EFFECTS OF LOW PROTEIN DIETS ON BROILERS' ENERGY BALANCE, CECAL MICROBIOTA COMPOSITION, PLASMA METABOLOMICS, AND OXIDATIVE STRESS DURING EXPERIMENTALLY INDUCED HEAT STRESS

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Abstract:

Heat stress is a pressing challenge for livestock production. In 2013, the poultry industry alone lost $125-165 million due to heat stress-related death. The objective of Study 1 was to assess the effects of low protein diets (LP) on feed intake, heat production and markers of feed intake and thermogenesis regulation in the gut and muscle in broilers under experimentally induced heat stress (HS). 200-day-old male broiler chicks were weight-matched and assigned into 36 pens with 5-6 chicks/pen followed by randomly subjecting pens into two treatments (18 pens/treatment) including: 1) thermoneutral (TN), 2) HS (35^{o}C for 7 hours/day). Within each treatment, the pens were randomized to receive two diets (9 pens/diet; 50 birds/diet): 1) normal protein (NP), 2) LP. The study lasted 6 weeks with 2 weeks of adaptation (starter phase) and 4 weeks of data collection (grower and finisher phases). Feed intake and body weight were measured daily and weekly, respectively. On week 6 birds were euthanized and blood, duodenum, ileum, cecal contents and leg muscle samples were collected. A subgroup of birds was euthanized for assessing the breast meat quality and body composition using a dual-energy X-ray absorptiometry. All data were analyzed with either general linear model or mixed procedure (SPSS®). Means within each treatment between NP and LP diets were separated by Student's t-test. The results of Study 1 provide evidence that LP diets mitigate the negative outcome of heat stress by reducing feed intake and heat production, which are regulated through factors expressed in the gut and skeletal muscle. Little is known whether the beneficial effects of LP diets on heat stress is associated with alterations in oxidative stress, plasma metabolomics and cecal microbiota. The objective of Study 2 was to investigate the effects of LP diets on broilers oxidative stress, plasma metabolomics and cecal microbiota composition during experimentally induced HS. The experimental design was the same as Study 1. The results of Study 2 showed that dietary protein content influenced the plasma metabolites with similar pattern during TN and HS, but that had differential effects on cecal microbiota composition under TN and HS.

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CHAPTER 1

INTRODUCTION

1. Heat Stress in the Poultry Production Industry

Heat stress (HS) is a major concern for modern broiler producers (Virden et al., 2009). Stress is described as the response of the body to abnormal conditions that potentially interrupt homeostasis or normal physiological equilibrium (Sugiharto et al., 2016). Stress can be induced by a variety of environmental stressors such as sunlight, thermal radiation, air temperature, humidity, and movement. Due to the increase in environmental temperatures, HS has been a massive concern for the livestock industry. HS occurs when the animal's internal body temperature increases above the thermoneutral zone due to high environmental temperature (Lara et al., 2013). Animals and in particular poultry are sensitive to environmental temperature changes which are why HS is becoming more of a concern as the environmental temperature continues to rise.

HS is estimated to impose a total of $1.69 to $2.36 billion economic losses to the U.S. livestock production industry, annually (St-Pierre, 2003). In 2013, the U.S livestock production industry reported that $125 to $165 million were lost in the poultry industry alone due to HS (Lara et al., 2013). Therefore, researchers are looking for effective dietary strategies to help poultry adapt and recover from HS.

2. Effect of Heat Stress on Coping Mechanisms and Physiological Parameters in Chickens

HS is typically classified into two categories: acute and chronic. Acute HS is when the animal experiences an increased heat for a short period, while chronic HS is when the animal experiences an increased heat for a long period (Gonzalez-Esquerra et al., 2006). HS can not only cause death in birds, but if they survive, it can affect their immunity, metabolism, microbiota composition, and performance (Nawab et al., 2018). It has been reported that HS can induce long-term biological effects in broilers experiencing HS in the early stages of life such as an increase of corticosterone secretion (Ericsson, 2015). When the bird encounters stress for the first time, the sympathetic-adrenal-medullary axis (SAM) also known as the "fight or flight" response which causes the release of catecholamines such as epinephrine and norepinephrine stimulated (Post et al., 2003; Virden et al., 2009). The release of these hormones instead will increase the blood pressure, muscle tone, nerve sensitivity, blood sugar, and respiration in an attempt to neutralize the effect of the stressor (Virden et al., 2009). If this would not be effective enough, the hypothalamic-pituitary-adrenal cortical system is activated immediately releasing a cascade of hormones such as adrenocorticotropic hormone (ACTH) and corticosterone which if this process lasts for long periods, then can alter the performance and growth of the bird by decreasing feed intake, body weight gain, innate immunity, changes in metabolism and nutrient digestibility (Mishra et al., 2019; Virden et al., 2007; Virden et al., 2009; Lin et al., 2006). During long-term stress, physiological and coping mechanisms will take place until the

bird can return to homeostasis or death occurs. When corticosterone concentration is elevated for long periods increased chances of cardiovascular disease, hypercholesterolemia, gastrointestinal lesions, alterations in immune system function, and changes in glucose and mineral metabolism can occur (Virden et al., 2007). During HS, the neuroendocrine system activates pathways that increase the synthesis of glucose for energy use and release of stress hormones such as epinephrine that will increase the heart rate of the bird. An increase in heart rate will also increase the body temperature (Nawab et al., 2018). Corticosterone, a major glucocorticoid found in circulation while birds are under stress. If secreted for a long period, corticosterone has been reported to cause depression, reduced immunity, cardiovascular problems, and breakdown of muscle (Nawab et al., 2018). During HS the immune cells count, such as lymphocytes and macrophages, will alter due to being suppressed by cortisol (Aggarwal et al., 2013). This can affect cell proliferation, cytokine secretion, and antibody production, which will in return affect the immunity of the bird (Lara et al., 2013).

When environmental temperatures continue to increase the bird will try to reduce body temperature by not only altering biological activity but by using different coping mechanisms or physiological responses to reduce body temperature. One of the first physiological responses seen is spending less time at the feeder and increasing water consumption (Nardone et al., 2010). By decreasing feed intake, the bird can reduce the heat increment that is generated by the digestion of carbohydrates, lipids, and proteins (Awad et al., 2017; Swennen et al., 2005; Syafwan et al., 2011). Heat increment is defined as the

specific dynamic effect or action (Musharaf et al., 1999). Due to the absence of sweat glands in birds, they will have to increase evaporation to reduce body temperature. The bird not only uses panting but also uses other mechanisms such as rapid oscillation of the floor of the mouth and upper part of the throat to reduce body temperature (Awad et al., 2017). During panting, birds can release over 60% of moist air from the body and decrease body temperature (Kapetanov et al., 2015). Lifting wings or wing spread is another way the bird tries to dissipate heat by increasing body surface area (Zmrhal et al., 2018). Further, panting lowers the temperature of a bird's respiratory system (Awad et al., 2017). An increase in panting for a long period results in a decrease in feed and water intake but more concerning it increases the carbon dioxide levels in the blood, which in turn can increase the blood pH and chance of respiratory alkalosis (Awad et al., 2017; Lara et al., 2013). Since the act of panting requires energy, if HS is experienced for a long period, it can cause long-term effects on the production of the bird (Kapetanov et al., 2015). Over time the bird will then decrease its movement such as walking and increase lying down to try to reduce the energy used. Birds experiencing HS for long periods have been seen to have a decreased feed intake which in return would cause poor growth performance, immunosuppression, hypoxia, and high mortality (Lara, 2013; Mishra et al., 2019).

3. Effect of Heat Stress on Feed Intake and Intake Related Markers in Chickens

In commercial poultry farms, temperature, humidity, and airflow of the facility are controlled to provide optimal development since feed intake and growth are affected by changes in temperature (Tickle et al., 2018). In poultry production, feed intake suffers during HS. During HS a substantial decrease in nutrient absorption is seen which results in reduced feed intake (Ferket et al., 2006, Furlan et al., 2004). Reduction of feed intake causes a reduction in growth performance which is a major factor in production. Hormones or gut peptides that regulate feed intake are either considered to be orexigenic (stimulate feed intake) or anorexigenic (inhibit feed intake) (Richards et al., 2003). One of the orexigenic peptides in birds is ghrelin (Richards et al., 2003; Song et al., 2012). Ghrelin is considered the only endogenous peripheral hormone that incudes hunger and increases feed intake in both poultry and mammalian species (Murphy et al., 2004). Anorexigenic hormones are also known as satiety hormones cause a reduction in feed intake by a variety of mechanisms including inhibiting or slowing gastric emptying (Huda et al., 2006). Some intake regulating peptides are known to be important for both poultry and mammalian species include glucagon-like peptide 1 (GLP1) (Kolodziejski et al., 2018), glucose-dependent insulinotropic polypeptide (GIP) (Kolodziejski et al., 2018; Wang et al., 2012), secretin (Wang et al., 2012), cholecystokinin (CCK) (Song et al., 2012), and peptide YY (PYY) (Aoki et al., 2016).

During HS, adipokines such as leptin and adiponectin, or gut peptides that stimulate the hypothalamic axis causing a change in feed intake are secreted (Slimen et al., 2016; Ferket et al., 2006). In an experiment investigating the effect of HS on laying hens feed

intake, it was observed that the mRNA expression of ghrelin increased and CCK decreased in HS hens compared to ones under thermoneutral (TN) condition which resulted in a decreased feed intake (Song et al., 2011).

4. Effect of Heat Stress on Heat Production and Thermogenesis Related Markers

During acute and chronic HS, the body prioritizes sending nutrients to specific major organs. Since broiler chickens are being bred for a faster growth rate, this has caused a higher feed intake and faster metabolism, which in return increases metabolic heat production (Bloch et al., 2019). In muscle, β-1 adrenergic receptor (β1-AR), peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α), AMP-activated protein kinase (AMPKα1), sirtuin 1, cytochrome c oxidase subunit IV (Cox IV), mammalian target of rapamycin (mTOR), and avian uncoupling protein (AvUCP) are involved in regulating body temperature (Jastrebski et al., 2017). β1-AR and UCP gene expression have been also seen in brown fat and participate in non-shivering thermogenesis in mammals (Joubert et al., 2010). AvUCP gene expression has been shown to play a key role in adaptive thermogenesis (Evock-Clover et al., 2002). β1-AR has been reported to up-regulate AvUCP gene expression in muscle (Joubert et al., 2010). During HS, β1-AR regulates AvUCP gene expression which has been shown to alter fatty acid metabolism, thermogenesis and the levels of reactive oxygen species (ROS) produced in the

mitochondria (Arain et al., 2008, Joubert et al., 2010). mTOR, Sirtuin, PGC-1α, AMPKα1, and Cox IV are known for being mitochondrial energy sensors that help the body regulate energy homeostasis. mTOR has been identified as a major component in the signaling pathway for cellular growth and metabolism through various mechanisms (Han et al., 2015).

Mammalian target of rapamycin, also known as mTOR, is an important signal pathway found in muscle tissue that not only regulates muscular hypertrophy and metabolism but also plays a vital role in mediating protein synthesis through downstream targets such as insulin-like growth factor 1 (IGF-1) which promotes protein synthesis and muscle hypertrophy and improves feed efficacy (Bodine et al., 2001; Bottje et al., 2014; Han et al., 2015; Ma et al., 2018). Although mTOR signaling pathways are well understood, the effect of diet and temperature on the expression of mTOR is little understood. In chronic HS conditions, broilers fed normal protein (NP) diets showed a reduction in mTOR mRNA expression which consequently reduced the expression of IGF-1, growth performance, and breast muscle tissue (Ma et al., 2018). The addition of branch chain amino acids (BCAA) in a low protein (LP) diet has been seen to promote the gene expression of mTOR in broilers under TN; thus, stimulating protein synthesis and increasing hypertrophy of breast tissue (Deng et al., 2014; Ospina-Rojas et al., 2018; Ospina-Rojas et al., 2020). Further research is required to understand the effect of diet and temperature on the expression of muscle mTOR gene expression in birds.

Sirtuins are associated with various cellular and metabolic processes during times of stress. Sirtuin plays a role in the regulation of redox balances and controlling cellular stress response while maintaining genome integrity and protein stability (Surai et al., 2019). Sirtuins regulate many biological processes such as cell growth and differentiation, apoptosis, energy transduction, and glucose homeostasis (Surai et al., 2019). Specifically, sirtuin 1 gene expression has been shown to decrease fatty acid synthesis and increase fatty acid oxidation and works in coordination with other enzymes such as PGC-1α in the regulation of antioxidant gene transcriptions (Shimao et al., 2019; Surai et al., 2019).

PGC-1α works with AMPK and sirtuin to not only regulate the activity of antioxidants but also to initiate the transcription of genes such as AvUCP (Joubert et al., 2010; Surai et al., 2019). PGC-1α also has specifically contributed to muscle fiber transformation and mitochondrial biogenesis leading to the belief that it plays a vital role in the thermogenesis of birds (Ueda et al., 2005).

AMPKα1 is an isoform of AMPK, which increases the energy supply. By switching on ATP-generating pathways, there will be an increase in energy which can alter the lipid metabolism. These changes include inhibiting fatty acids and cholesterol synthesis in the liver and lipolysis in adipocytes and stimulating fatty acids uptake and their oxidation in muscle (Proszkowiec-Weglarz et al., 2005). Not only does this cascade cause changes in metabolic enzymes, but AMPK also acts as a gage controlling cellular energy changes including alterations in extracellular nutrient levels such as glucose, fatty acids, and hormones, such as leptin and ghrelin (Carling et al., 2004; Proszkowiec-Weglarz et al.,

2005). Thus, AMPK is involved in the regulation of energy homeostasis and can change the hypothalamic neuron activity by both hormonal and nutrient signals which influence feed response (Xu et al., 2011). All of these markers provide a vital role in heat production, but little is known about the pattern of their changes and their function in birds under HS conditions.

5. Effect of Heat Stress on Body Composition and Meat Quality

HS not only causes poor appetite and reduced feed intake, but it also affects digestion and metabolism of feed (Sugiharto et al., 2016). A change of environmental temperatures such as sunlight, temperature, and humidity can affect different bird's metabolism and thermoregulation, which in return causes imbalances in the body (Nawab et al., 2018). HS has been considered to be detrimental to broilers due to its effect on meat quality and body composition (Zhang et al., 2012). Birds experiencing chronic HS have been seen to have many adverse effects on meat quality and body composition due to changes in aerobic and post-mortem glycolytic metabolism and intramuscular fat deposition, which results in pale meat color with low water-holding capacity (Zhang et al., 2012, Zaboli et al., 2019). Zhang et al. (Zhang et al., 2012) reported that when broilers experience diurnal cyclic HS, the protein content of breast meat is significantly decreased which could be due to changes in protein metabolism, decrease in protein synthesis and increase in catabolic rate. During HS, the plasma thyroid hormones, T_3 and T_4, concentration is increased as a physiological

response to the temperature which can influence the meat quality of broilers (Gonzalez-Rivas et al., 2019). The half-life of T_3, the biologically active form found in broilers, is shorter than that of T_4, which is the storage form (Bueno et al.,2020). HS has been reported to produce pale, soft, and exudative meat often referred to as PSE or 'atypical poultry meat' (Solomon et al, 1998). PSE characterized by low water holding capacity is often caused by the muscle hyper-metabolism mediated by the direct action of calcium channels causing an increase in the open state of these channels and release of calcium (Barbut et al., 2008; Gonzalez-Rivas et al., 2019). Since a substantial portion of broiler meat is used for further processing such as margination, tumbling, and cooking processes, PSE is detrimental to the poultry industry (Zaboli et al., 2018). It has been documented that when birds experience HS for long periodsthere is a rapid pH drop, higher lightness, changes in redness, high drip, and cooking losses in breast meat (Zaboli et al., 2018; Zhang et al., 2012; Aksit et al., 2006). When heat-stressed birds have an excess of protein in supply, the fat deposition will be increased as the body is unable to reduce the heat produced by the supply of protein (Geraert et al., 1996). It is important to find solutions to reduce the body temperature and improve broiler's meat quality under HS through higher protein synthesis and lower fat deposition.

