \leq 0.05$) associations between 2 OTUs and group. OTU 296 (Figure 6a), belonging to the genus SMB53, was associated with group one (Figure 3a), while OTU 55 (Figure 6b), belonging to the family Ruminococcaceae, was associated with group two. The NCBI BLAST function was able to further classify OTU 296 as *Papillibacter cinnamivorans*(Identity,% = 93.70%), and OTU 55 as *Holdemania massiliensis* (Identity, % = 86.96%).

Discussion

In this study, we found that β-AA supplementation and heat stress each affect specific bacterial OTUs and taxa, which may cause changes in post-ruminal fermentation. Notably there was no interaction between environment and supplementation on the microbial community, suggesting that β-AA in conjuncture with heat stress does not change the cecal microbial composition. Moreover, group had a significant impact on the overall bacterial community indicating that feeding of ammonium chloride to group two of the study impacted the cecal microbiota and potentially digestive function.

Heat Stress

In the core microbial community of the cecum, family Ruminococcaceae were more abundant in thermoneutral lambs than in heat stressed lambs. This is similar to studies performed in broilers where Ruminococcaceae were in higher abundance in the cecum of thermoneutral compared to heat-stress animals (Sohail et al., 2015).

Ruminococcaceae produce acetate and butyrate and are involved in the first step of microbiome-assisted carbohydrate metabolism by degrading polysaccharides (Flint et al., 2008), indicating their importance in energy provided to the animal. An increased abundance of Ruminococcaceae in our thermoneutral lambs compared to the lambs under heat stressed conditions indicates a possible impact on cecum carbohydrate metabolism.

Bacteria of the family Verrucomicrobiaceae were more abundant in heat stressed compared to thermoneutral lambs. In a previous study, Verrucomicrobiaceae populations were increased in corals undergoing thermal stress, similar to our lambs. The authors suggested that increased abundance of Verrucomicrobiaceae was due to invading microbes of the family taking advantage of the stressed corals (Lee et al., 2015). Additionally, after dehorning and castration of dairy calves, Verrucomicrobiaceae were in higher abundance in the light-weight, compared to heavier calves (Mir et al., 2019). This further indicates that Verrucomicrobiaceae may take advantage of stressed environments, allowing for their growth and survival.

β-AA Supplementation

Natural catecholamines such as norepinephrine and epinephrine promote bacterial growth in vitro (Lyte and Ernst, 1992), however, there is no known information available regarding the impact of ZH and RH on cecal bacterial communities. Similarly to Walker and Drouillard (2010), and our previous study evaluating β-AA supplementation on the rumen bacterial population (Chapter II), ZH and RH supplementation had little impact on the CMM. Moreover, when evaluating differentially abundant families, only one was differentially abundant in supplemented compared to control lambs. Spirochaetaceae

families, more abundant in ZH-supplemented lambs, have been associated with a decrease in G:F in beef cattle when isolated from ruminal contents. (Paz et al., 2018). However, current literature is not available that would explain the increase of Spirochaetaceae we saw in our lambs supplemented with ZH, which increases G:F (Vasconcelos et al., 2008; Montgomery et al., 2009; Hales et al., 2014).

When evaluating differentially abundant OTUs, *Harryflintia acetispora* (OTU 243) and *Prevotella ruminciola* (OTU 299) were in higher abundance in control lambs compared to both ZH- and RH-supplemented lambs. This suggests that β-AA may impact the ability for these species to survive causing their populations to decrease. *H. acetispora* is a novel species that has been isolated from the cecum of chickens (Petzoldt et al 2016). *H. acetispora* are non-motile, gram-negative, anaerobes that can form endospores (Petzoldt et al 2016). Given that *H. acetispora* is a novel species there is no available literature that would provide an explanation as to why it was more abundant in control lambs compared to those supplemented with β-AA. Additionally, *Prevotella ruminicola* were more abundant in non-supplemented lambs. The genus Prevotella is one of the most predominant genera in the rumen, with major roles in carbohydrate and nitrogen metabolism (Bladen et al. 1961; Gasparic et al., 1995). Specifically, *P. ruminicola* are saccharolytic species of gram-negative bacteria that ferment a wide range of carbohydrates. (Bryant *et al*.,1958). The increase of *P. ruminicola* in the control lambs compared to lambs supplemented with β-AA suggests that the use of β-AA impacts post-ruminal carbohydrate metabolism.

Group

The addition of ammonium chloride to the diet in group two caused changes to the cecal microbial community. Beta diversity indices of both the total and core microbial community showed group one and group two were significantly different. The single difference in treatment between group was the addition of ammonium chloride to the diet, conducted due to the formation of bladder stones in lambs of group one. The addition of ammonium chloride reduces urinary pH and thus prevents the formation of urinary calculi. Ammonium chloride has been suggested to be considered as a digestive tract irritant (Scientific Opinion on ammonium chloride for lambs for fattening, 2012), therefore the changes in the microbial community due to the addition of ammonium chloride are not surprising. However, the addition of ammonium chloride was not expected to have a greater impact on the cecal microbiome than that of the main study variables (environment and supplement).

Proteobacteria were higher abundance in group one than in group two, after addition of ammonium chloride. Proteobacteria is a major phylum of gram-negative bacteria consisting of a variety of pathogens such as *Escherichia* and *Salmonella*, as well as, bacteria responsible for ammonia and nitrogen oxidizing (Head et al. 1993; Teske et al., 1994). In humans, Proteobacteria are low abundance in healthy individuals but increased Proteobacteria can result in gut dysbiosis and disease (Shin et al., 2015). Therefore, the increase in Proteobacteria in group one is interesting due to the addition of ammonium chloride in group two. Additionally, the family Pasteurellaceae and species *Pasteurella aerogenes*, found within the phyla Proteobacteria, were more abundant in group one. *Pasteurella aerogenes* has been observed in individuals with ulcers and

wounds due to pig bites or handling (Ejlertsen et al.,1996). Additionally, *P. aerogenes* has the ability to hydrolyze urea, produce oxidase and catalase, decarboxylate ornithine and, to produce gas from glucose (Ejlertsen et al.,1996). Lambs from group one were not supplemented ammonium chloride, therefore it is possible that these lambs underwent damage to their digestive tract allowing for the growth of harmful *P. aerogenes*. Additionally, the family Peptostreptococcaceae was observed to be more abundant in group one. Peptostreptococcaceae were over-represented in the guts of colorectal cancer patients (Ahn et al., 2013). This finding further suggests that lambs in group one were experiencing dysbiosis in their cecum.

Papillibacter cinnamivorans, identified by the NCBI BLAST tool, was associated with animals in group one. *Papillibacter cinnamivorans* is a strictly anaerobic, Gram-positive, non-spore forming, mesophilic bacterium (Defnoun et al. 2000); the reason for its association with group one lambs remains unknown. OTU 55, which was associated with group two, was identified by NCBI BLAST as *Holdemania massiliensis*. However, due to identification parameters being low in confidence, the Greengenes classification given by QIIME1 was used for further evaluation. Greengenes classified OTU 55 belonging to the family Ruminococcaceae. As previously mentioned, Ruminococcaceae are involved in microbe-assisted carbohydrate metabolism (Flint et al., 2008), indicating their importance in energy production. Therefore, Ruminococcaceae being associated with group two may indicate a change in cecum carbohydrate metabolism associated with the administration of ammonium chloride.

Conclusion

Overall, group had a strong effect on the overall microbial community. This can be attributed to the addition of ammonium chloride to the diet of animals in group two. Interestingly supplementation with β-AA and heat stress conditions did not have a strong effect on the overall microbial community, but changes in particular OTUs did occur. The finding of different taxa in group one that have been associated with epithelial damage and gut dysbiosis indicates that animals in group one was affected by an agent that was negatively impacting the cecal microbiota. Treatment with ammonium chloride in group two, in addition to mediating the development of bladder stones, appeared to prevent these harmful taxa from invading the cecum. The observation of a significant difference between group one and two is interesting as similar analysis of the rumen microbiome from the same lambs did not have similar results. This suggests that the cecum microbial community may be more susceptible to infections compared to the rumen microbial community.

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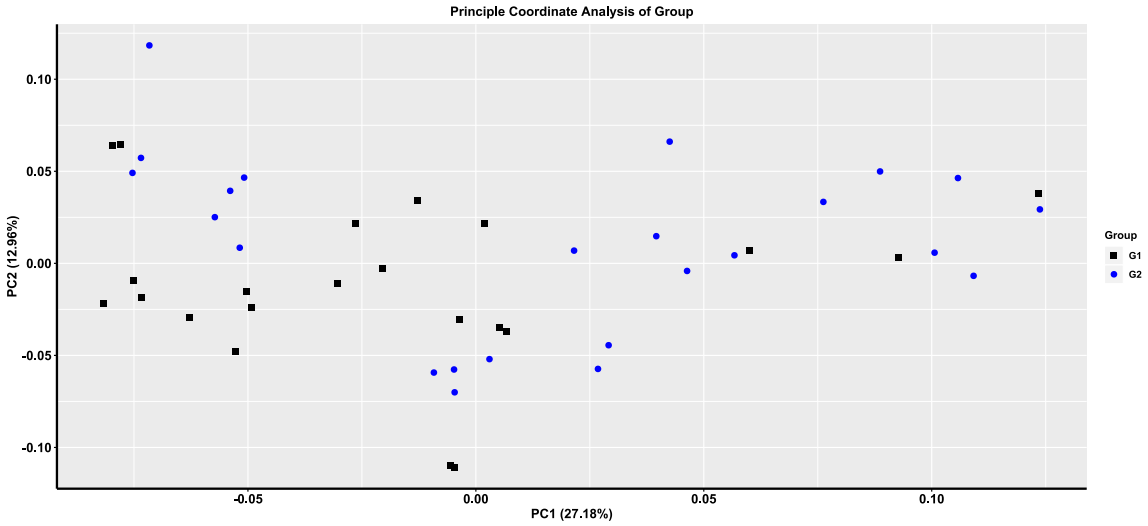


Figure 1. Principle Coordinate Analysis of the cecal bacterial community structuring between group one and group two. Each dot (black square or blue circle) represents a community from animal. The two axes provide a 2D visualization with variance between the axes measured. This figure shows slight clustering of group two towards the left portion of the figure.

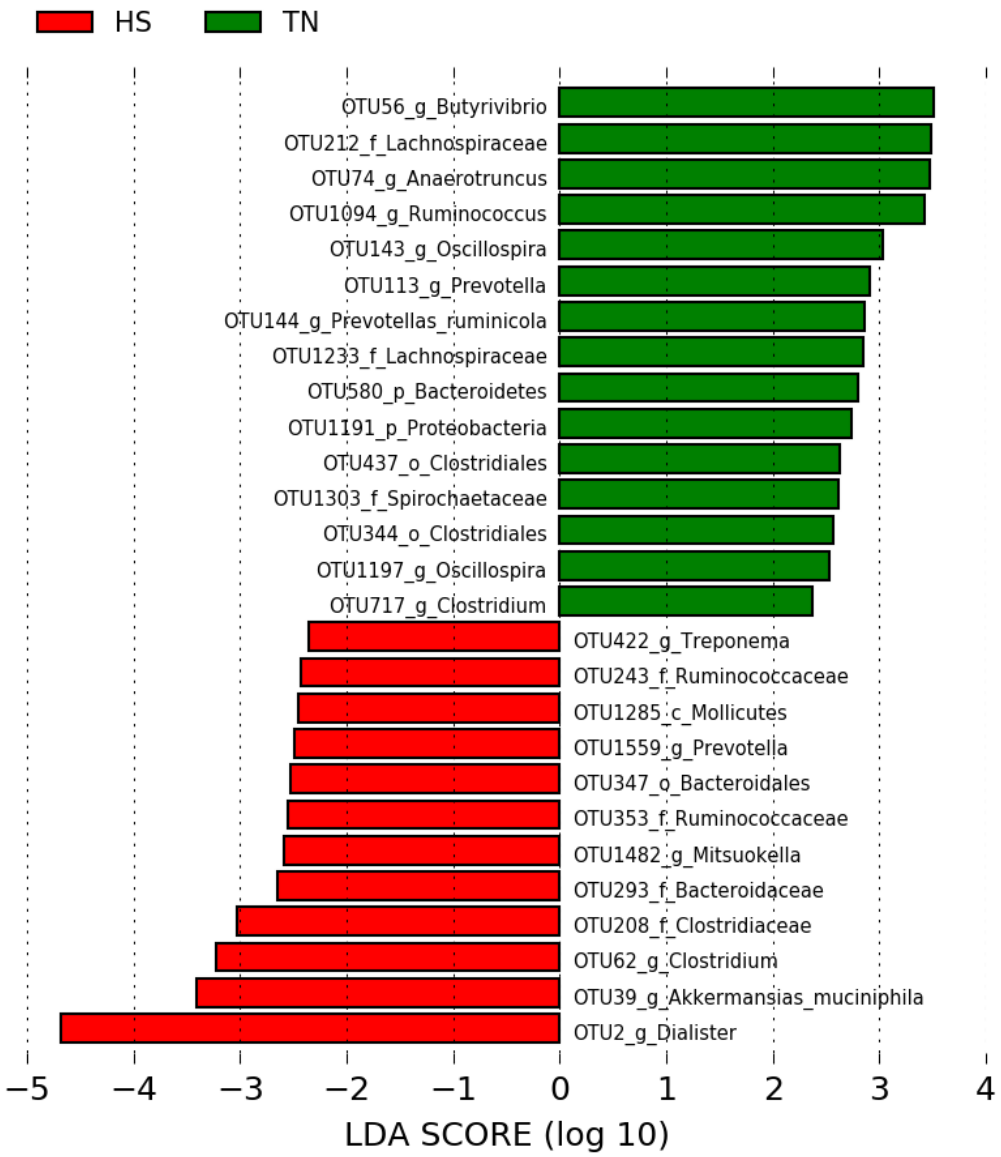


Figure 2. Differential analysis of microbiota community among environmental temperatures. The Linear discrimination analysis (LDA) coupled with effect size (LEfSe) was used to identify the most differentially abundant taxa among thermoneutral (TN) and heat stress (HS) environments. Only the results meeting an LDA significant threshold of >2 are shown.