6. Effect of Heat Stress on Oxidative Stress in Birds

In high temperatures, the structure and physiology of the cell are altered which can cause issues with processes such as transcription, RNA processing, translation, oxidative metabolism, and membrane structure and function which can increase the production of

ROS (Arain et al., 2008). Although ROS in low levels is not harmful, during HS the ROS levels exceed the level of antioxidants which can damage lipids, proteins, carbohydrates, and DNA which could lead to aging, loss of protein function, reduced enzyme activity, and development of PSE conditions (Estevez et al., 2015; Zaboli et al., 2019). In this situation, the system will try to protect cells from extreme damages by releasing antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). Heat shock proteins (HSP) along with antioxidants will be released to help protect the damaged cells and remove ROS (Lara et al., 2013).

7. Effect of Heat Stress on Gut Microbiota and Blood Metabolomics Profile in Birds

The microbiota population in the bird is very sensitive to changes in temperature and the health of the bird (Awad et al., 2018). The gastrointestinal tract (GIT), specifically, harbors more than 100 trillion microorganisms that play many physiological roles (Sohail et al., 2014). The microbiome of birds is composed of a variety of different bacteria and composition can be altered by genetics, age, diet composition, disease, medication, or environmental factors (Sohail et al., 2014; Cesare et al., 2019; Forsythe et al., 2009; Shi et al., 2019; Tian et al., 2019). The microbes in the bird are important for health and play a role in food absorption, electrolyte balance, immune development, and maintaining overall homeostasis (Sohail et al., 2014; Nawab et al., 2018; Tian et al., 2019). When the microbiota population balance is altered or not at equilibrium, the bird is more susceptible

to infectious pathogens such as *Salmonella* and *Campylobacter* (Lara et al., 2013). In times of environmental stress or disease in birds, the gut microbiome is compromised and that can influence feed intake, nutrient absorption, and gut integrity (Awad et al., 2018). HS, specifically, has been found to impact intestinal morphology, integrity, and mucosal immunity in poultry (Wang et al., 2018). When the microbiome is compromised it can result in decreased performance and increased occurrence rate of diseases which could increase chances of mortality (Nawab et al., 2018; Sohail et al., 2015). It has also been reported that by improving the microbial balance found in the GIT of the birds after being compromised, the body weight gain and growth can be improved(Lan et al., 2004). In several studies, there were significant differences in gut microbiota composition of birds under HS vs. TN, while the diversity of species was significantly affected by HS (Tian et al., 2019; Shi et al., 2019; Wang et al., 2018). Shi et al., 2019 reported that the HS may not only alter microbiota or dysbiosis but could also increase the gut permeability as well as immune and metabolic dysfunction. HS can also influence the community structure of the bacteria found in the GIT. For example, an increase in abundance of *Clostridium* and *Coprococcus*, which are associated with intestinal injury was observed in broilers under HS conditions while the abundance of *Faecalibacterium* known for its anti-inflammatory activity and maintenance of gut health was decreased (Shi et al., 2019, Sohail et al., 2015, Wang et al., 2018). It is important to understand how dietary factors can alter the compromised microbiome of birds during HS.

Blood metabolites profile can be also altered during HS. Metabolites in the blood are not only altered when the diet is changed but also when the temperature increases or decreases. When a bird is experiencing chronic HS, a reduction in feed intake increases its maintenance requirements and induces a negative energy balance, which can impact the metabolites profile (Lu et al., 2018; Geraert et al., 1996). In a study comparing broilers in HS and TN conditions, it was observed that there were seventy-eight levels of different metabolites that were altered due to HS which included pathways such as metabolism of amino acids (AA), glucose, and uric acid (Lu et al., 2018). In another study comparing acute and chronic HS in broilers, it was observed that the long-term HS caused tissue damage which was evident by the increased activity of plasma lactate dehydrogenase, glutamic-oxaloacetic transaminase, and creatine kinase (Xie et al., 2015). Others comparing different levels of chronic HS on broilers observed a decreased expression of Triiodothyronine(T_3) and chlorine levels, and significant effects of temperature on uric acid, glucose, lactic dehydrogenase, and creatine kinase (Lin et al., 2000). Overall, temperature not only affects feed intake and growth performance but also plays a huge role in shaping the blood metabolites and gut microbiome profile of birds. By understanding how diet could alter the microbiome and blood metabolomics during HS, we can hopefully find solutions to help bring the microbiome back to equilibrium thus increasing growth and performance.

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CHAPTER 2

Literature Review

Heat stress can affect the metabolism, immunity, and overall health of birds. Since HS has increasingly become a challenge to the poultry industry, scientists are looking for developing new dietary strategies to improve the survivability and growth performance of birds under HS. Some of the nutritional suggestions proposed to alleviate HS in birds include supplementations of fats, vitamins, minerals, and probiotics (Nawab et al., 2018). Further, LP diets have received a lot of attention as a dietary solution for HS. These diets can alleviate the adverse effects of HS and improve the protein efficiency ratio (Awad et al., 2017). The objective of this literature review is to discuss the effects of LP diets on growth performance, feed intake and heat production, body composition, and meat quality, gut microbiota and blood metabolomics profile, and oxidative stress of birds under TN and HS conditions.

1. Low Protein Diets and Their Benefits in Poultry Production

Low protein diets reduce feed costs (Awad et al., 2017; Marayat et al., 2018). To achieve a low CP level in LP diets, generally, the soybean meal (common protein source) is lowered. There is five limiting AA in low soybean and LP diets for birds, which include methionine (Met), lysine (Lys), threonine (Thr), tryptophan (Trp), and glycine (Gly). These AA are essential for the broilers to synthesize non-essential AA (Aftab et al., 2006). Lowing the CP in the diet without supplementation of these limiting AA decreases the

growth performance and feed intake, and changes the body composition of the bird (Marayat et al., 2018; Waldroup et al., 2005). Similar results have been reported when a high CP diet with an excess amount of supplemented essential AA is offered to birds (Daghir et al., 2009; Smith et al., 1998). Compromised growth performance and feed intake of birds fed with these types of diets have been attributed to an imbalance of AA.

Low protein diets have also been reported to reduce nitrogen excretion which results in lower environmental pollution (Van Harn et al., 2019). It has been demonstrated by Ferket that by lowering dietary CP and supplementing optimal amounts of synthetic AA nitrogen excretion can be dropped significantly which can decrease environmental pollution (Ferket et al., 2002). The reduction of nitrogen excretion reduces the moisture in the litter which also improves broiler welfare (Van Harn et al., 2019).

2. The Effect of Low Protein Diets on Survivability, Coping Mechanisms and Growth-Related Measures in Birds Under Heat Stress

LP has been shown to increase bird's survivability and biochemical parameters during HS (Ghasemi et al., 2020). LP diets supplemented with limiting AA have been suggested to be used for increasing growth performance and decreasing mortality rate in HS conditions due to the reduction of the heat increment generated by protein breakdown (Ghasemi et al., 2020; Daghir et al., 2009). In HS conditions, the reduction of feed intake often is the major factor that affects production, body weight, feed efficiency, and dietary digestibility which is detrimental to the poultry industry (Lara et al., 2013; Syafwan et al., 2011). In

one study, it was observed that birds provided with a LP diet had an increased foraging activity (Eriksson et al., 2014). To help the bird during HS conditions, the producer needs to balance the benefits of a faster growth rate and an increased risk of mortality (Syafwan et al., 2011). It has been shown that when the growth rate or size of a bird increases the chances of mortality under HS conditions also increases (Lara et al., 2013).

Eriksson et al. (2014) also suggested that when birds were nutritionally satisfied, they prefer to stay close to the water and food even though ample space is provided. In HS conditions birds try to improve thermoregulation and reduce the effects of HS by increasing respiratory rate, panting, wing spreading, increasing water intake, decreasing feed intake, and reducing movement (Mohammed et al., 2018; Lin et al., 2006). The goal of these physiological activities is to reduce the body temperature since the birds lack sweat glands. Increased respiratory rate or panting dissipates heat through evaporation while wing spreading increases heat loss by increasing the body surface (Mohammed et al., 2018; Lolli et al., 2009). It has been reported that there is a positive correlation between evaporating cooling and water consumption of birds under acute HS (Belay et al., 1993). When LP diets are fed, it is often suggested that the broiler's body temperature will increase due to the secretion of thyroid hormones (Furlan et al., 2004). Since there is a reduction of feed intake this will also cause a reduction of essential AA such as arginine, lysine, isoleucine, methionine, and tryptophan that are available to the bird (Furlan et al., 2004; Zulkifil et al., 2018). This suggests that if broilers are fed with LP diets, they should also receive supplemented AA typically found to be deficient during HS. This practice will result in the

reduction of thyroid hormone secretion and heat production by the bird. The LP diet will help the bird continue to decrease body temperature until returning to a thermoneutral state. In a recent In the study, it was shown that feeding broilers with LP diets supplemented with AA allowed for n optimal growth and maintained normal feed intake during HS (Attia et al., 2020).

3. The Effect of Low Protein Diets on Feed Intake and Intake Related Markers in Birds Under Heat Stress

As previously mentioned, feed intake is regulated by gut peptides that either stimulate feeding behavior (orexigenic) or inhibit it (anorexigenic) (Richards et al., 2003). The only known orexigenic peptide in birds is ghrelin (Richards et al., 2003; Song et al., 2012). Anorexigenic hormones, also known as satiety hormones, inhibit or slow gastric emptying and slow intestinal transport of nutrients which cause a reduction in feed intake (Huda et al., 2006). Peptides such as GLP1 (Kolodziejski et al., 2018), GIP (Kolodziejski et al., 2018; Wang et al., 2012), secretin (Wang et al., 2012), CCK (Song et al., 2012) and PYY (Aoki et al., 2016) are known as satiety hormones. Birds in HS conditions show a substantial decrease in nutrient absorption and gut motility due to stress response which results in reduced feed intake (Ferket et al., 2006; Furlan et al., 2004). In an experiment performed by Monir (2014), broilers under TN conditions were given diets with variable CP content ranging from 0% to 18%. They observed that expression of GLP-1 was decreased as the amount of protein intake decreased; thus, providing evidence that diet

impacts the expression of gut peptides (Monir et al., 2014). In another study, it was shown that the NPY expression was significantly decreased in broilers under HS compared to birds under TN suggesting that birds decrease feed intake by reducing NPY expression toincrease the chance of survival (He et al., 2019). It has also been shown that while corticosterone levels are increased during HS, mRNA abundances of CCK and ghrelin are reduced in the gut (Liu et al., 2012). Further research is required to better understand how the gut peptides are altered by diet and temperature in birds.

4. The Effect of Low Protein Diets on Heat Production and Heat Generation Related Markers in Birds Under Heat Stress

During digestion, absorption, and metabolism of nutrients heat is released. As described in earlier sections, feed intake of broilers is decreased during HS; thereby the generated heat may be reduced as well. It has been reported that protein has the highest heat increment compared to carbohydrates and fats due to protein requiring more energy used to yield an equivalent amount of useful energy to the animal (Furlan et al., 2004, Musharaf et al.,1999). Decreasing CP may help broilers under HS by reducing the amount of protein being digested, absorbed, and metabolized which would reduce the heat increment.

Tofully understand how birds, respond to environmental changes such as diet and temperature, understanding the role of molecules involved in thermogenesis is essential. Some of these specific molecules are Avian uncoupling protein (AvUCP), Beta-1 adrenergic receptors (β1-AR), sirtuins, peroxisome proliferator-activated receptor- gamma

coactivator (PGC-1α), AMP-activated protein kinase, (AMPK), and cytochrome C oxidase (Cox IV). The expression of these markers is regulated by various stimuli such as triiodothyronine (Awad et al., 2017), AA (Aftab et al., 2006) and generally speaking energy metabolism (Furlan et al., 2004). AvUCP is involved in fatty acid oxidation. AvUCP can increase heat production by changing the transmembrane gradient of protons (Evock-Clover et al., 2002; Marayat et al., 2018; Echtay et al., 2002). β1-AR has been shown to mediate the up-regulation of AvUCP (Joubert et al., 2010). Although little research is available on the effect of LP diets on the expression of β1-AR and AvUCP, it is believed that feeding birds with LP diets under HS will alter the expression of β1-AR and AvUCP (Arain et al., 2008; Joubert et al., 2010). In an experiment where broilers were exposed to HS and provided a LP diet supplemented with methionine, the expression of AvUCP was lower compared to the other diets (Marayat et al., 2018). mTOR, sirtuins, PGC-1α, AMPKα1, and Cox IV expressions are also involved in thermogenesis and energy metabolism regulation (Surai et al., 2019; Ueda et al., 2005; Xu et al., 2011). The effect of diet and temperature on the expression pattern of these molecules is still not clear.

5. The Effect of Low Protein Diets on Body Composition and Meat Quality in Birds Under Heat Stress

Often when broilers experience HS during periods of rapid growth it results in undesirable meat characteristics due to alterations in physiological and metabolism of muscle (St-Pierre et al., 2003, Lu et al., 2017). During HS, muscle moisture content and breast meat yield are

reduced, lipid oxidation is increased, and muscle pH is dropped which are all detrimental to the meat quality (Shakeri et al., 2019; Nawab et al., 2018; Daghir et al., 2009; Zaboli et al., 2019). Also, increased loss of muscle water holding capacity which increases the occurrences of PSE is associated with HS in birds (Barbut et al., 2007; Soloman et al., 1997; Zaboli et al., 2019). When dietary protein is reduced the amount of skeletal muscle deposition is also reduced. It has been reported that when broilers are fed LP diets as low as 15.3% CP, they had a higher body fat deposition (Filho et al., 2003). In TN conditions, reducing the dietary CP has been reported to reduce breast muscle proportion which also increases body fat accumulation. This is associated with amino acid deficiency and imbalance (Marayat et al., 2018). When LP diet supplemented with synthetic essential AA is offered under TN conditions, that could enhance the tenderness of meat by reducing the diameter of myofibers (Kobayashi et al., 2013). It has been reported that increasing the supplementation of AA such as lysine or arginine could improve weight gain and breast meat yield, and reduce the negative effects of HS on meat quality (Lin et al., 2019). Lysine has been used to promote muscle accretion in broiler chickens under TN conditions (Awad et al., 2017). If the thermoregulation of the birds under HS can be improved, then hormones released in response to HS will be reduced as well. This instead will decrease the muscle glycogenolysis and PSE and improve water holding capacity which alternatively improves the meat quality (Gonzalez-Rivas et al., 2020). LP diets supplemented with AA have been shown to improve protein efficiency and decrease nitrogen excretion without negative effects on performance and carcass yield in finishing broilers (Attia et al., 2020). Birds

showed no significant increase in abdominal fat during HS compared to those in TN conditions which proposes the idea that the birds used the AA and protein from the LP diet more efficiently during HS than TN (Attia et al., 2020).