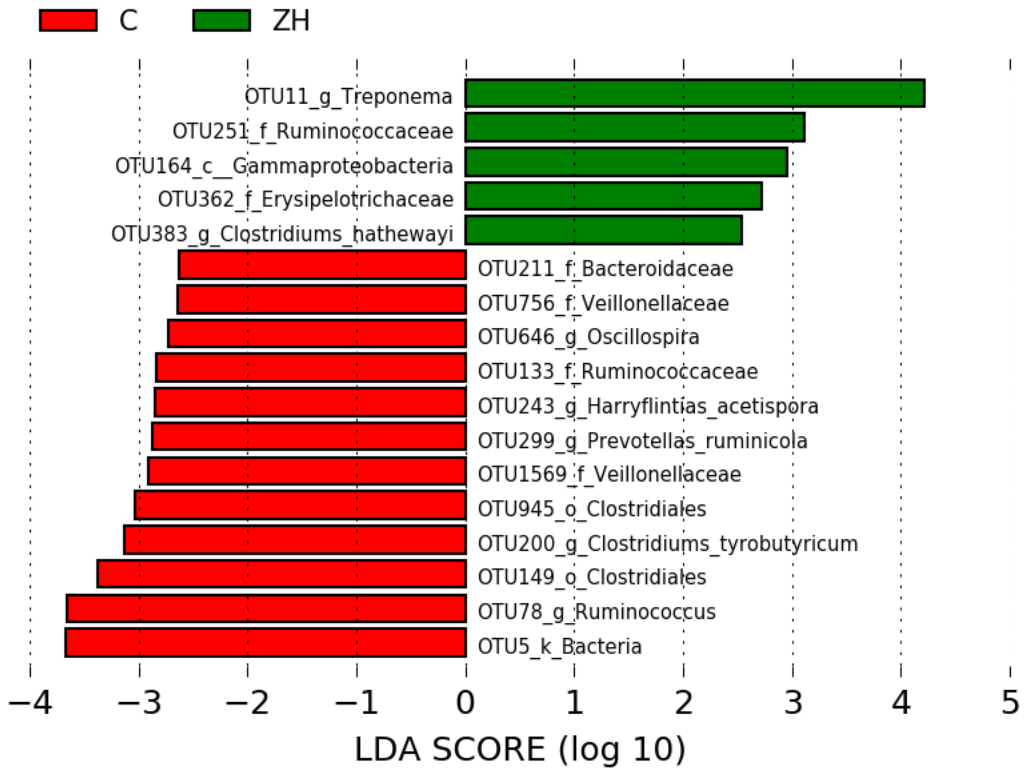


Figure 3. Differential analysis of microbiota community among dietary supplements. The Linear discrimination analysis (LDA) coupled with effect size (LEfSe) was used to identify the most differentially abundant taxa among non-supplemented (C) and ZH-supplements (ZH). Only the results meeting an LDA significant threshold of >2 are shown.

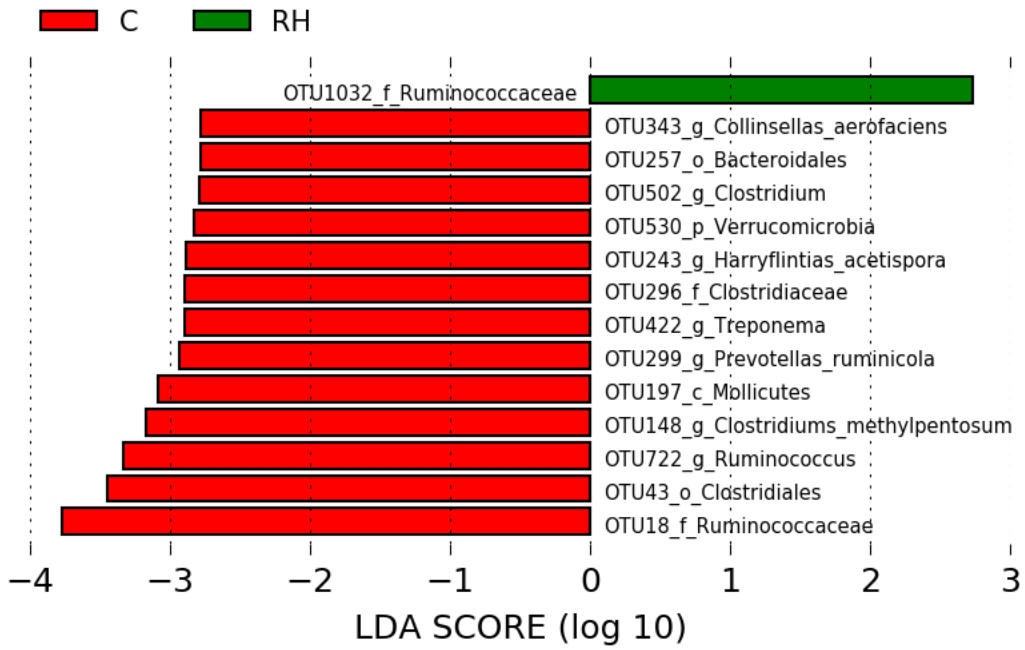


Figure 4. Differential analysis of microbiota community among dietary supplements. The Linear discrimination analysis (LDA) coupled with effect size (LEfSe) was used to identify the most differentially abundant taxa among non-supplemented (C) and RH-supplements (RH). Only the results meeting an LDA significant threshold of >2 are shown.

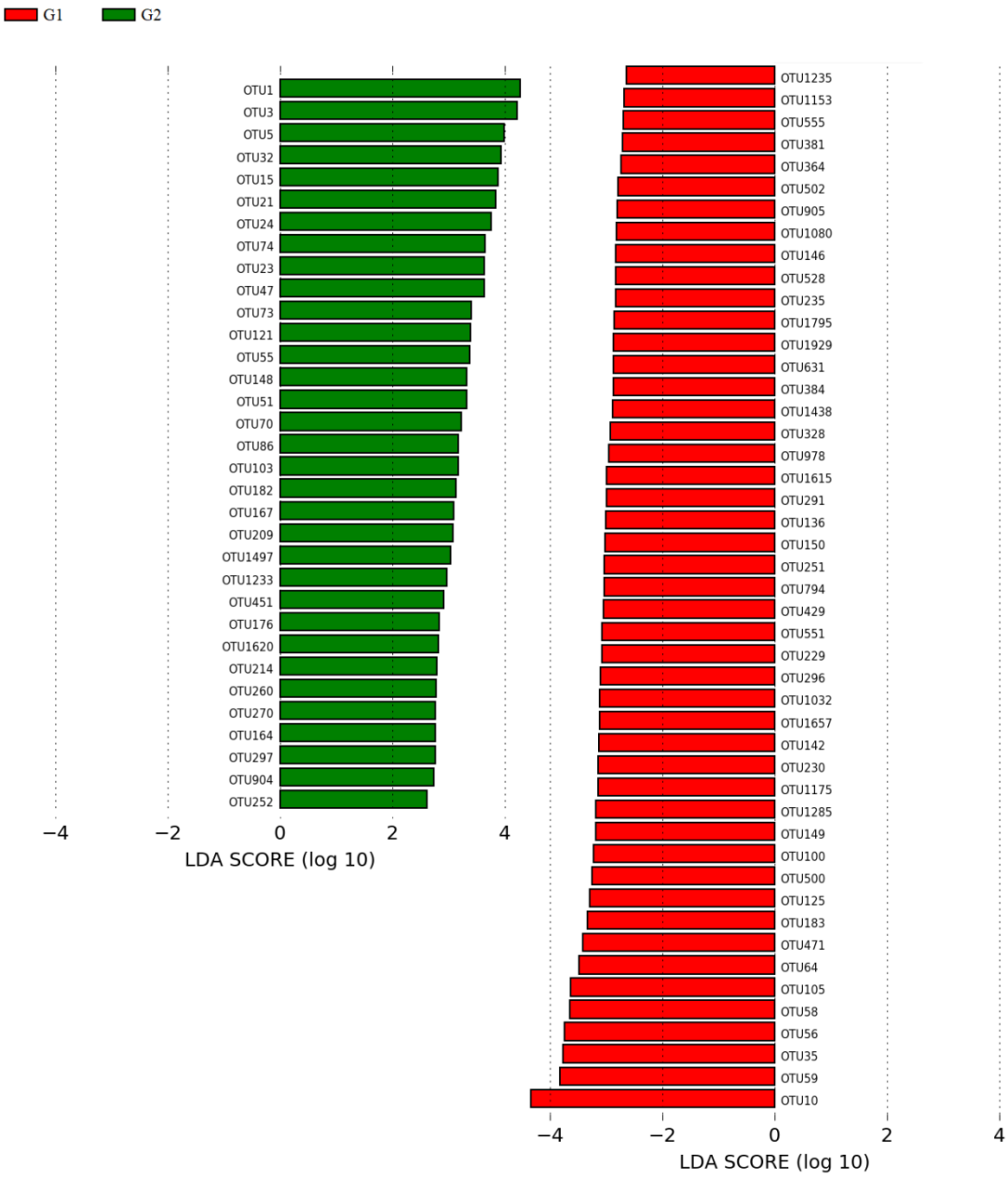
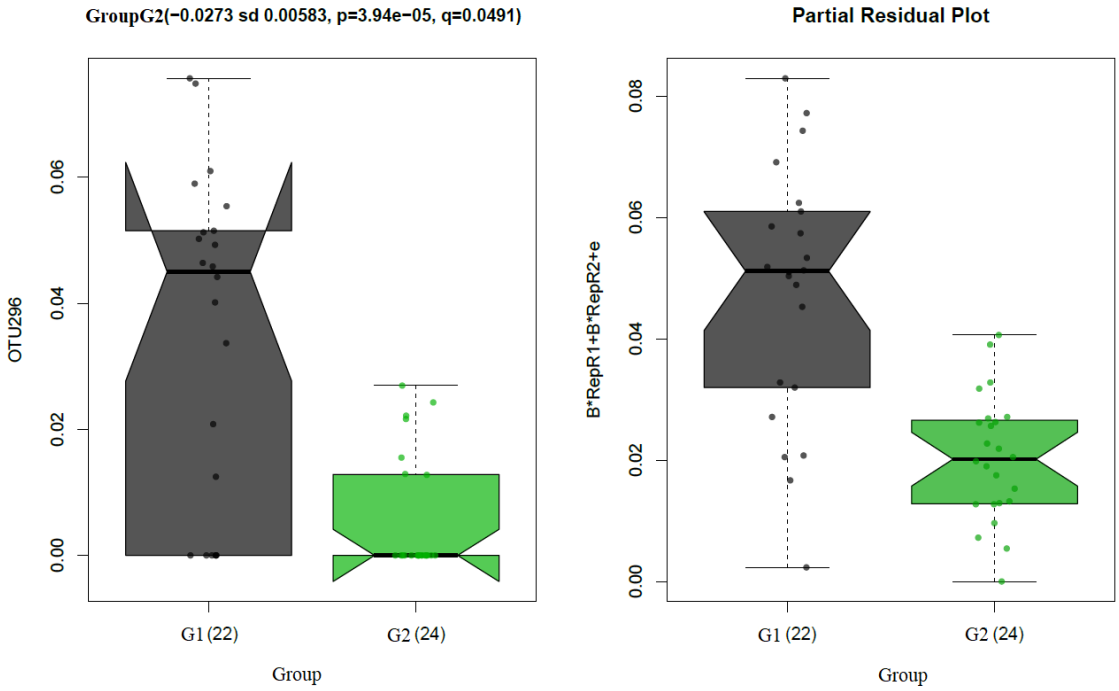
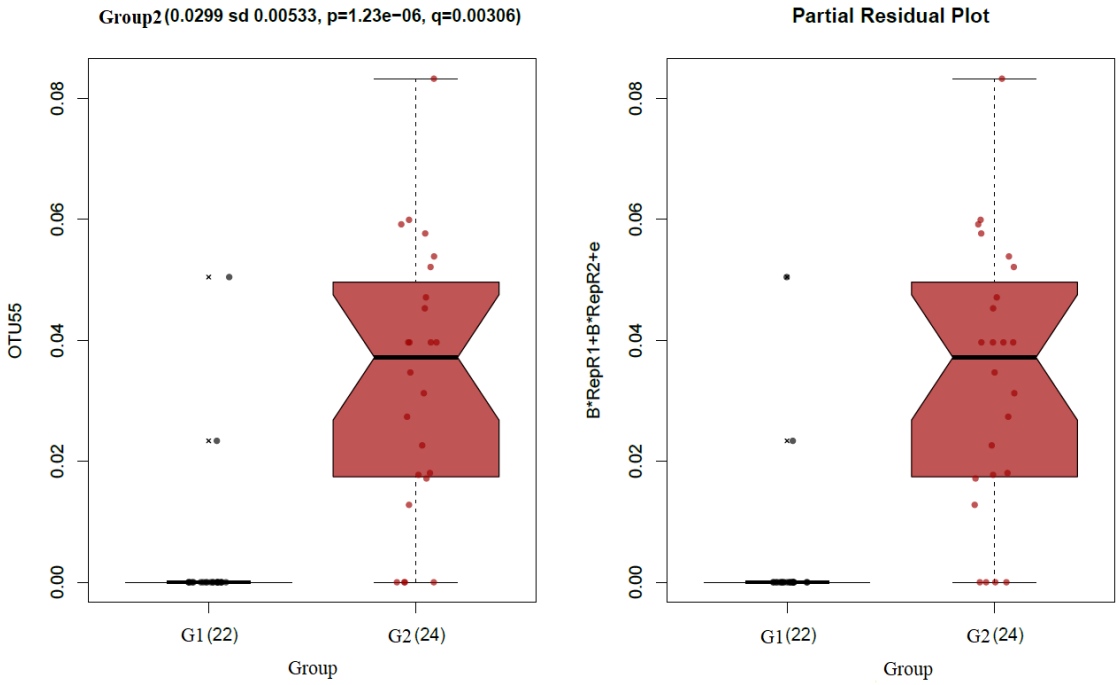