6. The Effect of Low Protein Diets on Gut Microbiota and Blood Metabolomics Profile in Birds Under Heat Stress

Heat stress has been shown to increase gut disorders due to the reduction of gut microbiota in broilers (Shi et al., 2019). Due to the proportionally shorter GIT in birds, microbiota and metabolites play essential roles in enhancing nutrient absorption and strengthening the immune system (Cesare et al., 2019). A reduction in microbial populations is important for bird's health may impair growth performance. In broilers, the GIT is predominantly occupied by Firmicutes, Bacteroidetes, and Proteobacteria (Sohail et al., 2014). Changes in stressors including internal and external stressors such as environmental temperature, pH, and water availability cause bacteria to alter their response and may also cause a challenge for survival (Traub-Dargatz et al., 2006). It has been shown that feeding pigs with very LP diets altered the microbiota composition and influenced the pig's performance due to the increased ability to degrade plant-based feed that contains components such as hemicelluloses and xylans (Spring et al., 2020). In another experiment, it was observed that providing a LP diet to birds not only improved feed conversion rate (FCR) but also increased bacteria such as *Lactobacillaceae* in the ceca which were associated with improved FCR and utilization of edible resources and reduced emissions

of nitrogen pollutants (Cesare et al., 2019). *Lactobacillaceae* have been suggested to improve gut health, reduce pathogens, increase antioxidant activity and promote efficient nutrient and energy extraction by the host (Cesare et al., 2019; Humam et al., 2019). Increasing the *Lactobacillacea* population may help balance the gut physiology in broilers under HS (Nawab et al., 2018). Once the microbiome is balanced it could improve food absorption, electrolyte balance, immune development, and improve overall performance and growth (Sohail et al., 2014; Nawab et al., 2018; Tian et al., 2019).

Diet plays a pivotal role in metabolism and endocrine functions causing changes in plasma concentrations of metabolites and hormones (Kamran et al., 2016). Diet has been shown to change biological processes such as fat deposition, nitrogen retention, and nitrogen excretion (Kamran et al., 2010; Swennen et al., 2005). In an experiment conducted by Hernandez (2011), plasma albumin and total protein were lower in the birds provided the LP diet (Hernandez et al., 2011). Under TN conditions, when LP diets were supplemented with glycine and other essential AA, altered concentrations of blood metabolites, such as AA and lipids were observed (Hofmann et al., 2019). In other experiments, it was observed that birds fed with LP diets had lower uric acid in the plasma and changed the thyroid hormone concentrations such as plasma T_3 and T_4 (Corzo et al., 2004; Hofmann et al., 2019; Kamran et al., 2010; Swennen et al., 2005). In an experiment where broilers were provided a LP diet in HS conditions, it was observed that the birds had lower triglyceride levels and high-density lipoprotein-cholesterol concentration which are key factors in lipid metabolism (Ghasemi et al., 2020). Birds fed with the LP diets have

been seen to have better efficiency at the absorption of AA compared to those fed with NP diet. Metzler-Zebeli (2009) found nutrient and energy shortage due to changes in feed intake in birds under normal conditions. This caused a variation in not only amino acid profiles but also in physiological and metabolism-related factors such as insulin signaling, muscle metabolism, and different types of fatty acid metabolism (Metzler-Zebeli et al., 2019; Hofmann et al., 2019). In both TN and HS conditions, broilers fed with LP diet showed enhanced glucose production due to the increase in feed intake of a low nutrient-dense diet which is suggested to aid in "fight or flight" response to the environmental temperatures which has been suggested to improve survivability (Zhao et al., 2009; Ghasemi et al., 2020). Although growth performance has been seen to be reduced when LP diets are offered to birds in HS conditions, there are beneficial effects for LP diets such as improved survivability and heterophile to lymphocyte ratio (Ghasemi et al., 2020). There is little information on the effect's LP diets on blood metabolites of birds under HS.

7. The Effect of Low Protein Diets on Oxidative Stress in Birds Under Heat Stress

In hyperthermia, oxidative stress is triggered when the liver responds by increasing the production of biochemical antioxidants (Jastrebski et al., 2017). Due to the rapid-growing advantages of broilers, they have become more susceptible to oxidative stress. Oxidative stress occurs when reactive species (RS) levels exceed the level of antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, ascorbate, and vitamin E in cells (Zaboli et al., 2018). Radicals and metabolic substances are described as potentially toxic

and are referred to as “reactive oxygen/nitrogen/chlorine species” and if left unbalanced, they can result in extensive tissue and cellular damage causing major physiological and coping mechanism responses which contribute to reducing productivity, immunity, and meat quality (Akbarian et al., 2016; Arain et al., 2018; Estévez et al., 2015). During extreme stress, if the mitochondrial homeostasis is unable to be achieved it can lead to decreased ATP synthesis, cellular calcium dyshomeostasis, and induction of the mitochondrial permeability transition, all of which predisposes cells to apoptosis or necrosis (Akbarian et al., 2016; Altan et al., 2010). It has been hypothesized that elevated body temperature increases RS and there is increased oxidative stress damage during high environmental temperatures (Zaboli et al., 2018). Damage caused by these radicals can be minimized by the antioxidants such as SOD, catalase, and GPx (Altan et al., 2010). During HS, excess RS decreases nutrient digestibility as well (Payne et al., 2005). In an experiment comparing the oxidative stress in rats fed with either NP or LP diets, there was an increase of lipid peroxidation in the intestines of rats fed with the LP diet (Darmon et al., 1993). There is little information on how LP diets alter antioxidant activity during HS in broilers.

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CHAPTER 3

GUT AND SKELETAL MUSCLE REGULATE THE FEED INTAKE AND THEMRMOGENESIS OF BROILER'S DURING EXPERIMENTALLY INDUCED HEAT STRESS

Abstract

The objective of this study was to assess the effects of low protein diets on feed intake, heat production and markers of feed intake and thermogenesis regulation in gut and muscle in broilers under experimentally induced heat stress. Two-hundred-day-old male broiler chicks were weight-matched and assigned into 36 pens with 5-6 chicks/pen followed by randomly subjecting pens into two treatments (18 pens/treatment) including: 1) thermoneutral (TN), 2) heat stress (HS; 35^{o}C for 7 hours/day). Within each treatment, the pens were randomized to receive two diets (9 pens/diet; 50 birds/diet): 1) normal protein (NP), 2) low protein (LP). The study lasted 6 weeks with 2 weeks of adaptation (starter phase) and 4 weeks of data collection (grower and finisher phases). Feed intake and body weight were measured daily and weekly, respectively. On week 6, birds were euthanized, and blood, duodenum, ileum, and leg muscle samples were collected. A subgroup of birds was euthanized for assessing the breast meat quality and body composition using a dual-energy X-ray absorptiometry. All data were analyzed with either general linear model or mixed procedure (SPSS®). Means within each treatment between NP and LP diets were separated by *Student's t-test*. There was no difference in final body weight and average daily gain between NP and LP diets in TN group, but LP significantly decreased these parameters during HS. In TN condition, LP tended to increase average daily feed intake (ADFI) compared to NP while during HS, LP was not different from NP in terms of ADFI. In TN condition, LP had a significantly higher thermal radiation compared to NP, but during HS the LP had a lower thermal radiation compared to NP. In support of ADFI data, in TN condition, LP had greater mRNA abundance of ghrelin in duodenum compared to NP, but during HS, LP tended to decrease the plasma ghrelin concentration compared to NP. Unlike TN condition, LP had a significantly lower muscle sirtuin and cytochrome c oxidase (COX IV) mRNA abundance than NP during HS. Relative to NP, the LP diet decreased the body lean mass, but increased the fat mass during both TN and HS. The breast meat of chickens fed with LP diet had higher indices of redness, chroma and

lightness during both TN and HS and had lower pH than those fed with NP during TN, but not during HS. Our data provide evidence that low protein diets mitigate the negative outcome of heat stress by reducing feed intake and heat production, which are regulated through factors expressed in the gut and skeletal muscle.

Introduction

The rate of increase in greenhouse gases production has been accelerated in last decade resulting an increased environmental temperature (Lindsey, 2019). The heat stress (HS) due to environmental factors is a pressing challenge for livestock production and when experienced for long periods of time, that affects immunity, metabolism, and gut microbiota composition and in severe cases leads to death (Nawab et al., 2018). In 2013 only, poultry industry lost $125-165 million due to heat-related death (Lara et al., 2013). During HS, the body applies a homeostatic effort to enhance the survivability by reducing the body temperature via decreasing feed intake and thermogenesis (Awad et al., 2017; Swennen et al., 2007; Richards et al., 2003; Nawab 2018; Lara et al., 2013). To improve the survivability of birds during HS, various dietary strategies such as supplementation of fats, vitamins, minerals, and probiotics have been proposed (Nawab et al., 2018). However, none of these strategies have been fully effective to mitigate the negative outcomes of HS on growth performance of birds (Daghir et al., 2009). Therefore, more effective dietary approaches should be considered for birds under HS.

Low protein (LP) diets balanced with limiting essential amino acids have been suggested as promising dietary solutions to reduce the HS and mortality in birds (Daghir

et al., 2009; Zulkifi et al., 2017; Waldroup 1982). Compared to carbohydrates and lipids, proteins have a high heat increment; hence offering LP diets supplemented with limiting amino acids may decrease the metabolic heat production (Awad et al., 2017; Swennen et al., 2007; Syafwan et al., 2011; Musharaf et al., 1999). LP diets have been shown to reduce feed intake (FI), heat production and mortality of broilers under HS (Ghasemi et al., 2020; Zulkifli et al., 2018; Suganya et al., 2015; Aftab et al., 2006; Furlan et al., 2004; Song, et al., 2012); however, high protein diets fed to broilers under HS conditions increase the heat production (Daghir et al., 2009; Smith et al., 1998). Reduced FI in response to LP diets during HS may also decrease the thermogenesis and body core temperature. Unlike HS condition, LP induced hyperphagia and thermogenesis have been reported for birds under TN conditions (Soares et al., 2019; Davidson, 1964; Buyse et al.,1992; Marayat, 2018). The hyperphagic response to LP diets in chickens has been explained as an effort to meet the protein needs, which can instead result in increased energy expenditure (Buyse et al., 1992). Therefore, it appears that LP diets induce differential effects on FI and heat production under TN or HS situations. Less is understood on the mechanisms by which LP diets regulate FI and thermogenesis during TN and HS conditions.

Changes in FI of birds fed with LP diets have been related with amino acid and micronutrient imbalances (Syawan et al., 2011), net and metabolic energy ratio (Aftab et al., 2006) and changes in circulating glucocorticoids concentrations such as corticosterone (Liu et al., 2012; Richards et al., 2010). While the role of gut peptides in regulation of feed intake in poultry is well known (Murphey et al., 2004; Richards et al., 2003; Richards et

al., 2010), the relative importance of these peptides in FI changes is little understood when LP diets are offered to birds under HS. Dietary protein likely induce GLP-1 secretion from ileal L cells in chickens via essential amino acids such as lysine and methionine (Monir et al., 2014; Nishimura et al., 2015). Therefore, LP induced increased FI is possibly due to reduced secretion of anorexigenic gut peptides like GLP-1. In one study, feeding broilers with LP diets under TN reduced the immunoreactive GLP-1 cells in the ileum (Monir et al., 2014). The effect of LP diets on the gene expression of gut peptides during HS is not known.

Changes in thermogenesis of birds fed with LP diets have been associated with alterations in concentration of hormones such as triiodothyronine (Awad et al., 2017), amino acids (Aftab et al., 2006) and energy metabolism (Furlan et al., 2004). Although the role of Beta-1 adrenergic receptors (β1-AR), and downstream mitochondrial biogenesis markers, sirtuin, peroxisome proliferator-activated receptor- gamma coactivator (PGC-1α), AMP-activated protein kinase, (AMPKα1), and cytochrome c oxidase subunit 4 (Cox IV) in non-shivering thermogenesis in mammals is well understood (Joubert et al., 2010), their importance in regulation of thermogenesis in birds fed with LP diets under HS is overlooked. LP diets have been shown to increase the expression of Avian uncoupling protein (AvUCP) and adenine nucleotide translocase in skeletal muscle of boilers under TN conditions (Li et al., 2013; Marayat et al., 2020). These studies suggest that LP induced thermogenesis is controlled by AvUCP in birds under TN. The effect of LP diets on muscle thermogenesis markers during HS is not known.

The objective of this study was to assess the effects of LP diets on FI, thermal radiation, markers of FI and thermogenesis regulation in gut and skeletal muscle, growth performance, body composition, and meat quality in broilers under experimentally induced HS.

Materials and Methods

Animals and Housing

All the experimental procedures performed in this study were approved by Oklahoma State University's Institutional Animal Care and Use Committee (Animal Care and Use Protocol # AG-19-5). A total of 200-day-old male broiler Cobb x Cobb chicks were obtained from Cobb commercial hatchery (Siloam Springs, Arkansas) and housed in a building with concrete floor pens covered with wood shavings, single hole stainless steel feeders, and nipple drinkers. The building was equipped with central heating, cooling, and ventilation systems. Ambient temperature and lighting program was according to breeding company recommendations (Cobb-Vantress, 2015). Briefly, room temperature was set at 32 ^{o}C, 29 ^{o}C, 27 ^{o}C, 25 ^{o}C, 22 ^{o}C and 20 ^{o}C for days 0-7, 8-14, 15-21, 22-28, 29-35 and 36-42, respectively. The light:dark periods were 23:1, 16:8, 17:7, 18:6, 19:5, 20:4, 21:2, 22:2 and 23:1 days 0-5, 6-22, 23, 24-36, 37, 38, 39, 40 and 41-42, respectively.

Diets, Experimental Design and Heat Stress Protocol

Upon arrival, chicks were randomly assigned into 36 pens with an average of 5 to 6 chicks per pen. After 2 weeks of acclimation period (*i.e.* starter phase), birds were weight-matched (472.2 ± 39.0 g) and subjected into four groups (9 pens/group; 50 birds/diet/group) for 4 weeks including: normal protein diet under TN (NPTN), LP diet under TN (LPTN), normal protein diet under HS (NPHS) and LP diet under HS (LPHS). The starter, grower, and finisher phase diets (2 weeks each) were prepared as recommended by Nutritional Requirements of Poultry by the National Research Council (NRC, 1994). Ingredients and composition of the diets are shown in Table 1. The crude protein (CP) level of LP diets was obtained by reducing the soybean meal and these diets were supplemented with limiting amino acids (*i.e.* lysine, methionine, threonine, tryptophan, isoleucine, valine and glycine) equal to the normal protein diets. Normal protein and LP diets were isocaloric, which was achieved by adjusting the corn levels after dropping the soybean for LP diets. Birds were fed once a day at 1800 and had *ad libitum* access to both feed and water throughout the study. Birds in the HS treatment experienced a cyclic HS at 35ºC for 7 hours each day as reported by others (Sohail et al., 2010) from 1100 until 1800. Broilers assigned to TN treatment were housed under ambient temperature according to breeding company recommendations as described earlier.