Figure 5. Differential analysis of microbiota community among groups. The Linear discrimination analysis (LDA) coupled with effect size (LEfSe) was used to identify the most differentially abundant taxa among group one (1, red) and group two (2, green). Only the results meeting an LDA significant threshold of >2 are shown.



OTU 296	Strain	Query cover %	E value	Identity, %
Papillibacter cinnamivorans	CIN1	100%	1e-105	93.70%
Clostridium Viride	T2-7	100%	2e-102	92.89%
Intestinimonas butyriciproducens	SRB-521-5-I	100%	4e-95	91.34%

Figure 6a. The association of the relative abundance of OTU 296 and group one (G1) or group two (G2), along with the complementary partial residual plot. The r (sd) coefficient effect size, and P and q value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.



OTU 55	Strain	Query cover %	E value	Identity, %
Holdemania massiliensis	AP2	100%	3e-76	86.96%
Holdemania filiformis	J1-31B-1	100%	3e-76	86.96%
Anaerorhabdus furcosa	T-301-A2	100%	1e-69	85.38%

Figure 6b. The association of the relative abundance of OTU 55 and group one (G1) or group two (G2), along with the complementary partial residual plot. The r (sd) coefficient effect size, and P and q value tests for significance, are shown above the figure. Below the figure, the top three microorganisms matching the OTU as identified by NCBI Standard Nucleotide BLAST (Bethesda, MD) are listed.

Table 1. Taxonomy classification of OTUs found to be differential abundant (Figure 5)

OTU	Taxonomy	OTU	Taxonomy
OTU1	*Asteroleplasma anaerobium*	OTU146	genus Adlercreutzia
OTU3	order Clostridiales	OTU528	genus Treponem
OTU5	kingdom Bacteria	OTU235	*Butyricicoccus pullicaecorum*
OTU32	family Mogibacteriaceae	OTU1795	order Bacteroidales
OTU15	class Mollicutes	OTU1929	family Ruminococcaceae
OTU21	kingdom Bacteria	OTU631	genus Oscillospira
OTU24	phylum Verrucomicrobia	OTU384	order Clostridiales
OTU74	genus Anaerotruncus	OTU1438	family Lachnospiraceae
OTU23	family Clostridiaceae	OTU328	genus Succiniclasticum
OTU47	family Coriobacteriaceae	OTU978	genus Prevotella
OTU73	family Mogibacteriaceae	OTU1615	order Bacteroidales
OTU121	genus Prevotella	OTU291	phylum Tenericutes
OTU55	family Ruminococcaceae	OTU136	family Ruminococcaceae
OTU148	*Clostridium methylpentosum*	OTU150	class Clostridia
OTU51	family Christensenellaceae	OTU251	family Ruminococcaceae
OTU70	genus Prevotella	OTU794	*Prevotella copri*
OTU86	family Victivallaceae	OTU429	genus Roseburia
OTU103	genus Ruminococcus	OTU551	genus Prevotella
OTU182	family Paraprevotellaceae	OTU229	family Lachnospiraceae
OTU167	order Bacteroidales	OTU296	family Clostridiaceae
OTU209	genus Prevotella	OTU1032	family Ruminococcaceae
OTU1497	order Clostridiales	OTU1657	family Lachnospiraceae
OTU1233	family Lachnospiraceae	OTU142	phylum Verrucomicrobia
OTU451	order Clostridiales	OTU230	family Ruminococcaceae
OTU176	order Clostridiales	OTU1175	family Mogibacteriaceae
OTU1620	genus Treponema	OTU1285	class Mollicutes
OTU214	order Bacteroidales	OTU149	order Clostridiales
OTU260	order Bacteroidales	OTU100	genus Clostridium
OTU270	order Bacteroidales	OTU500	family Desulfovibrionaceae
OTU164	class Gammaproteobacteria	OTU125	family Mogibacteriaceae
OTU297	family Ruminococcaceae	OTU183	order Burkholderiales
OTU904	family Rikenellaceae	OTU471	family Ruminococcaceae
OTU252	family Ruminococcaceae	OTU64	family Lachnospiraceae
OTU1235	family Coriobacteriaceae	OTU105	family Lachnospiraceae
OTU1153	family Ruminococcaceae	OTU58	family Peptostreptococcaceae
OTU555	family Ruminococcaceae	OTU56	genus Butyrivibrio
OTU381	family Ruminococcaceae	OTU35	genus Oscillospira
OTU364	family Ruminococcaceae	OTU59	order Clostridiales
OTU502	genus Clostridium	OTU10	*Pasteurella aerogenes*
OTU905	family Lachnospiraceae		

CHAPTER IV: INVESTIGATION OF THE RUMEN EPITHELIUM TRANSCRIPTOME IN LAMBS FED BETA-ADRENERGIC AGONISTS AND SUBJECTED TO HEAT STRESS

Introduction

The primary functions of the gastrointestinal epithelium are to provide the host protection from microorganisms, toxins and chemicals (Gäbel et al., 2002), and to aid in nutrient absorption, metabolism, and delivery of nutrients to other body tissues (Furness et al., 2013). The gastrointestinal tract plays a significant role in energetics by utilizing 20% of the oxygen in ruminants and accounting for 30% of metabolic and protein synthesis activities (Cant et al., 1996). Improving animal growth and efficiency are critical points of research as the world's population and demand for agriculture products increase. Therefore, adaptions or changes in the gut can are of interest to maximize growth efficiency and wellbeing of livestock.

Supplementation with dietary β-adrenergic agonists (β-AA) is one strategy used in the livestock industry to increase efficiency of growth in livestock; these supplements do so by decreasing adipose deposition while increasing protein accretion (Johnson et al., 2014). However, there is no current literature available that addresses the impact supplementation with β-AA has on the rumen epithelium. Although β-AA provide a positive impact on animal agriculture, it has been suggested that feeding supplement while animals are subjected to stressors such as heat stress can increase mortality (Grandin, 2008). Conversely, however, Dávila-Ramírez et al. (2014) reported feeding ZH to rams under heat stress had no negative impact on animal health. The differing reports

regarding heat stress and β-AA indicates a gap in the present knowledge of how these variables impact animal well-being.

Heat stress occurs when an animal's body temperature rises above its thermoneutral zone, at which point the heat load exceeds the animal's capacity for heat dissipation (Bernabucci et al., 2010) resulting in decreased feed intake and poor performance (Marai et al., 2007; Guo et al., 2018; Johnson, 2018). Additionally, feed intake ADG, and final body weight decrease due to heat stress (Beatty et al., 2006; Mitlöhner et al., 2002; Blaine and Nsahlai, 2011). With respect to the digestive system, animals also respond to heat by redistributing blood flow from gastrointestinal organs to the periphery (Sakurada and Hales, 1998). The reduction in blood supply to the gut can damage its epithelial lining impacting nutrient absorption and permitting endotoxin to enter the body (Cronjé, 2005). The decrease in animal performance and wellbeing attributed to heat stress affects economically important carcass and performance traits leading to millions of dollars in losses every year (Renaudeau et al., 2012).

Heat stress and β-AA supplementation have antagonistic effects on animal performance, however, there is a gap in knowledge in the understanding of how the two impact rumen function. Specifically, how these variables individually, and in concert, impact animal performance and wellbeing in regard to the rumen has yet to be elucidated. Therefore, the purpose of this study was to investigate the effects of β-AA supplementation, heat stress, and their interaction on the rumen epithelium using transcriptomic analyses

Materials and Methods

The following experimental procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and approved by the Institutional Animal and Care and Use Committee at the University of Nebraska - Lincoln (IACUC protocol #1300). Animal studies were performed at the University of Nebraska-Lincoln Animal Science Complex.

Experiment One

Forty-nine Suffolk x Rambouillet crossbred lambs (53.26 ± 3.68 kg) were utilized in a 2X3 factorial design. Upon arrival, lambs were weighed, rectal temperatures recorded, ear tagged with individual ID's, metaphylacticaly treated [Ivomec®; 10 mL/hd (Merial, Duluth, GA) and Draxxin®; 1 mL/hd (Zoetis, Parsippany, NJ)]. Lambs were stratified by body weight and divided into two groups (group one = 39.99 ±1.92 kg; group two = 37.35 ± 1.92 kg) to accommodate for the availability of thermal chambers (12 stalls).

Lambs were housed in one of two environmental conditions. The thermoneutral (TN) condition was approximately 21 °C and 25% humidity during both study periods. Heat stress (HS) was achieved by maintaining a temperature of approximately 34 °C and 35% humidity from 0800 to 2000 h, and 29 °C between 2000 and 0800 with temperature changing over a period of 2 h at the beginning and end of each heat cycle. In both environmental conditions, the light was on from 0630 to 2045 h.

Lambs assigned to group one were transitioned to a 90% concentrate finishing diet (49% SweetBran®; 37.8% dry-rolled corn; 8.3% chopped alfalfa hay; 4% mineral

supplementation; dosage of coccidiostat (20 g/ton)) over a period of 21 d. Lambs assigned to group two were held on a maintained diet (54.8% SweetBran®; 41.1% chopped alfalfa hay; 4% mineral supplementation; dosage of coccidiostat (20 g/ton)) at 2% of BW for 41 d prior to 21 d transition to the 90% concentrate finishing diet previously stated with the addition of ammonium chloride (10 mg/hd/d).

Lambs were supplemented with one of three supplemental treatments. Zilpaterol hydrochloride (ZH) was supplemented at 5.44 g/ton (2.5 mg/kg) and ractopamine hydrochloride (RH) was supplemented at 18.14 g/ton (40 mg/kg). The supplement was mixed into 200 g of fine ground corn with the control (C) receiving no supplement in 200g of fine ground corn. β-AA were supplemented into the diet beginning on d1 and mixed into 907.19 g of feed. The remaining amount of regular feed was given around 1400 h.

Within group, lambs were stratified by body weight and randomly assigned to one of six treatment groups: control/thermoneutral (CTN), Ractopamine HCl/thermoneutral (RHTN), Zilpaterol HCl/thermoneutral (ZHTN), control/heat stress (CHS), Ractopamine HCl/heat stress (RHHS), and Zilpaterol HCl/heat stress (ZHHS).

Lambs were harvested on d 21 of the trial. The harvest order was randomly assigned and 12 were harvested each d via captive bolt followed by exsanguination. Tissue samples were taken from full thickness sections of the rumen epithelium. All tissue samples collected were placed in 2 mL collection tubes and flash frozen in liquid nitrogen. During RNA isolation the rumen tissue samples were dissected from the muscle so that only the epithelium was isolated.