Table 1. Ingredients and chemical composition of experimental diets[1] (As-fed basis)

		Grower[3]		Finisher[4]	
Ingredients[5], %	**Starter[2]**	**NP**	**LP**	**NP**	**LP**
Corn, yellow dent	60.58	65.34	79.59	69.99	83.81
Soybean meal, 47.5% CP	33.05	28.94	13.26	25.04	9.58
Dicalcium phosphate 18.5%	2.09	1.71	2.12	1.50	1.98
Limestone	1.18	1.09	1.07	0.95	0.95
Salt	0.44	0.33	0.32	0.25	0.25
Choline Chloride 60%	0.12	0.08	0.08	0.03	0.03
Minerals and vitamins premix[6]	0.40	0.40	0.40	0.37	0.40
Magnesium oxide	0.83	0.72	0.70	0.75	0.75
L-Lysine HCl	0.19	0.17	0.64	0.10	0.57
DL-Methionine	0.23	0.12	0.19	0.08	0.14
L-Threonine	0.17	0.16	0.37	0.15	0.36
L-Tryptophan	-	-	0.09	-	0.10
L-Isoleucine	0.07	0.06	0.05	0.05	0.06
L-Valine	0.10	0.09	0.11	0.05	0.12
Glycine	0.55	0.49	0.72	0.37	0.60
Chromium oxide	-	0.30	0.30	0.30	0.31
Calculated Chemical Composition					
Dry matter, %	88.98	88.93	88.70	88.97	88.73
ME, kcal/kg	3256	3270	3274	3284	3285
Crude protein, %	21.80	20.09	14.75	18.39	13.17
Crude fiber, %	2.52	2.49	2.28	2.46	2.25
Crude fat, %	3.35	3.42	3.50	3.48	3.56
L-Lysine, %	1.11	1.00	0.99	0.85	0.85
L-Threonine, %	0.80	0.73	0.73	0.68	0.68
DL-Methionine, %	0.50	0.38	0.38	0.33	0.32
L-Tryptophan, %	0.23	0.21	0.21	0.19	0.19
Glycine, %	1.25	1.14	1.14	0.97	0.97
L-Isoleucine, %	0.80	0.73	0.47	0.66	0.42

L-Valine, %	0.89	0.83	0.60	0.73	0.55
L-Leucine, %	1.50	1.42	1.06	1.34	0.99
L-Histidine, %	0.65	0.59	0.37	0.54	0.32
L-Arginine, %	1.21	1.09	0.66	0.99	0.56
L-Phenylalanine, %	0.87	0.80	0.53	0.74	0.47
Calcium, %	1.04	0.90	0.93	0.80	0.85
Phosphorus, %	0.79	0.70	0.71	0.65	0.67
Analyzed Chemical Composition					
Dry matter, %	87.00	87.60	87.20	86.80	87.10
Crude protein, %	18.50	19.40	14.10	17.20	12.70
Crude fat, %	2.40	2.30	2.50	2.50	2.60
Calcium, %	1.33	1.34	1.07	1.08	1.15
Phosphorus, %	0.83	0.73	0.78	0.69	0.67

[1] NP: normal protein diet (NRC, 1994); LP: low protein diet

[2] Diets were provided from day 1 to 13

[3] Diets were provided from day 14 to 27

[4] Diets were provided from day 28 to 42

[5] Corn, soybean meal, dicalcium phosphate, limestone, salt, choline chloride, glycine, lysine, DL-methionine, and magnesium oxide were obtained from Nutra Blend, LLC (Neosho, MO). L-threonine (98.5%) and L-tryptophan (98%) were obtained from Ajinomoto (Overland Park, KS). L-isoleucine (98.5%) and L-valine (96.5%) were obtained from Ajinomoto Health & Nutrition North America, Inc. (Raleigh, NC). Chromium oxide was purchased from Fisher Scientific (Bartlesville, OK).

[6] Minerals and vitamins premix was obtained from Nutra Blend, LLC (Neosho, MO). The premix provided per kg of mix (MIN): manganese, 4.0%; zinc, 4.0%; iron, 2.0%; copper, 4,500 ppm; iodine, 600 ppm; selenium, 60 ppm; vitamin A, 3,086,474.19 IU; vitamin D_3, 1,102,317.07 ICU; vitamin E, 6,613.9 IU; vitamin B12, 4.41 mg; menadione, 330.70 mg; riboflavin, 2,645.55 mg; D-pantothenic acid, 2,645.54 mg; niacin, 11,023.12 mg; vitamin B6, 551.16 mg; folic acid, 275.58 mg; choline, 154,323.71 mg; biotin, 13.23 mg

Heat Stress Coping Mechanisms and Physiological Adaptations Measurements

During daily HS period, *i.e.* 1100-1800, heat stress coping mechanisms and physiological adaptations measurements of birds were recorded at three separate phases: 1100-1300, 1400-1600, and 1600-1800. Heat stress coping mechanisms observations included the percent of birds eating, drinking, wing spreading, and panting in each pen. Physiological observations recorded were respiratory rate (breaths /minute) and rectal temperature of randomly selected broilers in each pen. During above observations, pen temperature and room temperature/humidity were recorded using humidity/temperature chart recorder data logger and a digital weather station, respectively. Data logger was placed at pen level to obtain accurate temperature of the pen.

Feed Intake and Growth Performance

Feed intake and water intake were recorded daily at 1800. Pen body weight was measured weekly and divided by the number of birds in each pen. Based on the above records, average daily gain (ADG), average daily feed intake (ADFI), average daily water intake (ADWI), gain to feed (G:F), and water to feed (W:F) were calculated. Further, based on analyzed concentration of CP% in diets, the average daily protein intake (ADPI) and gain to protein (G:P) were calculated.

Thermal Images

Thermal images were captured from one randomly selected bird in each pen during HS period, *i.e.* 1100-1800 using FLIR C2 compact thermal camera with focal length of 1.54 mm and thermal accuracy of ±2°C (FLIR Systems, Boston, MA, USA). Emissivity coefficient was set at 0.86. Camera was positioned roughly 1 m above of each pen and each image included at least one bird positioned in the center of the image.

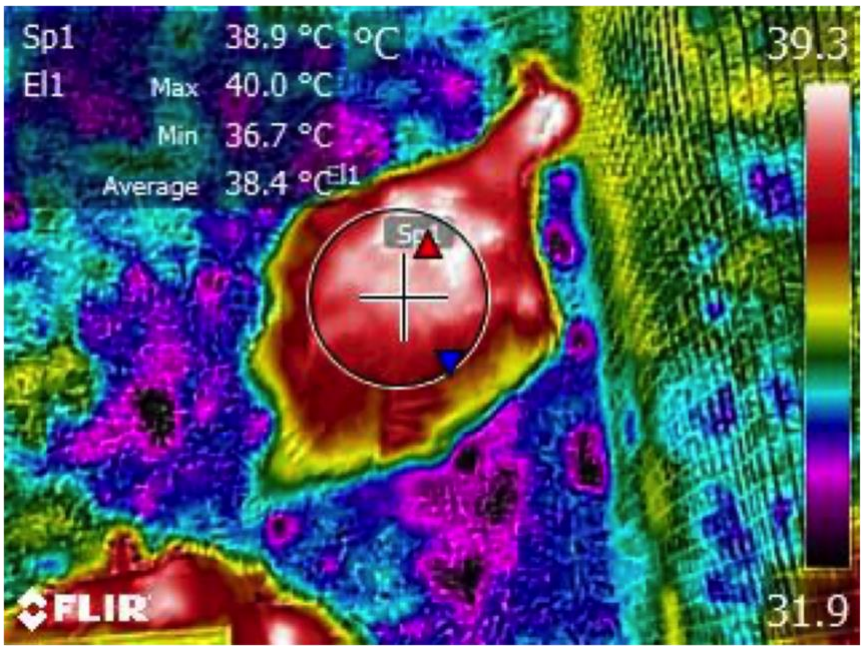

Figure 1. A representative screenshot of a thermal image from FLIR Research Studio.
The region of interest was determined by drawing a circle in the back of birds. The mean temperature data from the region of interest were extracted from birds fed with normal protein and low protein diets at different time points during thermoneutral and heat stress periods and used for data analysis. FLIR Research Studio (version 5.13.18031.2002).

Feed Samples Collection

Feed samples were collected during mixing diets. A feed sample (~50 g) was collected from each feedbag, pooled for each diet phase and subsamples for composition analysis. Feed samples were stored at −20°C until proximate analysis for feed composition.

Blood and Tissue Samples Collection

At the end of the finisher phase, all chickens were euthanized via CO_2 asphyxiation. Tissue samples including pectoralis major, duodenum, ileum, and cecal content were collected. All tissues and cecal contents collected were wrapped in foil, snap-frozen in liquid nitrogen and stored at −80 °C until further analysis. Blood samples were collected from 1 randomly selected bird/ pen (i.e. 8-9 chickens from each treatment). Blood samples were collected in a pre-labeled 3 mL EDTA coated blood collection tubes (BD Vacutainer®, Franklin Lakes, NJ) from the jugular anterior vena cava after euthanasia. Blood tubes were immediately placed on ice after collection, transferred to lab, centrifuged at 2,000 x g for 10 minutes at 4 °C and plasma was separated. The collected plasma was stored at −80 °C until further analysis.

Meat Quality

At the end of study, 10 birds from each treatment were randomly selected from all pens and used for meat quality analysis. Immediately after euthanasia, skin over the breast were exposed and placed on ice for an hour. Then, breast muscle was removed, and its pH, color, and lipid oxidation were measured.

The pH was measured in three regions of breast muscle including cranial, middle, and caudal, using the Hanna Portable Waterproof Meat pH Meter (HI 99163, Smithfield, RI). The pH meter was calibrated with standard buffer solutions at pH of 4 and 7 prior to use. The breast muscle was stored at 4 ^{o}C and its pH was measured again after 24 hours.

Meat color was measured using HunterLab Miniscan EX (MSEZ2514, Reston, VA). Before measuring, miniscan was standardized by correlated tiles of black glass and white tile. The color was read three times from cranial, middle, and caudal end of the breast muscle. Muscle then was stored at 4^{o}C and the procedure was repeated 24 hours later.

Lipid oxidation was measured by blending 3 grams of breast tissue with DI water and trichloroacetic acid. After filtration of the breast tissue, the filtrate was mixed with thiobarbituric acid and DI water and placed in a 100 ^{o}C water bath for 10 minutes. After being cooled for 5 minutes, samples were scanned using a spectrophotometer at 532 nm wavelength. Trichloroacetic acid and thiobarbituric acid were combined and used as blank.

Body Composition Analysis

At the end of the study, 8 broilers from each treatment (1 randomly selected bird per pen), were used for whole-body composition analysis. Following euthanasia, birds were scanned by dual-energy X-ray absorptiometry (DEXA) (Hologic, Discovery QDR series, Bedford, MA) to determine the bone mineral density, bone mineral content, lean mass and fat mass following published protocols (Shili et al., 2020; Fetterer et al., 2013).

Radiative Heat Transfer Analysis

Thermal images were analyzed using the FLIR Research Studio software (FLIR Systems, Boston, MA, USA). To obtain the dorsal surface body mean temperature, a region of interest was drawn in circle shape in the back of bird (Figure 1). Radiative heat transfer of the birds (W/m^2) was calculated using the following equation: $\sigma\varepsilon(T_s^4 - T_a^4)$ (Ferreira et al., 2011; Bakken et al., 1975), where σ is Stefan-Boltzmann constant (5.67×10^{-8} W/m^2K^4, W being defined as watts which is rate of energy transfer, m which is meters, and K which is defined as kelvin which is the base unit of temperature), ε is thermodynamic emissivity (0.86), T_s is body surface temperature (kelvin) and T_α is ambient temperature (kelvin).

Diet Composition Analysis

Diet composition analyses were performed by ServiTech (Dodge City, KS) for dry matter, CP, crude fat, crude fiber, calcium, and phosphorus as we previously described (Spring et al., 2020; Spring et al., 2020; Shili et al., 2020).

Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

RNA isolation and RT-qPCR was performed for cholecystokinin (CCK), ghrelin, gastric inhibitory polypeptide (GIP), and secretin in duodenum, peptide YY (PYY) in ileum, and β-1 adrenergic receptor (β1AR), AMP-activated protein kinase alpha (AMPKα), sirtuin, cytochrome c oxidase subunit 4 (COX IV) and peroxisome proliferator-activated receptor-gamma coactivator alpha (PGC1-α) in breast muscle following our published procedures (Pezeshki et al., 2012; Shili et al., 2021). After isolation, RNA concentration and ratio of absorbance at 260 and 280 (260:280) were measured by Nanodrop spectrophotometer (Nanodrop® Technologies, Wilmington, DE) and only samples with a 260:280 ratios of 1.9-2.1 nm were used for analysis. Complementary DNA was synthesized in a T100™ Thermal Cycler (Bio-Rad, CA, USA). The sequences for primers were obtained from other publications (Aoki et al., 2016; Han et al., 2015; Joubert et al., 2010; Kolodziejski et al., 2018; Shimao et al., 2019; Song et al., 2012; Wang et al., 2012; Xu et al., 2011) and cross-checked using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-

blast/). Using a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) the cycle threshold (Ct) values for target genes and β-actin, as a reference gene, was obtained and melt curve analysis was performed as we previously described (Pezeshki et al., 2012; Shili et al., 2021). The relative abundance of target genes was determined using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001; Schmittgen et al., 2008).

Statistical Analysis

Growth performance initial BW (body weight), final BW, ADFI, ADG, ADPI, ADWI, G:F, G:P, W:F), HS coping mechanisms observation (eating, drinking, panting, wing spread, respiratory rate, and rectal temperature), DEXA (BMC, BMC, BMD, lean mass, and fat mass), meat quality (redness, chroma, yellowness, darkness and pH) and qPCR data were analyzed using GLM procedure (IBM SPSS Statistics, Version 23, Armonk, NY, USA) with diet, temperature, and interaction of diet and temperature in the model. Phase (grower and finisher) related growth performance, meat quality, coping mechanisms, and radiative heat transfer data were analyzed using Mixed analysis procedure (IBM SPSS Statistics, Version 23, Armonk, NY, USA). Diet, temperature, phase, interaction of diet and temperature, diet and time, temperature and time, and diet, temperature and time were included in the model as fixed effects. The chicken was the random variable. Based on the smallest values of fit statistics for corrected Akaike's Information Criterion and Bayesian Information Criterion, the covariance structure of the repeated measurements for each

variable was modeled as either first-order antedependence, autoregressive, heterogenous autoregressive, compound symmetry, heterogenous compound symmetry or toeplitz. The paired ttest was used for comparing diet treatments within TN and HS groups. Differences were considered significant at $P \leq 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

Results

Heat Stress Coping Mechanisms Adaptations

The effect of temperature on eating, panting, wing spread, respiratory rate and rectal temperature was significant (Table 2). The eating was decreased (3.3 vs 9.6%, respectively), but panting, wing spread, respiratory rate and rectal temperature were increased during HS (88.0 vs. 2.9%, 27.9 vs. 0%, 199.7 vs. 66.1 breath/min and 42.2 vs 40.4^{o}C, respectively). The effect of diet on drinking was significant with LP having a lower drinking (%) than NP (6.1 and 8.1%, respectively). Compared to NPTN, LPTN reduced drinking by ~32%, increased panting by ~94% and tended to increase the respiratory rate by ~3%. Compared to NPHS, LPHS reduced drinking by ~16%. Although NPTN and LPHS were not compared statistically, numerically when compared to NPTN, LPHS showed an increased panting, wing spread, respiratory rate and rectal temperature (86.5 vs 1.8%, 28.3 vs 0.0%, 199.8 vs 65.2 breath/min, and 42.2 vs 40.3 ^{o}C, respectively) but a decrease in eating and drinking percent (3.6 vs. 9.0% and 6.8 vs 8.2%, respectively).