Experiment Two

Twenty-five crossbred Rambouillet lambs (44kg) were utilized in 2x2 factorial design. Upon arrival, lambs were weighed, rectal temperatures recorded, ear tagged with individual ID's, metaphylacticaly treated [Ivomec®; 10 mL/hd (Merial, Duluth, GA) and Draxxin®; 1 mL/hd (Zoetis, Parsippany, NJ)]. Lambs were housed in group pens and acclimated for 21-d. After 21 d, lambs were stratified by body weight and randomly assigned to one of four treatment groups: control/thermoneutral (CTN), Ractopamine HCl/thermoneutral (RHTN), control/heat stress (CHS), and Ractopamine HCl/heat stress (RHHS). Additionally, lambs were parsed into four groups (HS: A & B; TN: C & D) to allow for pair feeding (starting on d -5).

Lambs were housed in one of two environmental conditions; thermoneutral (controls; 25°C, 15% RH) or heat stress (40°C, 35% RH) for 30 d. Heat stress was achieved by maintaining a temperature of approximately 40°C and 35% humidity from 0800 to 2000 h, and 29°C between 2000 and 0800 with temperature changing over a period of 2 h at the beginning and end of each heat cycle. In both environmental conditions, the light was on from 0630 to 2045 h.

Lambs were fed a 16% protein, 2% fat diet (16% crude protein, 2% crude fat, 16% crude fiber; Lamb Grower-Finisher Complete B30 (Purina)) beginning on d -5 of the study. Orts, feed left over from the day before, were collected daily and recorded to evaluate average performance statistics (e.g., average daily gain, gain:feed), to determine the amount of feed to offer the following day as well as that to offer the pair fed counterparts. Supplemental treatment included either ractopamine hydrochloride given

via oral bolus at 60mg/d/animal, or no supplement (control), for which only an empty capsule was administered.

Lambs were harvested on d 30 of the study by an overdose of pentobarbital. The harvest order was randomly assigned and 6 were harvested each day, with the exception harvest d 1 and d 4 with 5 and 9 lambs being harvested, respectively. Rumen muscle and epithelium tissue samples were dissected out from each other, placed in 2 mL collection tubes, and flash frozen in liquid nitrogen.

RNA Extraction from Rumen Epithelium and Sequencing

Total RNA was isolated from the rumen epithelium from the 49 wethers in study one and 25 wethers in study two using the Direct-zol RNA MiniPrep Plus kit (Zymo Research) with modifications. Roughly 100 mg of tissue was weighed and minced quickly before being placed in 600uLof Trizol. The tissue was homogenized in Trizol using five bead-beating steps performed in a TissueLyser (Qiagen Inc., Valencia, CA, USA) for 45 sec at 30 Hz with a two-minute incubation in ice between bead-beating steps.

Following this bead beading step, tissue was further homogenized with the addition of 400 uL of Trizol. The homogenized tissue was incubated at room temperature for 5 min after which the samples were spun down to remove any excess tissue. After transferring the supernatant from this step to new tubes, a chloroform precipitation was performed where 200 uL of chloroform was added, vortexed for 30 sec and incubated at room temperature for 3 min. Following this incubation, samples were centrifuged for 15 min at 11,900 rpm and 4 ºC. The top aqueous layer was removed and placed in 600 uL of

100% ethanol and mixed. This mixture was placed on a spin column and washed twice using 400 uL of RNA pre-wash buffer. A 15 min DNase treatment was then performed using 75 uL of DNA digestion buffer and 5 uL of 6U/uL of DNase per sample. This treatment was followed by three washes with 700 uL of RNA wash buffer. RNA was eluted in 100 uL of RNase free water.

Once isolated, RNA was quantified, and integrity analyzed using the Agilent Bioanalyzer. RNA Integrity Number (RIN) scores above 8 were considered adequate. Samples from study one were sent to the University of California Davis' Genome Center (Davis, CA) for QuantSeq 3' mRNA library prep and 100 bp, single-end sequencing on an Illumina HiSeq 4000. Samples from study two were sent to the Oklahoma Medical Research Foundation (Oklahoma City, OK) for standard poly-A+ mRNA prep and paired-end sequencing was performed on an Illumina NovaSeq S1.

Bioinformatics Analyses

HTStream (Ver. htstream/e919d21) was used for preprocessing and quality control of sequence data by removing adapter sequences, trim 11 bp and remove 5' poly-A/T repeats. Trimmed reads were aligned to the Ovine Oar_v3.1 with STAR (ver2.5) alignment (Dobin & Gingeras, 2015). Differential expression analysis was performed comparing samples in HS to TN environments and supplement fed animals to the control using limma-voom (Law et al. 2014; Ritchie et al. 2015). Lowly-expressed transcripts (zero expression in half or more of the individuals in each treatment, or with <1 count per million in n libraries where n=number of individuals in the analysis) were removed. Transcripts with an adjusted $P \leq 0.10$ for study one and adjusted $P \leq 0.05$ for study two were considered differentially expressed (DE). DE transcripts were then analyzed using

Ingenuity Pathway Analysis (QIAGEN Inc.; IPA) to predict pathways altered. For genes without annotated gene IDs in the ovine genome, human or mouse orthologs were input when possible.

Results

Study One

An average of 4.7 million reads were obtained per sample with 58.49% of the reads uniquely mapping to the Ovine Oar_v3.1 genome. Of the 27,054 annotated transcripts, 5,632 were observed in these data; 3,623 remained after removing those lowly expressed. No interaction was found between temperature and supplement, therefore only the main effects were evaluated. Analyses identified 607 transcripts with P(raw)<0.05, with 7 DE (Adj. $P < 0.10$; Table 1) between TN and HS lambs. No genes were differentially expressed due to supplement.

Study Two

An average of 26.3 million reads were obtained per sample with 83% mapping uniquely to the genome. Data from one lamb (L15) identified him as an outlier and thus, that observation was removed from the analysis. Of the 27,054 transcripts evaluated, 9,690 remained after removing those that were lowly expressed. No interaction between environment and supplement was observed. Evaluation of the MDS plot generated showed that samples clustered by group as well as environment (Figure 1), therefore the model from which DE was identified included the pair fed groups with group A/C considered batch one and group B/D considered batch two. Resulting analyses identified 733 DE transcripts (Adj. $P \leq 0.05$) between TN and HS lambs (Appendix A). From these

results, 106 pathways were predicted to be altered due to environment (Table 2). No transcripts were differentially expressed in the control vs. RH comparison.

Discussion

In this study, we report that heat stress, but not β-AA supplementation, affects the transcriptome profile of the rumen epithelium. Additionally, no supplement-environment interaction was present suggesting that supplementation of β-AA during heat stress conditions does not exacerbate the effects of heat stress on the rumen epithelium. Interestingly, neither RH nor ZH had an effect on the rumen epithelial transcriptome, which was unexpected given the presence of β-AR in the epithelium. These results could suggest that β-AA does not affect the rumen epithelium and nutrient absorption, which would indicate that these supplements are acting elsewhere in the animal to achieve the desirable changes in carcass traits. Skeletal muscle strips from animals in study one to measure ex vivo glucose oxidation under basal and insulin-stimulated conditions. Supplementation of β2-AA increased muscle glucose oxidation by ~15% and addition of insulin to the media increased glucose oxidation by ~25%, while supplementation of β1-AA had no effects on muscle glucose oxidation (Barnes et al. 2017). Taken together, these data suggest B2-AA impact carcass traits through binding BAR in skeletal muscle and not through altering rumen epithelial characteristics. Semitendinosus muscle taken from the lambs in study one also found an impact of ZH; supplement with the B2-AA altered the expression of 8 transcripts at harvest, and 42 at biopsy (Kubik et al., 2018). However, RH again had no measurable impact on the skeletal muscle. These findings, especially the RH results, were surprising due to the known role β-AA play in improving carcass composition. Adipose tissue was taken from lambs in both studies but has yet to

be analyzed. Upon analysis, it could provide an insight into where RH may be working in the body if not in the skeletal muscle or rumen epithelium.

Seven transcripts from study one and 733 from study two were DE due to heat stress. The seven transcripts from study one include *SLC25A21, LIAS, CCDC17, HRNNPA3, ZBTB44, ACOX1*, and ENSOARG00000008612. Interestingly none of those DE transcripts overlapped between studies. In study two we utilized full length, paired end, traditional RNA sequencing while it study one we utilized 3'Tag-Seq which generates a single library molecule per transcript that is complementary to 3'end sequences. We speculate that some of the difference in results may be attributed to missing annotation of the 3' end of some transcripts in the current ovine reference genome; poor annotation of the 3' end of the gene would cause these transcripts to be absent in analysis while assaying the entire transcript as performed in study two allows for alignment across the larger genic region. Additionally, it is also possible that expression in transcripts associated with heat stress and β-AA supplementation in study one were altered during the period of time the lambs were held at ambient condition in the abattoir awaiting harvest. Moreover, animals were not given supplementation the day of harvest in study one which could have also altered expression of transcripts. Due to the minimal significance observed in study one, the remainder of the discussion will focus on the results from study two.

Protein Ubiquitination

Protein ubiquitination and the SUMOylation pathways were predicted to be impacted due to heat stress. During protein ubiquitination, ubiquitin is attached to the lysine residue of a protein, marking it for degradation and/or preventing its activity in

various interactions (Lecker et al., 2006). Heat stress causes protein damage (Schröder et al., 1993), therefore an increase in ubiquitination is expected. Although there was no directionality of activity identified by IPA (z-score = NaN), 23 of the 271 genes annotated as being involved in this pathway were upregulated, while 5 out of the 271 were downregulated; this could be interpreted as suggesting an increase in protein degradation. Heat shock induces an initially rapid increase in protein degradation in mammalian cells (Parag et al., 1987; Gropper et al., 1991), which would also explain why many transcripts involved with the protein ubiquitination pathway were upregulated. Under the heat stressed condition our lambs were undergoing increased protein damage which led to an upregulation in transcripts involved in protein ubiquitination to mark damaged protein for degradation.

Post-translational modification of proteins by members of the small ubiquitin-related modifier (SUMO) protein family regulates cellular processes, including transcription, replication, and DNA repair (Johnson, 2004; Geiss-Friedlander and Melchior, 2007). Modulation of SUMO conjugation is impacted by various environmental stressors such as osmotic, heat and oxidative stress (Saitoh and Hinchey, 2000). Oxidative and ethanol stress have also been reported to increase SUMOylation in yeast (Zhou et al., 2004), however these results are not simple as changes in SUMOylation depends on stress intensity and duration (Tempé et al., 2008). Low to moderate oxidative stress inhibits global SUMOylation with exposure to 1 mM H_2O_2 leading to deSUMOylation of cellular proteins within one hour (Bossis et al., 2006). In our study, the SUMOylation pathway was downregulated (z-score = -0.707), suggesting oxidative stress in the rumen was causing a delay in the heat stress response (Adachi et

al., 2009). Therefore, it is possible that by suppressing the heat stress response, cells experiencing DNA damage were not able to undergo DNA repair by SUMO proteins, leading to apoptosis. Alternatively, a downregulation in the SUMO pathway could be due to the lambs becoming acclimated to heat stress environment, resulting in a desensitization to oxidative stress and a decreased need for the response.

Oxidative Stress

Heat stress is a cause of oxidative stress (Akbarian et al., 2016; Alemu et al., 2018). The production of reactive oxygen species (ROS) is a key event of many inflammatory disorders, including disorders of the gastrointestinal tract. In the gut, disruption of the mucosal barrier will rapidly activate the innate immune system and induce an acute inflammatory response (Aviello et al, 2017). The production of ROS in the gastrointestinal tract due to the immune response may aid in the resolution of inflammation (Campbell et al., 2014). However, uncontrolled and persistent oxidative stress leading to an overproduction of reactive oxygen species will cause tissue injury (Rezaie et al., 2007).

The NRF2-mediated oxidative stress response was predicted to be upregulated (z-score = 0.905) in the lambs housed in a heat stress environment compared to the thermoneutral controls. This pathway is activated when oxidative stress is present and the genes involved act to eliminate and detoxify reactive oxidants (Nguyen et al., 2009). Similarly to the tissue samples in this study, when bovine granulosa cells were exposed to heat stress, there was an increase in NRF2-mediated signaling (Alemu et al., 2018). Additionally, specific genes expected to be involved in heat stress and oxidative stress, including DNAJ genes, *NQO1*, *ACTG2*, and *GAB1*, were upregulated in these lambs after

30 d of heat exposure. These lambs, therefore, may have responded to heat by excessive production of ROS, leading to an upregulation of this pathway to eliminate reactive oxidants that were causing intestinal injury. Increased intestinal injury in our lambs due to the overproduction of ROS, could allow for endotoxin to escape from the gut, leading to increase in the inflammation response. Additionally, intestinal tissue damage would have major impacts on an animal's ability to absorb nutrients, leading to a decline in animal growth and performance.