Table 2. Heat stress coping mechanisms of broilers fed with low protein diets during experimentally induced heat stress

Items	Treatments[1]				SEM[2]	*P*-Values		
	NPTN[2]	LPTN[2]	NPHS[2]	LPHS[2]		Diet	Temp[2]	Diet × Temp
Eating (%)	9.0	10.2	3.0	3.6	0.6	0.13	≤ 0.01	0.64
Drinking (%)	8.2	5.6[*]	8.1	6.8[*]	0.3	≤ 0.01	0.17	0.14
Panting (%)	1.8	3.5[*]	87.2	86.5	7.3	0.61	≤ 0.01	0.24
Wing spread (%)	0.0	0.0	27.4	28.3	2.5	0.72	≤ 0.01	0.72
Respiratory rate (breath/min)	65.2	67.0[#]	199.5	199.8	11.6	0.34	≤ 0.01	0.52
Rectal temperature (°C)	40.3	40.4	42.0	42.2	0.2	0.28	≤ 0.01	0.79

[1]The values are the mean; n=9 pens (5-6 birds/pen).

[2]NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress; SEM: standard error of means; Temp: temperature.

[*]Within rows, NPTN vs LPTN and NPHS vs LPHS $P \leq 0.05$

[#] Within rows, NPTN vs LPTN and NPHS vs LPHS $0.05 < P \leq 0.10$

When data was analyzed for each phase (i.e. grower and finisher phase; Supplementary Figure 1A-L), LPTN increased respiratory rate and panting (%) in the grower phase compared to NPTN (~3.8% and 85.3%, respectively). Compared to NPTN, LPTN increased eating (%) and panting (%) in the finisher phase (~25.1% and 103.4%, respectively). Compared to NPHS, LPHS decreased drinking (%) in the grower phase (~32.1%). Relative to NPHS, LPHS decreased rectal temperature, respiratory rate and drinking (%) in the finisher phase (~1.0%, 0.4% and 24.2%, respectively). Although NPTN and LPHS were not compared statistically, numerically when compared to NPTN in the grower phase, LPHS showed an increased panting, wing spread, respiratory rate and rectal temperature (~98%, 100%, 66% and 3%, respectively) but a decrease in eating and drinking percent (~66% and 36%, respectively). When compared to NPTN numerically in the finisher phase, LPHS showed an increased panting, wing spread, respiratory rate and rectal temperature (~98%, 100%, 69% and 6%, respectively) but a decrease in eating and drinking percent (~61% and 23%, respectively).

Growth Performance

Initial BW and final BW were not different among dietary groups (Table 3). The effect of diet on ADG, ADPI, ADWI and W:F ratio was significant with having lower value for all of them for LP compared to NP (60 vs. 66 g/d, 24 vs. 30 g/d, 269 vs. 346 mL/d, 1.8 vs. 2.1

g/g for LP vs. NP). The effect of temperature on ADG, ADFI, ADPI, ADWI, G:P ratio and W:F ratio was significant with having lower values for all of them for HS compared to TN, except ADWI, G:P ratio and W:F ratio (49 vs 76 g/d, 149 vs 175 g/d, 21 vs 31 g/d, 335 vs 280 mL/d, 4.2 vs 2.8 g/g and 2.3 vs 1.6 mL/g, respectively). The effect of diet × temperature on ADFI, ADPI, G:P and W: F was significant. Although no differences on ADFI were detected between NPTN and LPTN groups, LPHS tended to reduce the ADFI compared to NPHS. Compared to NPTN, LPTN reduced ADPI, ADWI, G:F and W:F ratio but increased G:P ratio (~25%, 24%, 20%, 32% and 20%, respectively). Compared to NPHS, LPHS decreased ADG and ADWI (~19% and 20%) and tended to decrease the ADFI and ADPI (~11% and 17%, respectively). Although NPTN and LPHS were not compared statistically, numerically when compared to NPTN, LPHS showed an increased initial BW, G:P, and W:F (~17%, 60% and 16%, respectively) but a decrease in final BW, ADG, ADFI, ADPI, ADWI, G:F (~31%, 44%, 17%, 44%, 7% and 40%, respectively).

Table 3. Overall growth performance of broilers fed with low protein diets during experimentally induced heat stress

Items	Treatments[1]				SEM[2]	*P*-Values		
	NPTN[2]	LPTN[2]	NPHS[2]	LPHS[2]		Diet	Temp[2]	Diet × Temp
Initial BW[3], g	471	516	548	553	10	0.14	0.38	0.21
Final BW[3], g	2737	2688	2185	1901	65	0.21	0.22	0.13
ADG[3], g/d	78	74	54	44[*]	3	≤ 0.01	≤ 0.01	0.11
ADFI[3], g/d	169	181	158	140[#]	4	0.63	≤ 0.01	0.03
ADPI[3], g/d	36	27[*]	24	20[#]	1	≤ 0.01	≤ 0.01	0.02
ADWI[3], mL/d	319	241[*]	373	298[*]	9	≤ 0.01	≤ 0.01	0.76
G:F[3], g/g	0.5	0.4[*]	0.3	0.3	0.01	0.11	0.06	0.47
G:P[3], g/g	2.5	3.0[*]	4.3	4.0	0.19	0.51	0.03	≤ 0.01
W:F[3], mL/g	1.9	1.3[*]	2.4	2.2	0.08	≤ 0.01	≤ 0.01	0.05

[1]The values are the mean; n=9 pens (5-6 birds/pen).

[2] NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress; SEM: standard error of means; Temp: temperature

[3] BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; ADPI: average daily protein intake; ADWI: average daily water intake; G:F: gain: feed; G:P: gain: protein; W:F: water: feed

[*] Within rows, NPTN vs LPTN and NPHS vs LPHS $P \leq 0.05$

[#] Within rows, NPTN vs LPTN and NPHS vs LPHS $0.05 < P \leq 0.10$

When data were analyzed separately for each phase (grower and finisher) LPTN reduced ADPI, ADWI and W:F ratio, but increased G:P ratio compared to NPTN in grower phase (~22%, 20%, 22% and 32%, respectively; Table 4). Relative to NPHS, LPHS decreased ADG, ADFI, ADPI and ADWI but increased G:P ratio in grower phase (~31%, 19%, 41%, 13% and 25%, respectively). In finisher phase, compared to NPTN, LPTN reduced ADPI, ADWI, W:F ratio, tended to reduce G:F ratio but increased G:P ratio (~24%, 27%, 30%, 9% and 23%, respectively). Relative to NPHS, LPHS tended to decrease ADG and decreased ADPI, ADWI and W:F but increased G:P ratio (~12%, 33%, 22%, 15% and 28%, respectively). Although NPTN and LPHS were not compared statistically, numerically when compared to NPTN in the grower phase, LPHS showed an increased G:P and W:F (~14% and 11%, respectively) but a decrease in ADG, ADFI, ADPI, and ADWI, (~46%, 17%, 40% and 7%, respectively). When compared to NPTN numerically in the finisher phase, LPHS showed an increased W:F (~10%) but a decrease in ADG, ADFI, ADPI, ADWI, G:F and G:P(~47%, 27%, 46%, 17%, 25% and 12%, respectively).

Table 4. Phase growth performance of broilers fed with low protein diets during experimentally induced heat stress

Items	Treatments[1]							
	NPTN[2]	LPTN[2]	NPHS[2]	LPHS[2]	SEM[2]	*P*-Values		
						Diet	Temp[2]	Diet × Temp
ADG[3], g/d								
Grower	68.0	67.3	53.3	36.9*	1.8	≤ 0.01	≤ 0.01	≤ 0.01
Finisher	84.5	78.7	50.8	44.7#	3.7	0.03	≤ 0.01	0.96
ADFI[3], g/d								
Grower	145.7	156.7	149.7	120.6*	4.4	0.25	0.05	≤ 0.01
Finisher	198.0	202.8	160.7	144.8	5.5	0.46	≤ 0.01	0.18
ADPI[3], g/d								
Grower	28.3	22.1*	29.0	17.0*	1.0	≤ 0.01	0.09	0.02
Finisher	34.1	25.8*	27.6	18.4*	1.1	≤ 0.01	≤ 0.01	0.68
ADWI[3], mL/d								
Grower	256.0	203.8*	272.8	238.4*	4.8	≤ 0.01	≤ 0.01	0.03
Finisher	382.8	278.2*	408.2	319.2*	9.6	≤ 0.01	≤ 0.01	0.33
G:F[3], g/g								
Grower	0.4	0.4	0.4	0.4	0.01	0.28	0.03	0.79
Finisher	0.4	0.4#	0.3	0.3	0.01	0.04	≤ 0.01	0.19
G:P[3], g/g								
Grower	2.2	2.9*	2.0	2.5*	0.09	≤ 0.01	0.03	0.55
Finisher	2.6	3.2*	1.8	2.3*	0.10	≤ 0.01	≤ 0.01	0.84
W:F[3], mL/g								
Grower	1.8	1.4*	1.9	2.0	0.06	0.23	≤ 0.01	≤ 0.01
Finisher	2.0	1.4*	2.6	2.2*	0.09	≤ 0.01	≤ 0.01	0.19

[1] The values are the mean; n=9 pens (5-6 birds/pen).

[2] NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress; SEM: standard error of means; Temp: temperature.

[3] ADG: average daily gain; ADFI: average daily feed intake; ADPI: average daily protein intake; ADWI: average daily water intake; G:F: gain: feed; G:P: gain: protein; W:F: water: feed. The *P*-values for the overall model effect for diet, temp, phase, diet × temp, diet × phase, temp × phase and diet × temp × phase for ADG were 0.59, 0.62, 0.05, 0.03, 0.58, 0.84, 0.17, for ADFI were 0.26, ≤ 0.01, ≤ 0.01, 0.02, 0.68, ≤ 0.01, 0.26, for ADPI were ≤ 0.01, ≤ 0.01, ≤

0.01, 0.11, 0.79, ≤ 0.01, 0.05, for ADWI were ≤ 0.01, ≤ 0.01, ≤ 0.01, 0.11, ≤ 0.01, 0.31, 0.89, for G:F were 0.08, ≤ 0.01, ≤ 0.01, 0.71, 0.92, ≤ 0.01, 0.33 for G:P were ≤ 0.01, ≤ 0.01, 0.66, 0.57, 0.70, ≤ 0.01, 0.64 for W:F were ≤ 0.01, ≤ 0.01, ≤ 0.01, ≤ 0.01, ≤ 0.01, ≤ 0.01 and 0.06.

[*] Within rows, NPTN vs LPTN and NPHS vs LPHS $P \leq 0.05$

[#] Within rows, NPTN vs LPTN and NPHS vs LPHS $0.05 < P \leq 0.10$

Body Composition Analysis

The effect of diet on lean mass, lean percent, fat mass and fat percent were significant (Table 5). Compared to NP, LP fed birds had lower lean mass and lean percent and had higher fat mass and fat percent (2247.7 vs. 2505.2g, 90.1 vs. 94.3%, 212.5 vs. 122.2g, 8.3 vs. 4.5%, respectively). The effect of temperature on BMC, BMC percent, BMD, lean mass, lean percent, fat mass, and fat percent was significant (Table 5). Compared to TN, birds under HS had lower BMC, BMC percent, BMD, lean mass, fat mass, and fat percent (25.1 vs 34.8g, 1.1 vs 1.2%, 0.14 vs 0.15 g/cm^2, 2123.0 vs 2629.9g, 126.08 vs 208.7g, 5.6 vs 7.2%, respectively. BMC, BMC percent and BMD were not different among dietary groups (Table 5). Compared to NPTN, LPTN reduced lean mass and lean percent, but increased fat mass and fat percent (~8%, 7%, 110% and 123%, respectively). Compared to NPHS, LPHS decreased the lean mass and lean percent but increased fat mass and fat percent (~12%, 2%, 30%, 45%, respectively). The effect of diet × temperature on lean percent, fat mass and fat percent were significant, which is due to more significant effect of diet on changing these parameters under TN than HS. Although NPTN and LPHS were not compared statistically, numerically when compared to NPTN, LPHS showed an increased fat mass and fat percent (~6% and 49%, respectively) but a decrease in BMC, BMC percent, BMD, lean mass and lean percent (~32%, 5%, 7%, 28% and 2%, respectively).

Table 5. Bone and body characteristics of broilers fed with low protein diets during experimentally induced heat stress

Items	Treatments[1]				SEM[2]	*P*-Values		
	NPTN[2]	LPTN[2]	NPHS[2]	LPHS[2]		Diet	Temp[2]	Diet × Temp
BMC[3] (g)	35.40	34.14	26.09	24.04	1.10	0.21	≤ 0.01	0.76
BMC (%)	1.18	1.24	1.09	1.12	0.02	0.27	0.02	0.65
BMD[3] (g/cm^2)	0.15	0.15	0.14	0.14	0.002	0.97	0.04	0.17
Lean mass (g)	2747.85	2526.96*	2262.61	1983.51*	64.47	≤ 0.01	≤ 0.01	0.76
Lean (%)	94.40	88.00*	94.33	92.25*	0.65	≤ 0.01	≤ 0.01	≤ 0.01
Fat mass (g)	134.79	282.53*	109.64	142.51*	14.72	≤ 0.01	≤ 0.01	≤ 0.01
Fat (%)	4.46	9.96*	4.57	6.64*	0.64	≤ 0.01	≤ 0.01	≤ 0.01

[1] The values are the mean; n=9 pens (5-6 birds/pen).

[2] NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress; SEM: standard error of means; Temp: temperature.

[3] BMC: Bone mineral content; BMD: Bone mineral density

*Within rows, NPTN vs LPTN and NPHS vs LPHS $P \leq 0.05$

Within rows, NPTN vs LPTN and NPHS vs LPHS $0.05 < P \leq 0.10$

Radiative Heat Transfer

The overall effect of temperature and diet × temperature on the thermal radiation was significant (Figure 2). Birds under HS had lower thermal radiation than those kept under TN (14.6 vs 39.1 W/m^2, respectively). While LPTN had higher thermal radiation than NPTN (40.6 vs 37.5 W/m^2, respectively), LPHS had lower thermal radiation than NPHS (13.0 vs 16.1 W/m^2, respectively) (Figure 2A and 2B). LPTN had higher thermal radiation on days 23, 25, and 27 (~30%, 14% and 6%, respectively; Figure 2A). Compared to NPHS (Figure 2B), LPHS had lower thermal radiation on days 21, 22, 23, 24, 25, and 27 (~52%, 32%, 15%, 18%, 21% and 23%, respectively).