Another pathway that was altered by heat stress in the study was colanic acid building blocks biosynthesis (P = 2.7E-06). Colanic acid (CA) is an exopolysaccharide structure, produced under stress that is associated with the surface of a variety of bacterial species (Grant et al., 1969). Colanic acid is associated with Enterobacteriaceae, specifically *Escherichia coli*, and acts as a protective agent against environmental stressors (Navasa et al., 2009). Colanic acid is normally lowly produced, however, large amounts can be synthesized in response to specific mutation or environmental factors. The biosynthesis of colonic acid is up regulated in response to several environmental factors that modulate or damage cell structure including temperature (Navasa et al., 2009) and oxidative stress (Chen et al., 2004). Additionally, CA regulates mitochondrial dynamics of the host and unfolded protein response (Han et al., 2017). Because heat stress impacts mitochondrial function (Huang et al., 2015) it is possible that increased secretion of CA by bacteria in the gut worked to combat damage host mitochondria underwent. Therefore, the upregulation of this pathway in our study is evidence that the bacteria of the rumen microbiome were producing increased levels of CA to combat heat stress.

Additionally, SAPK/JNK signaling (*P* = 4.17E-03; z-score = 1.667; Figure. 2) and ERK/MAPK (*P* = 3.28E-03; z-score = 0.577; Figure. 3) were identified by IPA as likely to have been altered by heat stress. c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs), are characterized by their activation in response to cell stress by playing a vital role in cell proliferation, apoptosis, and differentiation (Dhanasekaran et al., 2008). Whether the activation of JNKs leads to apoptosis or cell differentiation is dependent on the stimuli and the cell-type involved (Lin and Dibling, 2002; Liu and Lin, 2005). The extracellular-signal-regulated kinase (ERK) pathway is one of the major signaling cassettes of the mitogen activated protein kinase (MAPK) signaling pathway. The ERK cascade can be activated by cellular stressors to induce cell growth, proliferation, differentiation and apoptosis (Lu et al., 2006).

The upregulation of these two pathways in our study are consistent with other reports evaluating the ERK and JNK pathways. Considering the effect of oxidative stress on intestinal epithelial cell apoptosis, Zhou et al. (2005), suggested that activation of ERK and JNK may be involved in the H_2O_2-induced intestinal epithelial cell apoptosis since the inhibition of either pathway resulted in reduction of the apoptotic response. Due to heat stress being associated with oxidative stress the lambs in our study may have been undergoing rumen epithelial cell apoptosis due to overproduction of ROS which is suggested by these two pathways being upregulated. In terms of heat stress, cells respond to elevated temperatures by activation of *p38 MAPK*, *ERK1/2*, and *JNK*, as well as by increasing the production of heat shock proteins (Mearow et al., 2002; Gourgou et al., 2010; Murai et al., 2010). Although, upregulation of HSP transcripts involved in these pathways were not observed, *RAS* and *MAPK* transcripts were predicted to be

upregulated. Both *RAS* and *MAPK* can induce apoptosis (Cox and Der, 2003; Wada and Penninger; 2004); additionally, *RAS* can also stimulate the production of ROS (Ozaki et al., 2000). Upregulation of these transcripts in response to heat stress further suggests increased apoptosis of cells damaged due to heat and oxidative stress.

Immune Response

Animals exposed to heat stress have a suppressed immune response, which could include serious health implications because an effective immune response is critical for maintenance of health and productive function (Ajuwon and Hua, 2015). Nuclear factor-κB (NF-κB) is an important intracellular signaling protein that controls the transcription of genes involved in cell growth, survival, apoptosis, and inflammatory responses (Baldwin, 2001). Interestingly the NF-κB signaling pathway was downregulated (z-score = -0.58) in the lambs of this current study. Similar results were observed in the semitendinosus of female pigs subjected to heat stress in which it was shown that heat stress did not increase inflammatory signaling through the NF-κB pathway, suggesting NF-κB pathway quiescence (Montilla et al., 2014). Additionally, a variety of heat shock response inducers are reported to prevent activation of NF-κB (Rossi et al.,1997). A study utilizing HS-Sultan cells showed that heat stress was able to inhibit constitutive NF-κB DNA-binding activity, resulting in apoptosis (Belardo et al., 2009). The downregulation of NF-κB signaling due to heat stress seen in the current study could indicate increased cell apoptosis as well as decreased immune function which could have negative impacts on animal health and viability in a livestock production system.

Cell Cycle and DNA Damage

Heat stress induces DNA damage responses in S, G1, and G2-phase cells (Velichko et al., 2012). Additionally, severe heat stress may lead to cell death through apoptosis (Velichko et al., 2013), downregulate DNA repair genes (Rockett et al., 2001), and induce senescence-like cell cycle arrest (Velichko et al., 2013). In the lambs of study two, both the G2/M DNA damage checkpoint regulation (P = 4.81E-03) and nucleotide excision repair (NER; P = 1.86E-03) pathways were projected to be upregulated by heat stress. DNA damage arrests the cell cycle to allow for DNA repair to occur before DNA replication or mitosis begins (Harper and Elledge, 2007; Ciccia and Elledge, 2010; Hu et al., 2016). In our lambs the DNA checkpoint pathway was upregulated (z-score = 0.45), suggesting an increase in cell cycle arrest to repair damaged DNA. Although previous pathways discussed regarding this study suggest an increase in apoptosis, the upregulation in DNA repair in our lambs may indicate that epithelial cells were trying to preserve cell viability through DNA repair. The NER pathway was also upregulated (z-score = 1.667) in our lambs. The NER pathway recognizes DNA helix-distorting lesions (Marteijn et al. 2014). The upregulation of the NER pathway further indicates DNA damage that was occurring due to heat stress and DNA repair was increased through NER and DNA damage regulation.

Conclusion

Feedlot cattle finished in the summer months are often adversely affected by heat stress which can impact production, costing the industry and impacting animal well-being. Further, β-AA supplementation is common in livestock production to improve animal performance. Although no interaction between environment and supplement was

identified, nor were any transcripts differentially expressed due to β-AA, transcripts found to be differentially expressed due to heat stress revealed potential mechanisms through which heat stressed ruminants respond to environmental stressors.

No impact of either β-AA supplement, RH or ZH, was observed. β-AAs therefore appear to have little effect on the epithelial lining of the rumen; thus, they likely have an alternate mode of action to achieve the desirable change in carcass weight and composition observed. ZH appears to be acting on the skeletal muscle (Barnes et al, 2017; Kubik et al., 2018), however mechanisms behind the RH mode of action are still unclear, reinforcing the need for additional molecular studies to elucidate the specific impacts and mode of action of β-AA. Many of the transcripts and pathways identified to be altered due to heat stress were associated with oxidative stress and apoptosis. Therefore, understanding how these pathways impact rumen function and the ability to derive and absorb nutrients will be critical in improving animal wellbeing and performance while under heat-stressed conditions.

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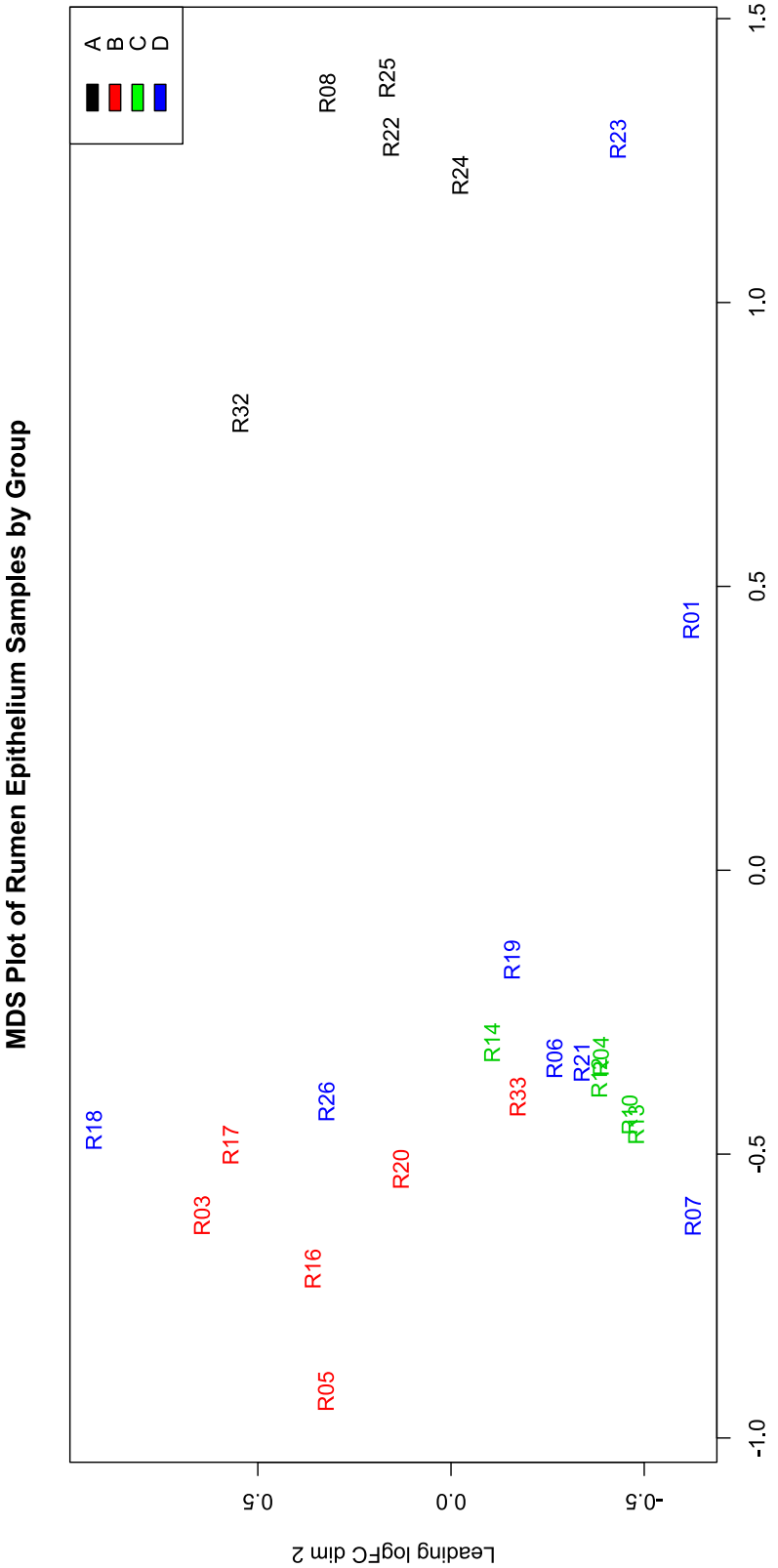

Figure 1. MDS plot of lamb rumen epithelium RNA samples. Lambs are clustering by group.

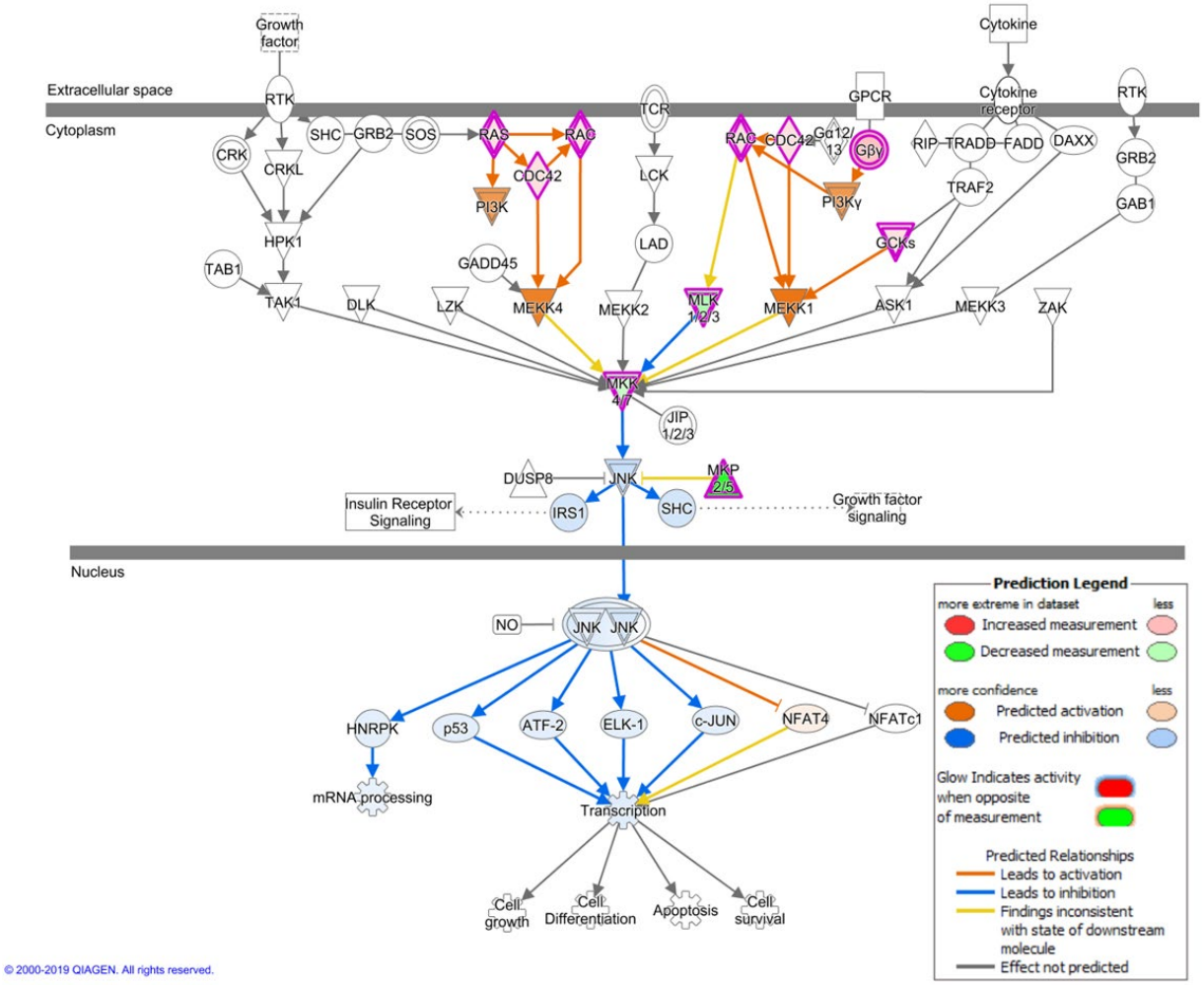

Figure 2. SAPK/JNK Signaling (IPA): The JNK signaling pathway was predicted to be upregulated in animals in the heat stress environment.

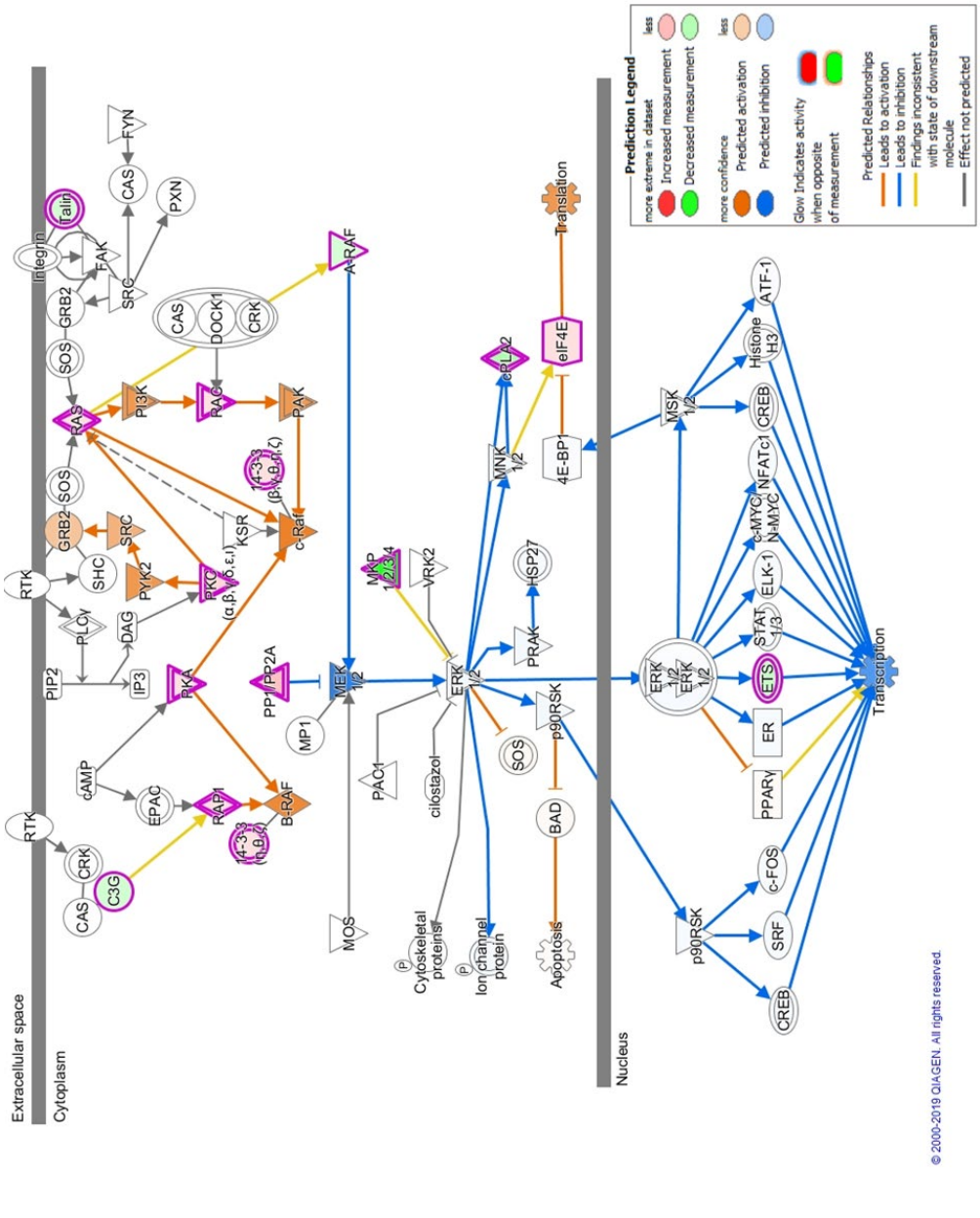

Figure 3. ERK/MAPK Signaling (IPA): the ERK signaling pathway was predicted to be upregulated in animals in the heat stress environment.

Table 1. Transcripts differentially expressed in study one due to heat stress (adj *P*-value < 0.1) in the rumen epithelium.

Gene Name	logFC	Adj. P.Value
SLC25A21	-1.5126	0.04
LIAS	0.947	0.05
CCDC17	0.7333	0.06
HNRNPA3	1.1341	0.06
ZBTB44	0.7533	0.06
ACOX1	0.6781	0.08
ENSOARG00000008612	0.6725	0.08

Table 2. Top canonical pathways altered in the rumen epithelium due to heat stress (*P*-value < 0.05, -log(p-value) > 1.3).

Canonical Pathways	P-value	z-score
Protein Ubiquitination Pathway	7.00E-08	NaN
NRF2-mediated Oxidative Stress Response	1.00E-06	0.905
Colanic Acid Building Blocks Biosynthesis	2.70E-06	2.449
Sumoylation Pathway	2.81E-05	-0.707
Sirtuin Signaling Pathway	3.07E-05	-0.258
Heme Biosynthesis II	1.24E-04	1
LPS/IL-1 Mediated Inhibition of RXR Function	1.33E-04	0
Germ Cell-Sertoli Cell Junction Signaling	1.37E-04	NaN
Tetrapyrrole Biosynthesis II	3.21E-04	NaN
Acyl-CoA Hydrolysis	4.37E-04	1
Bile Acid Biosynthesis, Neutral Pathway	6.15E-04	1
GDP-mannose Biosynthesis	6.27E-04	NaN
Adenine and Adenosine Salvage I	1.05E-03	NaN
Integrin Signaling	1.33E-03	1.807
Mismatch Repair in Eukaryotes	1.45E-03	NaN
Vitamin-C Transport	1.45E-03	NaN
Estrogen Receptor Signaling	1.68-03	NaN
Adenosine Nucleotides Degradation II	1.85E-03	-1
NER Pathway	1.86-03	1.667
Virus Entry via Endocytic Pathways	1.92E-03	NaN
Xenobiotic Metabolism Signaling	2.17E-03	NaN
Glucocorticoid Receptor Signaling	2.47E-03	NaN
ILK Signaling	2.86E-03	1.897
Macropinocytosis Signaling	2.94E-03	1.633
Sertoli Cell-Sertoli Cell Junction Signaling	3.01E-03	NaN
Semaphorin Signaling in Neurons	3.14E-03	NaN
Renal Cell Carcinoma Signaling	3.16E-03	0
ERK/MAPK Signaling	3.28E-03	0.577
Regulation of Actin-based Motility by Rho	3.40E-03	2.121
FcÎ³ Receptor-mediated Phagocytosis in Macrophages and Monocytes	3.40E-03	0.333
Granzyme A Signaling	3.48E-03	NaN
Purine Nucleotides Degradation II (Aerobic)	3.48E-03	-1
Cardiac Hypertrophy Signaling	3.92E-03	1.941
Actin Cytoskeleton Signaling	4.11E-03	1.941
SAPK/JNK Signaling	4.17E-03	1.667
Androgen Signaling	4.74E-03	0.816
Signaling by Rho Family GTPases	4.76E-03	1.155

Cell Cycle: G2/M DNA Damage Checkpoint Regulation	4.81E-03	0.447
Cholecystokinin/Gastrin-mediated Signaling	5.31E-03	1.89
IL-6 Signaling	5.58E-03	-0.333
RhoGDI Signaling	5.89E-03	-1.265
Pyrimidine Deoxyribonucleotides De Novo Biosynthesis I	5.89E-03	2
Guanosine Nucleotides Degradation III	5.96E-03	NaN
BER pathway	5.96E-03	NaN
Arsenate Detoxification I (Glutaredoxin)	6.05E-03	NaN
CXCR4 Signaling	6.74E-03	2.828
HGF Signaling	7.07E-03	0
Paxillin Signaling	7.07E-03	1.89
Urate Biosynthesis/Inosine 5'-phosphate Degradation	7.57E-03	NaN
Caveolar-mediated Endocytosis Signaling	7.99E-03	NaN
tRNA Charging	8.10E-03	2.236
Glioma Invasiveness Signaling	8.72E-03	2
Oxidative Phosphorylation	8.92E-03	3
NF-Î²B Signaling	9.01E-03	-0.577
Cell Cycle Control of Chromosomal Replication	9.24E-03	NaN
Androgen Biosynthesis	9.40E-03	NaN
Galactose Degradation I (Leloir Pathway)	9.80E-03	NaN
Estrogen Biosynthesis	1.00E-02	0.447
Breast Cancer Regulation by Stathmin1	1.05E-02	NaN
Epithelial Adherens Junction Signaling	1.12E-02	NaN
Hereditary Breast Cancer Signaling	1.22E-02	NaN
Agrin Interactions at Neuromuscular Junction	1.31E-02	1.89
Stearate Biosynthesis I (Animals)	1.34E-02	1.342
Thrombin Signaling	1.41E-02	2.449
Tec Kinase Signaling	1.44E-02	1.134
HMGB1 Signaling	1.50E-02	0.378
LXR/RXR Activation	1.69E-02	0
Glutathione-mediated Detoxification	1.90E-02	2
Thioredoxin Pathway	1.97E-02	NaN
Phospholipase C Signaling	2.07E-02	2.111
IL-8 Signaling	2.32E-02	1.897
MIF-mediated Glucocorticoid Regulation	2.34E-02	0
Retinoate Biosynthesis I	2.34E-02	1
Mitochondrial Dysfunction	2.36E-02	NaN
Remodeling of Epithelial Adherens Junctions	2.41E-02	NaN
The Visual Cycle	2.55E-02	NaN
GÎ±q Signaling	2.55E-02	1.134
Inflammasome pathway	2.55E-02	NaN
Adenine and Adenosine Salvage III	2.57E-02	NaN
Nucleotide Excision Repair Pathway	2.57E-02	NaN

Actin Nucleation by ARP-WASP Complex	2.89E-02	2
PAK Signaling	2.91E-02	1.633
Acute Phase Response Signaling	3.16E-02	-0.333
VEGF Signaling	3.19E-02	1.633
Glutamine Biosynthesis I	3.24E-02	NaN
Purine Ribonucleosides Degradation to Ribose-1-phosphate	3.24E-02	NaN
Xanthine and Xanthosine Salvage	3.24E-02	NaN
Asparagine Biosynthesis I	3.24E-02	NaN
D-mannose Degradation	3.24E-02	NaN
Prolactin Signaling	3.28E-02	NaN
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	3.41E-02	-1.134
Protein Kinase A Signaling	3.71E-02	1
CCR3 Signaling in Eosinophils	3.85E-02	1.342
LPS-stimulated MAPK Signaling	3.98E-02	1.633
Hepatic Fibrosis / Hepatic Stellate Cell Activation	4.00E-02	NaN
VDR/RXR Activation	4.06E-02	0
Adrenomedullin signaling pathway	4.11E-02	1.265
Mechanisms of Viral Exit from Host Cells	4.28E-02	NaN
Aryl Hydrocarbon Receptor Signaling	4.31E-02	-1
TR/RXR Activation	4.36E-02	NaN
PPARÎ±/RXRÎ± Activation	4.40E-02	1.667
Leukocyte Extravasation Signaling	4.50E-02	2.714
RAR Activation	4.50E-02	NaN
Retinol Biosynthesis	4.61E-02	1
RhoA Signaling	4.66E-02	2.121
GÎ±12/13 Signaling	4.81E-02	2.121

APPENDIX A: DIFFERENTIALLY EXPRESSED RUMEN EPITHELIUM TRANSCRIPTS IN LAMBS FED BETA-ADRENERGIC AGONISTS AND SUBJECTED TO HEAT STRESS FOR 30 DAYS

Table 1. Transcripts differentially expressed in study two due to heat stress (adj P-value < 0.05) in the rumen epithelium.