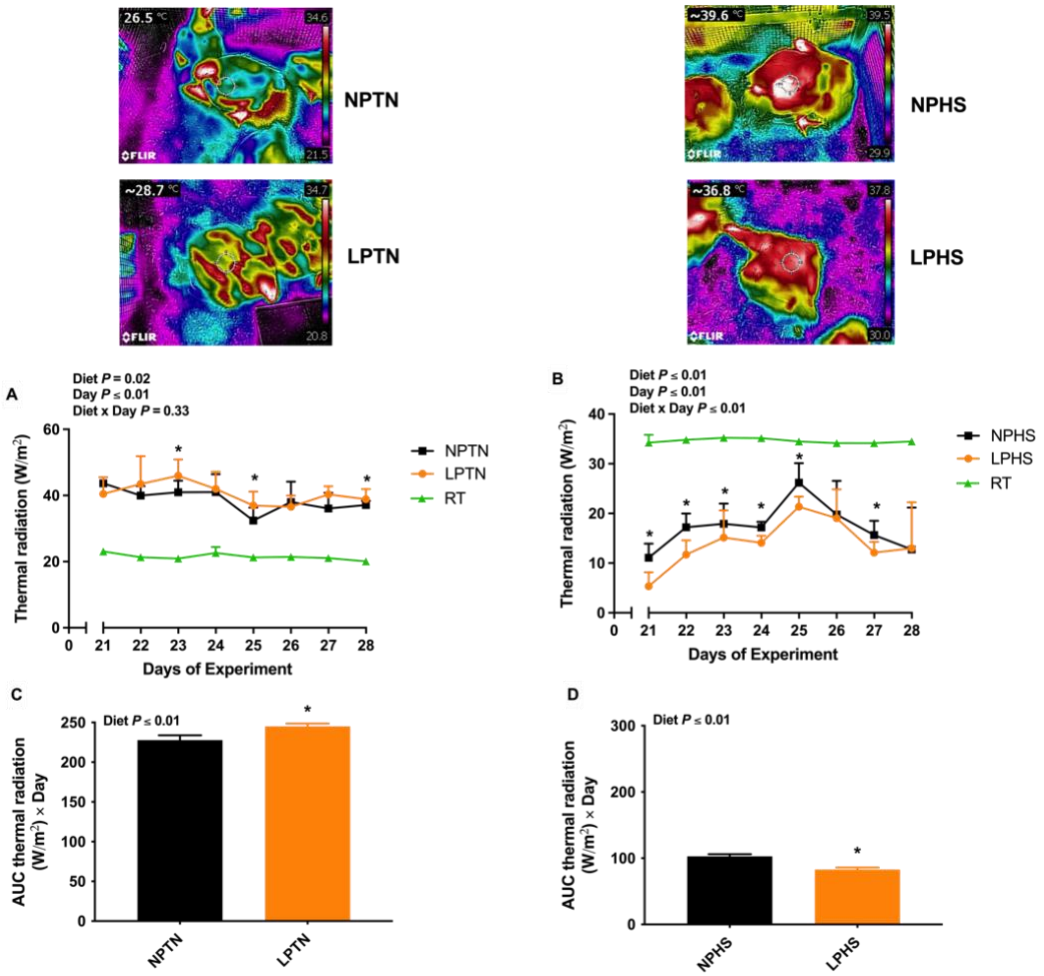

Figure 2. Thermal radiation of broilers fed with low protein diets during experimentally induced heat stress

Effect of low protein diets on thermal radiation (A and B) and area under the curve (AUC) of thermal radiation (C and D) of broilers during thermoneutral (TN) (A and C) and heat stress (HS) (B and D). NPTN: normal protein diet under TN; LPTN: low protein diet under TN; NPHS: normal protein diet under HS; LPHS: low protein diet under HS. RT: room temperature. The *P*-values for the overall model effects of diet, temperature (temp), day, diet × temp, diet × day, day × temp and diet × temp × day for thermal radiation were 0.99, ≤ 0.01, ≤ 0.01, ≤ 0.01, 0.56, ≤ 0.01, 0.03, respectively. The *P*-values for the overall model effects for diet, temp and diet × temp for AUC thermal radiation were 0.75, ≤ 0.01 and ≤ 0.01,

respectively. Asterisks (*) in the bar plots indicate significant difference ($P \leq 0.05$, t-test). The values are the mean ± standard errors of means; n=9.

When thermal radiation was expressed as area under the curve (AUC), the effect of temperature and diet × temperature on the thermal radiation AUC was significant. HS group had a lower thermal radiation AUC than that in TN group (92.8 vs 236.5 (W/m^2) × day, respectively). Compared to NPTN, LPTN increased the thermal radiation AUC by ~8% (Figure 2C), but relative to NPHS, LPHS decreased thermal radiation AUC by ~19% (Figure 2D).

Meat Quality

Overall, the effect of diet on redness, chroma, darkness and yellowness was significant with LP having higher redness, chroma and yellowness, but lower darkness compared to NP (14.9 vs 13.0, 25.8 vs 23.5, 21.0 vs 19.5 and 58.4 vs 61.1, respectively). The effect of temperature on darkness, redness, pH and chroma was significant with having higher darkness for HS, but lower redness, pH and chroma for HS compared to TN (62.3 vs 57.2, 13.4 vs 14.5, 5.7 vs 7.00 and 24.0 vs 25.3, respectively). In the grower phase, the effect of diet on redness, chroma, yellowness and darkness were significant with higher redness, chroma, yellowness but lower darkness for LP compared to NP (16.4 vs 14.0, 28.2 vs 24.6, 22.9 vs 20.2 and 57.0 vs 60.0, respectively). In the grower phase, the effect of temperature on redness, darkness and pH was significant with having lower redness and pH and higher

darkness or HS compared to TN (14.8 vs 15.6, 5.6 vs 6.8 and 61.1 vs 55.9 for HS vs. TN, respectively). Compared to NPTN, LPTN reduced meat pH, but increased redness, chroma and yellowness in the grower phase (~4%, 23%, 17% and 14%, respectively). Compared to NPHS, LPHS decreased meat darkness, but increased redness, chroma, yellowness, and pH in the grower phase (~9%, 13%, 11%, 9% and 5%, respectively). The effect of diet on meat redness and darkness during finisher phase was significant with having higher redness and lower darkness for LP vs. NP (13.3 vs 11.9 and 59.7 vs 62.39, respectively). In the finisher phase, the effect of temperature on redness, chroma, darkness and pH were significant with having lower redness, chroma and pH, but higher darkness for HS relative to TN (12.0 vs 13.5, 22.1 vs 23.8, 5.8 vs 7.2 and 63.5 vs 58.0, respectively). Compared to NPTN, LPTN reduced meat pH, but increased redness and chroma in the finisher phase (~3%, 16% and 8%, respectively). Compared to NPHS, LPHS decreased meat darkness, but increased pH in the finisher phase (~5% and 2%, respectively). Although NPTN and LPHS were not compared statistically, numerically when compared to NPTN in the grower phase, LPHS showed an increased redness, chroma, yellowness and darkness (~11%, 10%, 8% and 4%, respectively) but a decrease in pH, (~17%). When compared to NPTN numerically in the finisher phase, LPHS showed an increased darkness (~6%) but a decrease in redness, chroma, yellowness and pH (~1%, 3%, 4% and 19%, respectively).

Table 6. Meat quality traits in broilers fed with low protein diets during experimentally induced heat stress

Items	Phases	Treatments[1]				SEM[2]	*P*-Values		
		NPTN[2]	LPTN[2]	NPHS[2]	LPHS[2]		Diet	Temp[2]	Diet × Temp
Redness[3]									
	Grower	14.0	17.2*	13.8	15.6*	0.3	≤ 0.01	0.05	0.13
	Finisher	12.5	14.5*	11.6	12.4	0.2	≤ 0.01	≤ 0.01	0.09
Chroma[3]									
	Grower	24.7	28.9*	24.5	27.1*	0.5	≤ 0.01	0.12	0.22
	Finisher	22.8	24.7*	22.0	22.2	0.3	0.09	≤ 0.01	0.18
Yellowness[3]									
	Grower	20.4	23.2*	20.2	22.1*	0.4	≤ 0.01	0.33	0.44
	Finisher	19.1	20.0	18.6	18.4	0.3	0.52	0.07	0.29
Darkness [3]									
	Grower	56.1	55.4	64.1	58.5*	0.7	≤ 0.01	≤ 0.01	≤ 0.01
	Finisher	58.6	57.3	65.1	61.9*	0.6	≤ 0.01	≤ 0.01	0.13
pH									
	Grower	7.0	6.7*	5.5	5.8*	0.1	0.91	≤ 0.01	≤ 0.01
	Finisher	7.3	7.1*	5.8	5.9*	0.1	0.58	≤ 0.01	≤ 0.01

[1]The values are the mean; n=10 birds per treatment.

[2] NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress; SEM: standard error of means; Temp: temperature.

[3] Color assessment was measured using a^* (redness), b^* (yellowness), CIE L^* (darkness) and Chroma: a^* and b^* were used to calculate chroma as $(a^2+b^2)^{1/2}$. The *P*-values for the overall model effect for diet, temp, time, diet × temp, diet × time, temp × time and diet × temp × time for redness were ≤ 0.01, ≤ 0.01, ≤ 0.01, 0.02, 0.08, 0.30 and 0.92, for chroma were ≤ 0.01, ≤ 0.01, ≤ 0.01, 0.06, 0.02, 0.45 and 0.95, for yellowness were ≤ 0.01, 0.06, ≤ 0.01, 0.21, 0.02, 0.62 and 0.90, for darkness were ≤ 0.01, ≤ 0.01, ≤ 0.01, ≤ 0.01, 0.38, 0.98, 0.15, and for pH were 0.77, ≤ 0.01, ≤ 0.01, ≤ 0.01, 0.59, ≤ 0.01 and ≤ 0.01, respectively.

*Within rows, NPTN vs LPTN and NPHS vs LPHS $P \leq 0.05$

#Within rows, NPTN vs LPTN and NPHS vs LPHS $0.05 < P \leq 0.10$

Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Table 7. Quantitative PCR (qPCR) primer sequences, location on template, length, and GenBank accession number used in this study

Genes[1]	Sequence (5' → 3')	GenBank accession No.	Location on template (bp)	Amplicon length
Ghrelin	F-CCTTGGGACAGAAACTGCTC R- CACCAATTTCAAAAGGAACG	NM_001001131.1	199-218 382-401	203
CCK	F-CAGCAGAGCCTGACAGAACC R-AGAGAACCTCCCAGTGGAACC	NM_001001741.1	162-181 309-329	168
PYY	F-AGGAGATCGCGCAGTACTTCTC R-TGCTGCGCTTCCCATACC	NM_001361182.1	144-166 205-222	78
Secretin	F-TGAGTTGGCTGAGAGTACAG R-CTTCACATCTGTCACCAGCT	NM_001024833.2	3-22 570-589	587
GIP	F-CGCAGTGAGTGACCAAAGC R-TAGGAGCCATGCAAGGAAGT	NM_001080104.1	366-384 413-432	67
β1-AR	F-CTGGCACCTAGCACAATGAA R-CTGCTTGCTGATCCACATCT	NM_205518.1	1026-1045 1129-1148	123
PGC-1α	F-GGGACCGGTTTGAAGTTTTTG R-GGCTCGTTTGACCTGCGTAA	NM_001006457.1	2072-2092 2203-2222	151
AMPKα1	F-ATCTGTCTCGCCCTCATCCT R-CCACTTCGCTCTTCTTACACCTT	NM_001039603.1	1337-1356 1439-1461	125
β -Actin	F- CAATGGCTCCGGTATGTGCA R- AGGCATACAGGGACAGCACA	NM_205518.1	101-120 482-501	401
Sirtuin 1	F-GATCAGCAAAAGGCTGGATGGT R-ACGAGCCGCTTTCGCTACTAC	NM_001004767.1	1932-1953 2054-2074	143
Cox IV	F-CTTTCCACCTCCATCTGTGTGA R-TGCTGGATGGCTGAAATCG	NM_001030577.1	83-104 139-157	75

[1]CCK: cholecystokinin, PYY: peptide YY, GIP: gastric inhibitory polypeptide, β1-AR: β1-adrenergic receptor, PGC-1α: peroxisome proliferator-activated receptor-gamma coactivator, AMPKα1: AMP-activated protein kinase α1, β-Actin: beta actin, Cox IV: cytochrome c oxidase subunit IV

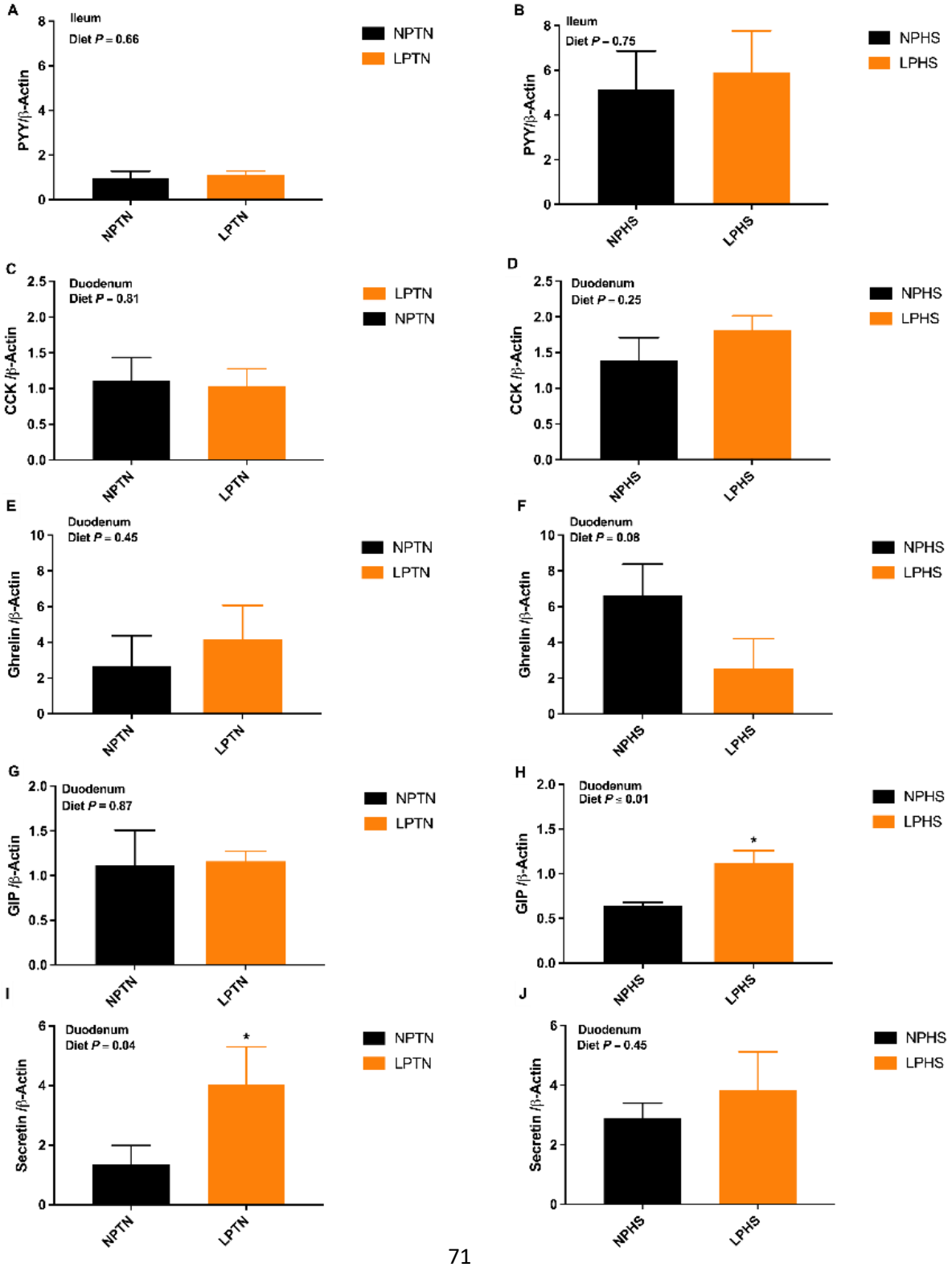
A
Ileum
Diet P = 0.66
PYY/β-Actin
NPTN
LPTN
B
Ileum
Diet P = 0.75
PYY/β-Actin
NPHS
LPHS
C
Duodenum
Diet P = 0.81
CCK /β-Actin
LPTN
NPTN
D
Duodenum
Diet P = 0.25
CCK /β-Actin
NPHS
LPHS
E
Duodenum
Diet P = 0.45
Ghrelin /β-Actin
NPTN
LPTN
F
Duodenum
Diet P = 0.08
Ghrelin /β-Actin
NPHS
LPHS
G
Duodenum
Diet P = 0.87
GIP /β-Actin
NPTN
LPTN
H
Duodenum
Diet P ≤ 0.01
GIP /β-Actin
NPHS
LPHS
I
Duodenum
Diet P = 0.04
Secretin /β-Actin
NPTN
LPTN
J
Duodenum
Diet P = 0.45
Secretin /β-Actin
NPHS
LPHS