Gene Name	logFC	Adj.*P*.Val
DES	8.768	0.000
SORBS2	4.687	0.000
FLNC	3.207	0.001
SH3BGR	3.027	0.001
MAP1A	3.122	0.001
PFDN1	0.432	0.001
KCNMA1	3.943	0.001
CETN2	0.513	0.001
LBHD1	0.923	0.001
CNN1	4.559	0.001
SYNPO2	3.319	0.001
WFIKKN2	3.912	0.002
NEXN	2.183	0.002
MYCBP	0.578	0.002
PARK7	0.419	0.002
REXO2	0.553	0.002
JPH2	2.797	0.003
COX17	0.502	0.003
PCP4	6.621	0.003
CELSR1	-0.477	0.003
RBPMS2	2.770	0.003
DUOXA1	0.746	0.003
ACTN2	2.180	0.003
LMOD1	2.162	0.003
TNFRSF6B	1.265	0.003
NEK1	-0.435	0.004
USP14	0.319	0.004
CLMP	2.815	0.004
ACTA1	5.849	0.004
PLAT	-0.727	0.004
GFER	0.431	0.004
RDH11	0.529	0.004
PPP1R14A	1.484	0.004
TXN	0.524	0.004
TMEM86A	-1.505	0.004
ECI2	0.610	0.005
SAMD8	-1.330	0.005
MRGPRF	2.510	0.005
PLIN4	3.237	0.005
DNASE2	0.535	0.005
ASTN2	-0.986	0.005
RNF44	-0.521	0.005
THEM6	0.789	0.005
ARHGEF25	1.961	0.005
FAM13B	-0.407	0.005
NDUFB3	0.355	0.005
UBE2S	0.349	0.005
NQO1	0.812	0.005
GALK1	0.638	0.005
Tomm5	0.506	0.005
PSMA7	0.427	0.005
HAGH	0.280	0.005
CALML4	1.641	0.005
DNPH1	0.574	0.005
ITGA7	2.004	0.005
VEZF1	-0.377	0.005
GREB1	-1.047	0.005
MIEN1	0.446	0.006
DDX17	-0.304	0.006
TIMM50	0.338	0.006

YIF1B	0.421	0.006
DST	-0.652	0.006
MAP2K7	-0.396	0.006
PLXND1	-0.777	0.007
DTYMK	0.499	0.007
GNG11	1.498	0.007
SWI5	0.381	0.007
HIST1H2BN	0.426	0.007
SMYD1	5.029	0.007
PDLIM3	2.048	0.007
JMJD7	0.593	0.007
MFAP5	1.712	0.007
NT5C2	-0.403	0.007
ITPRIPL2	-0.568	0.007
RAB34	0.431	0.007
Eloc	0.338	0.007
ACOT1	-0.919	0.007
SIGMAR1	0.419	0.007
ACTG2	3.831	0.007
MCUR1	0.401	0.007
RAC3	0.624	0.007
SLITRK2	2.778	0.008
HSPB6	1.752	0.008
SCEL	-0.803	0.008
ARHGAP21	-0.345	0.008
EIF2S2	0.338	0.008
DELE1	-0.286	0.008
ENAH	0.476	0.008
CYP2B6	-2.411	0.008
LDHB	0.485	0.008
H2AFZ	0.399	0.009
MYO9B	-0.299	0.009
PTGIS	2.197	0.009
STK36	-0.347	0.009
PDF	0.378	0.009
ZACN	-1.234	0.009
MYL12B	0.336	0.009
PATL1	-0.477	0.009
SMPX	4.493	0.009
GPX2	1.134	0.009

STK10	-0.376	0.009
CUL7	-0.274	0.009
RAPGEF1	-0.307	0.009
SPCS3	0.361	0.009
SLC7A5	0.713	0.009
ANXA1	0.332	0.009
CMC2	0.547	0.010
ID1	0.747	0.010
FAM20C	0.639	0.010
ESD	0.673	0.010
SYNM	2.587	0.010
MELK	0.562	0.010
LAMB3	-0.643	0.010
APRT	0.574	0.010
SLC25A5	0.385	0.010
COL17A1	-0.365	0.010
AL162171.1	-0.507	0.010
MTHFD2	0.525	0.010
DNAJB5	1.155	0.010
IL1R2	-0.542	0.010
KPNA2	0.306	0.010
PAPLN	-0.721	0.010
AGTPBP1	0.349	0.010
ELP1	-0.272	0.010
DHX57	-0.251	0.010
MOXD1	1.258	0.011
MRPL40	0.402	0.011
RRAS	0.766	0.011
GNMT	0.625	0.011
ATP8B1	-0.738	0.011
LY6D	1.010	0.011
FOXA1	-0.321	0.011
ANP32E	0.362	0.011
SLC20A1	-0.463	0.011
SAE1	0.269	0.011
PLS1	-0.987	0.011
PLCB4	1.376	0.011
CAP2	0.899	0.011
NR2C2	-0.323	0.011
SPC24	0.520	0.011

KIAA0930	-0.535	0.011
NCBP2	0.301	0.011
HYPK	0.404	0.012
GLCE	-0.467	0.012
GSTM3	1.356	0.012
ELL2	0.565	0.012
HMOX1	-1.116	0.012
RNASEH2C	0.523	0.012
TP63	-0.357	0.013
CBY1	0.358	0.013
KLHL41	1.268	0.013
SH2B1	-0.318	0.013
SPTBN1	-0.267	0.013
SSNA1	0.418	0.013
TGFB1I1	1.439	0.013
NDUFAF5	0.418	0.013
ENPP2	-0.772	0.013
SLC23A1	-0.647	0.013
SERPINB3	0.975	0.013
PGP	0.412	0.013
ADA	-1.070	0.014
OTUD1	-0.414	0.014
RNF20	-0.219	0.014
SCD	0.950	0.014
PCLAF	0.583	0.014
MBD6	-0.415	0.014
TMEM126A	0.366	0.014
N4BP3	0.403	0.015
PLEKHA4	0.429	0.015
H1FX	0.331	0.015
IL36G	0.810	0.015
DUSP4	-1.615	0.015
GGPS1	0.355	0.015
ILVBL	-0.357	0.015
IGF2R	-0.370	0.015
GAB2	-0.455	0.015
ASF1B	0.387	0.015
NFU1	0.366	0.015
ITGB4	-0.408	0.015
CNIH4	0.302	0.015

TSTA3	0.296	0.015
LSM4	0.416	0.016
TK1	0.605	0.016
CKB	0.339	0.016
CFAP36	0.388	0.016
CNTNAP1	1.499	0.016
MYBL2	0.504	0.016
POFUT2	-0.262	0.016
TUBB3	1.018	0.016
RRM2	0.582	0.016
USP36	-0.302	0.016
TP53I13	0.584	0.016
NDST2	-0.308	0.016
POLR2G	0.301	0.016
DNASE1L1	0.318	0.016
LSM7	0.367	0.016
SMDT1	0.328	0.016
BEX3	0.822	0.017
MRPS26	0.423	0.017
TEF	-0.325	0.017
FIZ1	-0.386	0.017
KIF3B	-0.317	0.017
SLC15A4	-0.359	0.017
HSD17B8	0.327	0.017
TIMM17B	0.410	0.017
DENND1B	-0.503	0.017
RHOBTB2	-0.393	0.017
SUGT1	0.294	0.018
GMPPB	0.403	0.018
PTTG2	0.369	0.018
GLUL	-0.886	0.018
STARD10	0.572	0.018
GFPT1	0.468	0.018
LAMC2	-0.438	0.018
ST6GALNAC2	0.640	0.018
USP54	-0.622	0.018
SIPA1L3	-0.415	0.018
CFL2	1.179	0.019
NUAK2	0.393	0.019
FABP7	2.553	0.019

TMEM184B	-0.286	0.019
CIAO2B	0.306	0.019
Akr1c18	0.554	0.019
MYEF2	0.373	0.019
BAHCC1	-0.438	0.019
RFC2	0.378	0.020
RF00100	0.772	0.020
COL16A1	-0.474	0.020
Akr1c14	0.790	0.020
ATP6V1F	0.370	0.020
VBP1	0.318	0.020
TXNDC17	0.481	0.021
CEBPB	0.551	0.021
GALT	0.577	0.021
CACNA1C	1.737	0.021
POLR1A	-0.259	0.023
GLCCI1	-1.020	0.023
CRCP	0.317	0.023
PITPNA	0.279	0.023
AC118754.1	-0.738	0.023
CENPU	0.481	0.023
PRRC2B	-0.407	0.023
TSR3	0.302	0.023
F8	-0.652	0.023
SMIM11A	0.460	0.023
CD14	0.600	0.023
FAM114A2	0.210	0.023
TUBA4B	0.313	0.024
CDC42	0.226	0.024
POMT2	-0.289	0.024
C11orf71	0.655	0.024
TIMM8B	0.384	0.024
POP5	0.367	0.024
SPRYD3	0.206	0.024
SMG6	-0.259	0.024
CMPK1	0.291	0.024
DAAM2	1.262	0.025
COX20	0.380	0.025
MCM6	0.369	0.025
ARHGEF40	1.170	0.025

TRIT1	0.314	0.025
SAV1	-0.297	0.025
DCXR	0.410	0.025
ALOXE3	-1.161	0.025
CDCA3	0.442	0.025
RAB25	0.344	0.026
TRIO	-0.278	0.026
ERCC6L	0.617	0.026
PTPRF	-0.344	0.026
KCP	0.730	0.026
CTDSPL2	-0.270	0.026
UBTD2	-0.428	0.026
NCAPG	0.519	0.026
TSHZ1	-0.283	0.026
CCDC149	-0.466	0.026
PSMD14	0.270	0.026
TRMT112	0.390	0.026
BZW2	0.456	0.026
Akr1c21	0.567	0.026
RGL2	-0.344	0.026
PPOX	0.360	0.026
PRR14L	-0.300	0.026
RGP1	-0.348	0.026
SNRPF	0.435	0.026
NEK7	-0.379	0.026
ADHFE1	-0.459	0.026
SRA1	0.336	0.026
NDUFB4	0.287	0.026
CYP2D6	-0.443	0.026
APOE	-0.651	0.026
DLG5	-0.441	0.026
MFSD3	-0.534	0.026
STARD5	0.385	0.026
YWHAZ	0.310	0.026
AMACR	-0.479	0.026
POMP	0.280	0.026
ETS1	-0.423	0.026
GTF2F2	0.238	0.027
TTC17	-0.275	0.027
PLXNB1	-0.285	0.027

SLC35E3	0.295	0.027
TRAP1	0.240	0.027
POLB	0.305	0.027
LFNG	-0.628	0.027
TBC1D13	-0.258	0.027
PSPC1	0.394	0.027
TPP1	-0.534	0.027
CNPY4	0.345	0.027
PTMA	0.341	0.027
ACOT8	0.610	0.027
ASAP1	-0.561	0.027
E2F1	0.431	0.027
GIGYF2	-0.250	0.027
VPS26B	-0.297	0.028
AKAP12	1.382	0.028
RPP25L	0.280	0.028
DHRS9	-1.541	0.028
KBTBD4	-0.614	0.028
CENPW	0.561	0.028
FKBP4	0.456	0.028
IMMP1L	0.412	0.028
THEM5	0.796	0.028
LRP4	-0.611	0.028
MRPL20	0.416	0.028
MRPS21	0.351	0.028
UBR4	-0.289	0.029
CD93	-0.730	0.029
MKLN1	0.217	0.029
CRIM1	-0.256	0.029
PYCR1	0.707	0.029
BOLA3	0.353	0.029
DYNC2LI1	0.459	0.029
SNX3	0.247	0.029
KLC3	0.383	0.030
OXLD1	0.367	0.030
UCHL5	0.306	0.030
FBXO32	-0.944	0.030
ZNF318	-0.347	0.030
PNP	0.477	0.030
TMEM69	0.345	0.030

B4GALT1	-0.300	0.030
IGFBP3	-0.938	0.030
ANKRD28	-0.253	0.030
DPH1	-0.244	0.030
FAM120B	-0.213	0.030
MYL6	0.316	0.030
GTF3C2	-0.214	0.030
POLR3H	0.329	0.030
LYNX1	0.557	0.030
GPAT3	-0.938	0.030
Mxra7	0.842	0.030
LIMS2	1.282	0.030
KANK2	1.151	0.030
ANP32A	0.450	0.030
NFIL3	0.384	0.031
ERMARD	-0.378	0.031
BCL7C	0.371	0.031
Akr1c20	0.527	0.031
PDXK	0.301	0.031
RAPGEF5	-0.450	0.031
CHCHD7	0.312	0.031
ATG3	0.237	0.031
RAP1A	0.214	0.031
SURF6	0.364	0.031
DESI1	0.282	0.031
ARHGAP35	-0.313	0.031
DNAJB4	0.414	0.031
UBE3B	-0.200	0.031
P3H4	0.471	0.031
MRPS15	0.208	0.031
CCT6A	0.422	0.031
SARNP	0.295	0.031
GSTA1	0.766	0.031
Cenpx	0.354	0.031
RANBP1	0.351	0.031
GMNN	0.379	0.032
TXNRD1	0.695	0.032
CYP2D6	-0.407	0.032
CD164L2	0.381	0.032
ZBTB38	-0.320	0.032