Figure 3. mRNA abundance of feed intake markers in duodenum or ileum of broilers fed low protein diets during experimentally induced heat stress

Effect of low protein diets on mRNA abundance of peptide YY (PYY) (A and B), cholecystokinin (CCK) (C and D), ghrelin (E and F), gastric inhibitory polypeptide (GIP) (G and H), and secretin (I and I) in duodenum or ileum of broilers during thermoneutral (TN) (A, C, E, G and I) and heat stress (HS) (B, D, F, H, J). NPTN: normal protein diet under TN; LPTN: low protein diet under TN; NPHS: normal protein diet under HS; LPHS: low protein diet under HS. The *P*-values for the overall model effects of diet, temperature (temp), and diet × temp for PYY were 0.74, ≤ 0.01, 0.82, for CCK were 0.45, 0.03, 0.29, for ghrelin were 0.37, 0.42, 0.07, for GIP were 0.05, 0.05, 0.10 and for secretin were 0.04, 0.42, 0.29. Asterisks (*) in the bar plots indicate significant difference ($P \leq 0.05$, t-test). The values are the mean ± standard errors of means; n=8.

The effect of diet on mRNA abundance of GIP and secretin in duodenum was significant with LP group having higher values than NP (Figure 3 G-J). The effect of temperature on mRNA abundance of PYY in ileum and CCK in duodenum was significant with HS having higher mRNA abundance of PYY and CCK than TN (Figure 3A-3D). The effect of diet × temperature on mRNA abundance of ghrelin in duodenum and GIP in duodenum tended to be significant. Although no differences were detected between mRNA abundance of ghrelin in NPTN vs LPTN, LPHS tended to decrease that by ~62% in comparison with NPHS (Figure 3E and F). No differences in abundance of GIP was detected between NPTN and LPTN groups, but LPHS had higher (~75%) GIP transcript than NPHS (Figure 3 G and 3H). No differences on transcript abundance of ileum PYY and duodenum CCK and ghrelin were detected when NPTN vs LPTN or NPHS vs LPHS were compared (Figure 3A-F). Although NPTN and LPHS were not compared statistically, numerically when

compared to NPTN, LPHS showed an increased PYY, CCK and secretin (~84%, 63% and 182%, respectively) but a decrease in ghrelin and GIP (~5% and 0.1%, respectively).

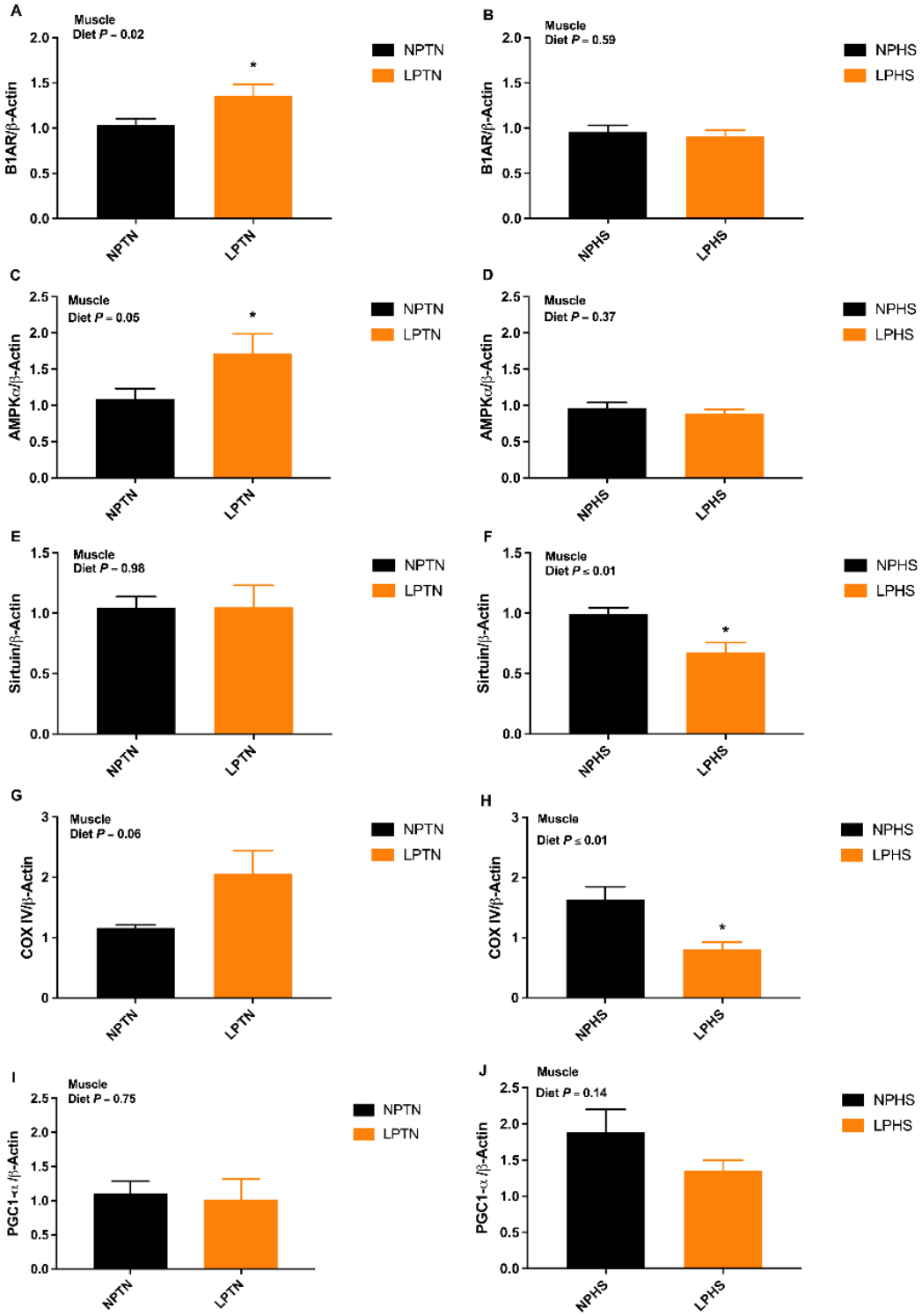
A
Muscle
Diet P = 0.02
B1AR/β-Actin
NPTN
LPTN
B
Muscle
Diet P = 0.59
NPHS
LPHS
C
Muscle
Diet P = 0.05
AMPKα1/β-Actin
D
Muscle
Diet P = 0.37
E
Muscle
Diet P = 0.98
Sirtuin1/β-Actin
F
Muscle
Diet P ≤ 0.01
G
Muscle
Diet P = 0.06
COX IV/β-Actin
H
Muscle
Diet P ≤ 0.01
I
Muscle
Diet P = 0.75
PGC1-α1/β-Actin
J
Muscle
Diet P = 0.14

Figure 4. mRNA abundance thermogenesis markers in muscle of broilers fed low protein diets during experimentally induced heat stress

Effect of low protein diets on mRNA abundance of β-1 adrenergic receptor (β1AR) (A and B), AMP-activated protein kinase alpha (AMPKα) (C and D), sirtuin (E and F), cytochrome c oxidase subunit 4 (COX IV) (G and H), and peroxisome proliferator-activated receptor- gamma coactivator alpha (PGC1-α) (I and J) in the muscle in broilers during thermoneutral (TN) (A, C, E, G and I) and heat stress (HS) (B, D, F, H and J). NPTN: normal protein diet under TN; LPTN: low protein diet under TN; NPHS: normal protein diet under HS; LPHS: low protein diet under HS. The *P*-values for the overall model effects of diet, temperature (temp), and diet × temp for β1AR were 0.08, ≤ 0.01, 0.02, for AMPKα were 0.07, ≤ 0.01, 0.02, for sirtuin were 0.07, 0.02, 0.06, for COX IV were 0.88, 0.12, ≤ 0.01, and for PGC1-α were 0.17, 0.02, 0.32. Asterisks (*) in the bar plots indicate significant difference ($P \leq 0.05$, t-test). The values are the mean ± standard errors of means; n=8

The effect of diet on mRNA abundance of muscle β1AR, AMPKα and sirtuin tended to be significant with LP group having higher values for β1AR and AMPKα and lower value for sirtuin compared to NP (Figure 4A-F). The effect of temperature on mRNA abundance of muscle β1AR, AMPKα, sirtuin and PGC1-α was significant with HS having lower values for β1AR, AMPKα and sirtuin, but higher value for PGC1-α than TN (Figure 4A-F and 4I-J). The overall effect of diet × temperature for muscle β1AR, AMPKα and COX IV (Figure 4A-D and 4G-H) was significant. Compared to NPTN, LPTN increased muscle β1AR and AMPKα transcript (~30% and 58%, respectively), but no differences were detected when NPHS vs LPHS were compared. Although LPTN tended to increase the mRNA abundance muscle COX IV in comparison with NPTN, relative to NPHS, that was decreased in LPHS by ~ 50%. The overall effect of diet × temperature for muscle sirtuin (Figure 4E and 4F) was significant. While no differences were detected on muscle sirtuin transcript between NPTN and LPTN, relative to NPHS, that was decreased in LPHS by ~ 32%. No differences on transcript abundance of muscle PGC1-α was detected when

NPTN vs LPTN or NPHS vs LPHS were compared (Figure 4I-J). Although NPTN and LPHS were not compared statistically, numerically when compared to NPTN, LPHS showed an increased PGC1-α (~22%) but a decrease in β1AR, AMPKα, sirtuin and COX IV (~12%, 18%, 36% and 30%, respectively).

Discussion

Heat stress has been reported to not only cause death but impact immunity, metabolism, microbiota composition, growth, and performance. It is important to identify dietary strategies that could help alleviate or improve survivability of birds (Nawab et al., 2018). LP diets not only improve survivability, but also reduce heat production (Ghasemi et al., 2020, Daghir et al., 2009), feed costs (Awad et al., 2017; Marayat et al., 2018) and improve thermoregulation which can reduce the negative effects of HS on the birds (Mohammed et al., 2018, Lin et al., 2006). The objective of this study was to assess the effects of LP diets on FI, thermal radiation, markers of FI and thermogenesis regulation in gut and skeletal muscle, growth performance, body composition, and meat quality in broilers under experimentally induced HS. Our study revealed several key findings: (1) LP diet reduced ADFI, which could be due to higher mRNA abundance of duodenal GIP and secretin in this group, (2) HS reduced ADFI, which could be related to higher transcript of ileum PYY and duodenal CCK, (3) HS reduced thermal radiation, which could be linked with decreased mRNA abundance of muscle β1AR, AMPK and sirtuin, (4) LP diet tended to

reduce the ADFI during HS, but not during TN. This could be due to lower mRNA abundance of duodenal ghrelin and higher GIP in LP group during HS, (5) LP diet increased the thermal radiation during TN, but reduced it during HS. This could be due to higher mRNA abundance of muscle β1AR and AMPK during TN, but not HS. Further, LP increased the mRNA abundance of COX-IV during TN, but reduced COX-IV and sirtuin abundance during HS. In summary our data provides evidence that LP diets mitigate the negative outcome of heat stress by reducing feed intake and heat production, which are regulated through factors expressed in the gut and skeletal muscle.

During HS, birds decrease their final BW and ADG. The results of our study on overall final BW of birds under HS agree with previous research (Laudadio et al., 2012). Unlike our study where we noticed the differential effects of LP diets on feed intake during TN and HS, in an experiment performed by Gonzalez-Esquerra et al., 2005 it was observed that dietary protein did not affect feed intake of birds under both TN and HS. The discrepancy in results of the later mentioned research and our study could be due to differences in supplementation of limiting amino acids that are essential for growth and development. Reduction of feed intake in birds under HS agree with previous studies (Smith et al., 1987, Alleman et al., 1997 and Yahav et al., 2009). Little is known on the role of gut peptides in feed intake regulation in poultry. Under HS conditions, LP fed birds had a higher mRNA abundance of GIP and lower ghrelin transcript in gut. In line with our data, Song et al., 2012 showed a decrease in ghrelin mRNA expression in the duodenum of laying hens fed with LP diets under HS conditions. Also, they (Song et al., 2012) showed

a decrease in ADFI of hens fed with LP diets under HS which was in agreement with our data.

In our study, overall HS reduced the thermal radiation, which could be linked with reduced mRNA expression of β1AR, AMPKα1 and sirtuin. Similar to our data Zhang et al., (2015) found that birds under HS conditions decreased sirtuin expression. Birds fed with LP diets under TN conditions had a higher thermal radiation than NP fed birds while under HS conditions, LP birds had a lower thermal radiation than NP fed birds. This might be due to changes in feed intake during TN and HS periods. In TN conditions LP had a higher ADFI compared to NP fed birds while in HS conditions birds fed LP diets had a lower ADFI than NP fed birds. In TN conditions, the transcript of muscle β1AR and AMPKα1 was higher in LP fed birds compared to NP while under HS conditions, mRNA abundance of muscle sirtuin and COX IV was decreased in LP fed birds compared to NP. In a study in pigs (Li et al., 2017) compared to normal protein fed group, the expression of AMPKα in muscle of pigs fed with very low protein diets was severely suppressed. This was not in line with our data possibly due to differences in dietary protein content and differences present across species. Others found that COX IV expression is decreased in birds fed LP diets under HS (Yang et al., 2010), which was consistent with our findings.

Compared to TN, birds under HS had lower BMC, BMC percent, BMD, lean mass, fat mass, and fat percent. Others (Zhang et al., 2012) also reported similar results and suggested that the increase in fat deposition could be related to the reduction in basal

metabolism and physical activity in birds under constant HS. Compared to TN, HS reduced meat redness, pH and chroma. During HS, broilers increase rate of anaerobic glycolysis to generate energy causing an increased lactic acid production, which might explain the lower meat pH (Zaboli et al., 2019) and the risk of PSE conditions in meat during HS.

Conclusion

Our data provide evidence that low-protein diets mitigate the negative outcome of heat stress by reducing feed intake and heat production, which are regulated through factors expressed in the gut and skeletal muscle, and HS coping mechanisms adaptions. Further, low-protein diets decrease the body lean mass and increase the fat mass during both thermoneutral and heat stress. The breast meat of chickens fed with low-protein diet had higher indices of redness, chroma and lightness during both thermoneutral and heat stress and had lower pH than those fed with normal protein diet during thermoneutral, but not during heat stress.