TLN2	-0.312	0.032
RFC3	0.342	0.032
MRPL19	0.246	0.032
ELMO2	-0.285	0.032
AP3D1	-0.221	0.032
HSPD1	0.262	0.032
CHDH	-0.754	0.032
PDIA6	0.278	0.032
TACC1	0.694	0.032
MMP9	1.129	0.032
SLC27A4	0.384	0.032
AR	-0.323	0.032
MFN2	-0.338	0.032
APOO	0.261	0.033
ANP32D	0.552	0.033
SF3B5	0.409	0.033
MYLK	1.800	0.033
ZNF862	-0.294	0.033
PROSER1	-0.311	0.033
HIKESHI	0.313	0.034
NUDT22	0.350	0.034
AGO2	0.267	0.034
FAM204A	0.258	0.034
RPS4X	0.424	0.034
FERMT1	-0.277	0.034
TLR4	-0.585	0.034
PPP2R3C	0.249	0.034
Akr1c6	0.594	0.034
AK1	0.547	0.034
PSMG3	0.247	0.034
CACTIN	-0.176	0.034
EP300	-0.389	0.034
PEX26	-0.340	0.034
WWC1	-0.324	0.034
NUBP2	0.274	0.035
NUP58	-0.172	0.035
IL1B	-0.744	0.035
RPA3	0.320	0.035
COPG2	-0.269	0.035
HCFC1	-0.573	0.035

SLC20A2	-0.492	0.035
DBP	-0.447	0.035
PCNA	0.381	0.035
TMEM231	-0.329	0.035
NCBP2-AS2	0.361	0.035
CTSO	-0.368	0.035
PRDM2	-0.385	0.035
AP1M2	0.271	0.035
MYO19	0.279	0.035
PSMD1	0.249	0.035
AGRN	-0.423	0.035
EBP	0.421	0.035
LAMA3	-0.299	0.035
TNFRSF12A	0.767	0.036
DMPK	1.116	0.036
LTBP3	0.357	0.036
CHERP	-0.253	0.036
SKP1	0.249	0.036
IFT22	0.409	0.036
MRPL42	0.240	0.036
GLRX5	0.390	0.036
TRIP13	0.422	0.036
GRID1	1.881	0.036
CRY2	-0.333	0.036
RNF216	-0.279	0.036
DPP3	0.244	0.036
LUZP1	-0.268	0.036
COMTD1	0.388	0.036
BNIP3L	-0.404	0.036
RAPGEFL1	-0.313	0.036
AP002956.1	1.209	0.036
PDSS1	0.297	0.036
PSMA5	0.344	0.036
KALRN	0.590	0.036
BCL9	-0.304	0.036
ENDOG	0.376	0.036
PLA2G6	-0.544	0.036
AARSD1	0.250	0.036
CPSF7	-0.263	0.036
TIMM10B	0.379	0.036

SEC14L1	-0.450	0.036
TRAPPC12	-0.201	0.037
NDUFA4	0.262	0.037
IL27RA	0.486	0.037
CEP78	0.335	0.037
DRG1	0.278	0.037
DUSP14	-0.662	0.037
FAM83A	0.474	0.037
ETV4	0.714	0.037
ARL3	0.327	0.037
SLC50A1	0.514	0.037
ZNRF1	0.329	0.037
COPA	-0.188	0.037
ZNF592	-0.302	0.037
USP11	-0.220	0.037
RAD51	0.365	0.037
NR3C1	-0.247	0.038
PSMD6	0.227	0.038
NDUFB2	0.295	0.038
NMRAL1	0.297	0.038
SDF2L1	0.475	0.038
CCSER2	-0.428	0.038
PEX11G	0.346	0.038
ADGRF4	0.283	0.038
LTA4H	0.358	0.038
KIAA1211L	-0.211	0.038
DRAM2	-0.276	0.038
CKS2	0.363	0.038
USP2	-0.458	0.038
PHTF2	-0.630	0.038
Anapc11	0.261	0.038
HAUS1	0.281	0.039
MND1	0.422	0.039
MRTFB	-0.380	0.039
IL6ST	-0.306	0.039
JAG2	-0.338	0.039
ZC3H12C	-0.497	0.039
TEC	0.247	0.039
PRKCB	1.024	0.039
DYRK2	-0.241	0.039

WDHD1	0.390	0.039
FARSB	0.280	0.039
C1QBP	0.200	0.039
IMPA2	0.216	0.039
PNKP	0.239	0.039
MED16	-0.206	0.039
PKMYT1	0.673	0.039
NDUFA12	0.237	0.039
THRB	-0.339	0.039
TBC1D22B	-0.233	0.039
DPH7	0.256	0.039
ABO	1.261	0.039
SLC29A1	0.526	0.039
LGALS7	0.399	0.039
DSG2	-0.490	0.040
ATP5MC1	0.276	0.040
MIB1	0.184	0.040
ENOSF1	-0.360	0.040
EMC9	0.356	0.040
A2M	-0.609	0.040
CHST2	-0.858	0.040
MRPL24	0.332	0.040
MAP3K9	-0.582	0.040
FCHO2	-0.361	0.040
FAM98C	0.343	0.040
IL19	-1.837	0.040
CARS2	0.255	0.040
GTF2IRD2	-0.432	0.040
PET100	0.346	0.040
ALAD	0.285	0.040
SNRPA1	0.353	0.040
DRAP1	0.265	0.040
ISOC2	0.329	0.040
PSMB10	0.378	0.040
NRDE2	-0.396	0.040
ZFHX3	-0.370	0.040
UBL4A	0.284	0.040
PEPD	-0.332	0.040
ME2	0.362	0.040
DDX54	0.190	0.040

RERE	-0.431	0.040
GOLGA3	-0.238	0.040
MTMR4	-0.264	0.040
Akr1c18	0.503	0.040
RBM3	-0.551	0.040
ACOT7	0.754	0.040
ARAF	-0.272	0.040
CYB5R4	0.249	0.040
TMEM258	0.216	0.040
BBOX1	-0.581	0.040
CMTR1	-0.288	0.040
CCNH	0.217	0.040
MRPL32	0.261	0.040
ZZEF1	-0.347	0.040
HARS2	0.166	0.040
NRBP1	-0.194	0.040
YEATS2	-0.246	0.040
ZNF512B	-0.388	0.040
ACPP	-0.522	0.040
B9D1	0.414	0.040
HEATR4	-0.665	0.040
BCOR	-0.363	0.040
UBN1	-0.217	0.040
AXL	-0.570	0.040
OGDH	-0.199	0.040
RAPH1	-0.506	0.040
PECAM1	-0.596	0.040
LRIF1	0.264	0.040
CHMP6	0.284	0.041
P2RX4	0.371	0.041
SF1	-0.211	0.041
TSTD1	0.314	0.041
SPAG9	-0.372	0.041
CRYAB	1.314	0.041
MRPL41	0.352	0.041
BRAT1	-0.480	0.041
LTB4R	0.490	0.041
MAGED1	-0.312	0.041
BTF3L4	0.204	0.041
HIVEP2	-0.301	0.041

BUD23	0.226	0.041
PTPMT1	0.379	0.042
LRRC24	-0.304	0.042
HECTD1	-0.271	0.042
ATG5	0.192	0.042
ANAPC13	0.304	0.042
TPP2	-0.334	0.042
RB1CC1	-0.238	0.042
CSRP1	0.998	0.042
SEL1L	-0.339	0.043
KNTC1	0.372	0.043
MAP4K2	0.266	0.043
HMBS	0.223	0.043
CASC3	-0.205	0.043
PRPF8	-0.233	0.043
MICOS10	0.282	0.043
TIMM22	0.285	0.043
PIH1D1	0.235	0.043
PIGF	0.312	0.043
WWC3	-0.466	0.043
HMGXB3	-0.245	0.043
PRKACA	0.197	0.043
ARID1B	-0.278	0.043
RRNAD1	-0.268	0.044
MCM2	0.349	0.044
PRDX2	0.278	0.044
HEBP2	0.272	0.044
SIPA1L2	-0.287	0.044
DPM3	0.408	0.044
ENHO	0.825	0.044
ATP5MD	0.304	0.044
PLEKHA8	0.256	0.044
RAB19	0.336	0.044
RYK	0.217	0.044
MPI	0.363	0.044
MXD3	0.353	0.044
SRRM1	-0.225	0.044
CDCP1	-0.241	0.044
DENND4B	-0.366	0.044
UBE2C	0.336	0.044

FTSJ1	-0.458	0.044
C8orf33	0.267	0.044
CHAMP1	-0.224	0.044
FOXN1	0.499	0.044
MET	-0.289	0.045
MRPL55	0.361	0.045
APCDD1	-0.299	0.045
RUSC1	0.280	0.045
NDUFA2	0.306	0.045
TPX2	0.249	0.045
SMARCD1	-0.228	0.045
MRPL57	0.302	0.045
TBCB	0.279	0.045
EIF4E	0.293	0.045
WDR81	-0.390	0.045
SEPT7	0.250	0.045
OSBPL1A	-0.297	0.045
WDR83	0.346	0.045
SEC22B	0.202	0.045
MLF2	0.277	0.045
SUSD1	-0.456	0.045
ZEB1	0.734	0.045
HIST1H1D	0.535	0.046
BUB3	0.336	0.046
RGS2	0.615	0.046
TGIF2	0.239	0.046
CNST	-0.496	0.046
DAP3	0.215	0.046
TMEM94	-0.237	0.046
CDK5RAP3	0.164	0.046
SIVA1	0.394	0.046
HMGCS1	0.306	0.046
EXOSC1	0.276	0.046
TRAPPC2B	0.249	0.046
POLR2A	-0.435	0.046
ATRAID	0.308	0.046
CDC20	0.387	0.046
FRMD4A	-0.309	0.047
SEC61G	0.258	0.047
TGFBRAP1	-0.233	0.047

MBNL1	0.351	0.047
TAF4	-0.278	0.047
ANKMY2	0.286	0.047
DNAJB6	0.278	0.047
ATPAF2	0.223	0.047
UBAP2L	-0.229	0.047
NUP98	-0.207	0.047
EWSR1	0.195	0.047
ANKS3	0.234	0.047
BNIP1	0.301	0.047
ATP9B	-0.199	0.047
BAG2	0.621	0.047
CAMK1	0.500	0.047
ALAS1	-0.373	0.047
EXO1	0.566	0.047
STAT6	-0.310	0.047
PLIN3	0.298	0.047
FBP2	-0.407	0.047
CDH13	-0.430	0.047
Akr1cl	0.468	0.047
PDLIM2	0.295	0.047
NCOA6	-0.328	0.047
ABCG1	-0.824	0.047
MRPL58	0.262	0.047
TARS	0.215	0.047
SUB1	0.374	0.047
CNNM4	-0.219	0.047
CCT5	0.327	0.047
MTRR	-0.681	0.047
ESCO2	0.496	0.047
KIF13B	-0.488	0.047
IMP4	0.294	0.047
CLUH	-0.224	0.047
EEF1E1	0.347	0.047
SMARCAD1	-0.265	0.047
PMM1	0.303	0.047
CD276	-0.275	0.047
EMILIN1	1.253	0.047
PSMD4	0.218	0.047
MGST2	0.353	0.047

VPS29	0.268	0.048
TNRC6C	-0.397	0.048
DACT3	0.921	0.048
PARP4	-0.274	0.048
NEO1	-0.199	0.048
SGSH	-0.305	0.048
TTK	0.462	0.048
CCDC88C	-0.301	0.048
EPRS	0.236	0.048
KLHDC8B	0.584	0.048
CDC6	0.537	0.048
CBS	0.366	0.048
ICE1	-0.207	0.048
ACSS1	0.773	0.048
SMCR8	-0.345	0.048
NCOA2	-0.376	0.049
TAPBPL	0.226	0.049
RHOD	0.472	0.049

SLC9A8	-0.352	0.049
NCCRP1	1.464	0.049
NR2C2AP	0.337	0.049
AOX1	-0.345	0.049
ASB8	-0.226	0.049
C6orf132	-0.344	0.050
ASNS	0.487	0.050
MXRA5	-0.664	0.050
GSTO1	0.345	0.050
TUBA1B	0.260	0.050
SAP18	0.196	0.050
RPL26L1	0.264	0.050
DBNDD2	0.327	0.050
TPM4	0.420	0.050
SBF1	-0.215	0.050