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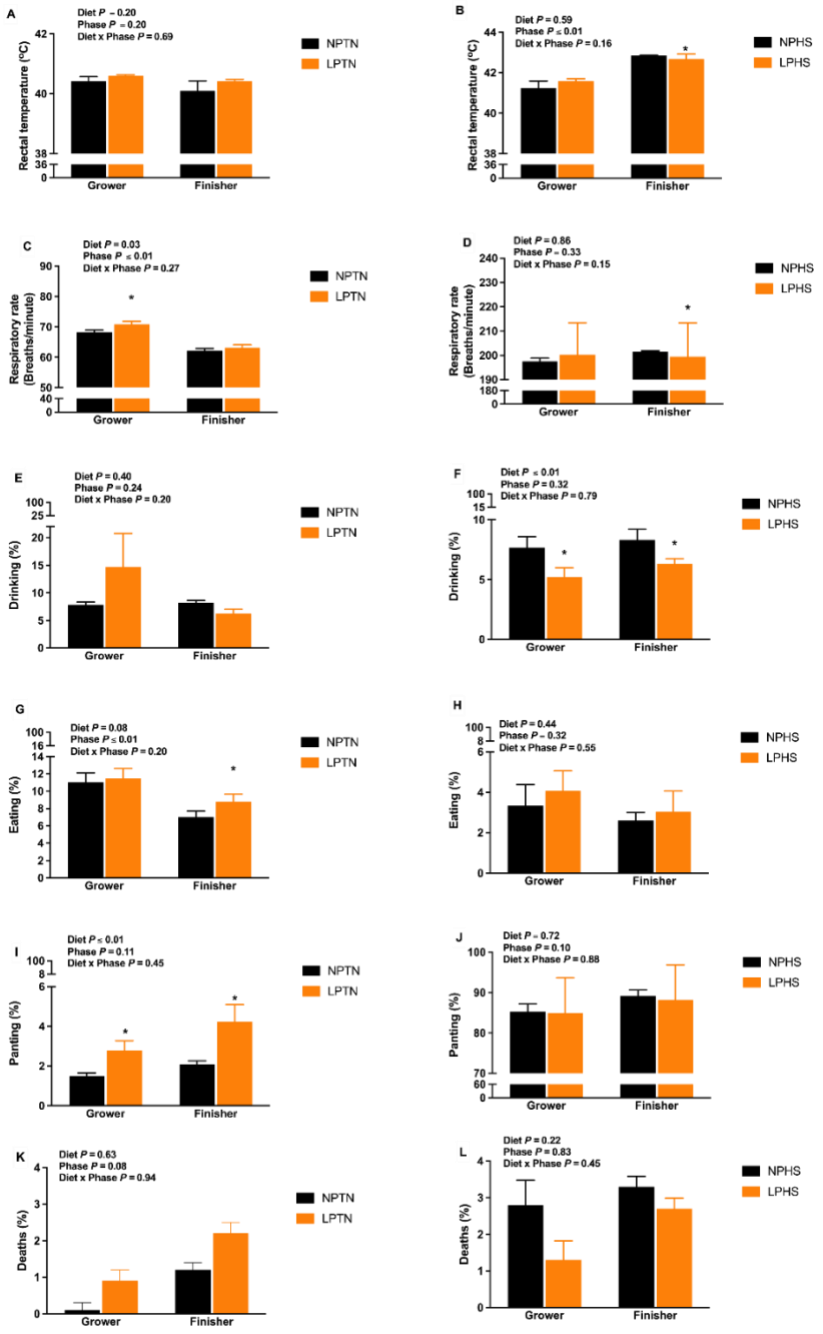
Supplementary Figures


A
Diet P = 0.20
Phase P = 0.20
Diet x Phase P = 0.69
Rectal temperature (°C)
Grower
Finisher
NPTN
LPTN
B
Diet P = 0.59
Phase P ≤ 0.01
Diet x Phase P = 0.16
Rectal temperature (°C)
Grower
Finisher
NPHS
LPHS
C
Diet P = 0.03
Phase P ≤ 0.01
Diet x Phase P = 0.27
Respiratory rate (Breaths/minute)
Grower
Finisher
NPTN
LPTN
D
Diet P = 0.86
Phase P = 0.33
Diet x Phase P = 0.15
Respiratory rate (Breaths/minute)
Grower
Finisher
NPHS
LPHS
E
Diet P = 0.40
Phase P = 0.24
Diet x Phase P = 0.20
Drinking (%)
Grower
Finisher
NPTN
LPTN
F
Diet P ≤ 0.01
Phase P = 0.32
Diet x Phase P = 0.79
Drinking (%)
Grower
Finisher
NPHS
LPHS
G
Diet P = 0.08
Phase P ≤ 0.01
Diet x Phase P = 0.20
Eating (%)
Grower
Finisher
NPTN
LPTN
H
Diet P = 0.44
Phase P = 0.32
Diet x Phase P = 0.55
Eating (%)
Grower
Finisher
NPHS
LPHS
I
Diet P ≤ 0.01
Phase P = 0.11
Diet x Phase P = 0.45
Panting (%)
Grower
Finisher
NPTN
LPTN
J
Diet P = 0.72
Phase P = 0.10
Diet x Phase P = 0.88
Panting (%)
Grower
Finisher
NPHS
LPHS
K
Diet P = 0.63
Phase P = 0.08
Diet x Phase P = 0.94
Deaths (%)
Grower
Finisher
NPTN
LPTN
L
Diet P = 0.22
Phase P = 0.83
Diet x Phase P = 0.45
Deaths (%)
Grower
Finisher
NPHS
LPHS

Supplementary Fig 1. Heat stress coping mechanisms of broilers fed with low protein diets during experimentally induced heat stress

Effect of low protein diets on rectal temperature (Aand B), respiratory rate (C and D), percent of birds drinking (E and F), percent of birds eating (G and H), percent of birds panting (I and J) and percent death (K and L) in broilers during thermoneutral (TN) (A, C, E, G, I and K) and heat stress (HS) (B, D, F, H, J and L). NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress. The *P*-values for the overall model effects of diet, temp, time, diet × temp, diet × time, time × temp, diet × temp × time for rectal temperature were 0.19, ≤ 0.01, ≤ 0.01, 0.55, 0.47, ≤ 0.01, 0.20, for respiratory rate were 0.34, ≤ 0.01, ≤ 0.01, 0.52, 0.07, ≤ 0.01 and 0.44, for percent of birds drinking were 0.93, 0.09, 0.33, 0.10, 0.20, 0.14, and 0.16, for percent of birds eating were 0.13, ≤ 0.01, ≤ 0.01, 0.64, 0.71, 0.08 and 0.56, for percent of birds panting were 0.61, ≤ 0.01, 0.05, 0.24, 0.95, 0.26 and 0.74 and percent death were 0.47, 0.56, 0.66, 0.22, 0.53, 0.94, and 0.50. The values are the mean ± SEM; n=9 pens (5-6 birds/pen).

CHAPTER 4

EFFECTS OF LOW PROTEIN DIETS ON BROILERS' ENERGY BALANCE, CECAL MICROBIOTA COMPOSITION, PLASMA METABOLOMICS AND OXIDATIVE STRESS DURING EXPERIMENTALLY INDUCED HEAT STRESS

Abstract

We have previously shown that low-protein (LP) diets mitigate the negative outcome of heat stress (HS) by reducing feed intake and heat production. Little is known whether the beneficial effects of LP diets on heat stress is associated with alterations in oxidative stress, plasma metabolomics and cecal microbiota. The objective of this study was to investigate the effects of LP diets on broilers oxidative stress, plasma metabolomics and cecal microbiota composition during experimentally induced HS. Two-hundred-day-old male broiler chicks were randomly assigned into 36 pens with of 5-6 chicks/pen. All birds were weight-matched and randomly subjected into either thermoneutral (TN) or heat stress (HS; 35^{o}C for 7 hours/day) (18 pens/treatment). Within each treatment, the birds were randomized to receive either normal protein (NP) or low protein (LP) diets (9 pens/diet; 50 birds/diet). The study lasted 6 weeks with 2 weeks of adaptation (starter phase) and 4 weeks of data collection (grower and finisher phases). On week 6, birds were euthanized and blood and cecal samples were collected. Under TN, birds fed with LP had a lower plasma superoxide dismutase activity and lipid peroxidation compared to NP, but no differences on these measurements were detected during HS. Birds fed with LP diet had a higher lipid hydroperoxides than NP under HS. Principle component analysis (PCA) showed a clear separation for plasma metabolites found between NP and LP diets under TN and HS. Dietary protein content impacted plasma metabolites related with metabolism and biosynthesis of alanine, aspartate, glutamate and phenylalanine during TN and HS. Compared to NP, chickens fed with LP diet had higher abundances of *p_Tenericutes*, *c_Mollicutes*, *c_Mollicutes_RF9*, and *f_tachnospiraceae* under HS. Thus, dietary protein content influenced the plasma metabolites with similar pattern during TN and HS, but that had differential effects on cecal microbiota composition under TN and HS.

Keywords: low-protein diet, heat stress, plasma metabolomics, cecal microbiota, broiler chickens

Introduction

Heat stress (HS) is a major environmental concern that adversely affects performance and body composition of broilers (Kumar et al., 2021). Various dietary interventions have been tested to mitigate the negative effects of heat stress in poultry (Nawab et al., 2018); however, no single dietary strategy has been identified to sustain the optimal growth performance of birds during HS (Daghir et al., 2009). Therefore, further research is needed to develop effective and novel strategies to improve the growth performance of birds under HS.

Low-protein (LP) diets supplemented with essential amino acids have been proposed as a dietary strategy to mitigate HS and related mortality in birds (Daghir et al., 2009; Zulkifi et al., 2017; Waldroup 2005). Due to high heat increment of dietary proteins; reducing the diet crude protein (CP) content may decrease the metabolic heat production and alleviate the HS signs (Awad et al., 2017, Swennen et al., 2007, Syafwan et al., 2011; Musharaf et al., 1999). Further, LP diets may mitigate the negative outcome of HS by reducing feed intake that can contribute to reduced heat production as well (Ghasemi et al., 2020; Zulkifli et al., 2018. Suganya et al., 2015; Aftab et al., 2006; Furlan et al., 2004, Song, et al., 2012). Little is known whether beneficial effects of LP diets on survivability of birds under HS are associated with changes in the gut microbiota composition and blood metabolites profile.

The gut microbiota composition is a function of genetics, disease, medication, diet and environmental factors (Shi et al., 2019). Changes in gut microbiota composition may alter nutrients digestion and absorption, energy extraction, immune response, and disease resistance (Tian et al., 2020). In healthy chickens in thermoneutral (TN) conditions, the composition of intestinal microbiota remains stable (Lan et al., 2004). Heat stress however, alters microbiota or dysbiosis which can influence the immunity, metabolism, and growth of the animals (Shi et al., 2019). Alterations in diet composition can influence the population of gastrointestinal tract's microorganisms (Cesare et al., 2019; Spring et al., 2020; Shi et al., 2019). Under TN conditions when birds fed with LP diet, they had an increased abundance of *Lactobacillaceae* in the ceca, which are associated with better feed conversion rate (Cesare et al., 2019). Little is understood on whether LP diets can alter and increase the population of beneficial bacteria in the gut of bird under HS conditions.

In addition to changes in gut microbiota composition, HS induces changes in the profile of blood metabolites such as plasma free and essential amino acids (Ostrowski-Meissner et al., 1981), glucose and thyroid hormones (Xie et al., 2015) in birds. Diet plays a pivotal role on metabolism and endocrine functions causing changes in plasma concentrations of metabolites and hormones such as triglyceride, uric acid and thyroid hormones such as T_3 and T_4 (Corzo et al., 2004; Hofmann et al., 2019; Kamran et al., 2010; Swennen et al., 2005). Under TN conditions, birds fed with LP diets altered the concentration of blood metabolites such as amino acids and lipids (Hofmann et al., 2019). Further, broilers provided with a LP diet under HS exhibited lower triglyceride levels and

high-density lipoprotein cholesterol concentrations (Ghasemi et al., 2020). The metabolomics profile of birds fed with LP diets under HS has not been fully characterized.

Oxidative stress may occur during HS due to increased levels of reactive species that cannot be neutralized by limited levels of natural antioxidants (Jastrebski et al., 2017). These reactive species can result in extensive tissue and cellular damage causing major physiological and coping mechanism responses which contribute to reducing productivity, immunity, nutrient digestibility, and meat quality (Akbarian et al., 2016; Arain et al., 2018; Estévez et al., 2015). It is thought that diet plays an influential role on increasing the available antioxidants to combat the free radicals produced by stress (Surai et al., 2019). Although LP diets have been reported to cause oxidative stress in rats under TN conditions (Darmon et al., 1993), the effect of LP diets on oxidative stress in broilers during HS is unknown.

The objective of this study was to assess the effects of LP diets on cecal microbiota composition, plasma metabolomics profile, and oxidative stress in broiler chickens under experimentally induced HS.

Methods and Materials

Animals and Experimental Design

The experimental procedures used in this study were in accordance with FASS Guide for the Care and Use of Agricultural Animals in Research and Teaching (McGlone, 2010) and approved by Oklahoma State University's Institutional Animal Care and Use Committee (Animal Care and Use Protocol # AG-19-5). The experimental design, husbandry and diets composition were previously described (Chapter 3). Briefly, 200 day-old male broiler Cobb x Cobb chicks (Cobb commercial hatchery, Siloam Springs, Arkansas) transported to Poultry Research Center at Oklahoma State University (OSU). Following 2 weeks of adaptations period (*i.e.* starter phase), birds were weight-matched and subjected into four groups (9 pens/group; 50 birds/diet/group) for 4 weeks including: normal protein diet under TN (NPTN), LP diet under TN (LPTN), normal protein diet under HS (NPHS) and LP diet under HS (LPHS) (Chapter 3). Birds in HS treatment were exposed with cyclic HS at 35^{o}C for 7 hours each day from 1100 until 1800 while broilers in TN treatment were housed under ambient temperature (Chapter 3). At the end of the study, blood samples were collected, processed and plasma stored at −80 ^{o}C. Then, birds were euthanized via CO_2 asphyxiation and cecal content were collected, snap-frozen in liquid nitrogen and stored at −80 ^{o}C until further analysis (Chapter 3).

Plasma Oxidative Stress Biomarkers

Superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, lipid peroxidation of malondialdehyde (MDA) and lipid hydroperoxide (LPO) in plasma were measured by using SOD Assay Kit (ab65354), Glutathione Assay Kit (ab102530), Lipid Peroxidation (MDA) Assay Kit (ab118970), and Lipid Hydroperoxide (LPO) Assay Kit (ab133085), respectively according to the manufacturer's instructions (Abcam, Cambridge, Massachusetts). The optical density for SOD, GPx, MDA and LPO kits was read at 450, 340, 532 and 500 nm wavelengths, respectively using Epoch microplate spectrophotometer (BioTek® Instruments, Inc. Highland Park, VT). The intra assay CV for SOD, GPx, MDA and LPO was 2.9%, 0.1%, 1.3%, and 1.1%, respectively. The inter CV for SOD and GPx was 2.7% and 0.1%, respectively.

Plasma Metabolomics

Plasma metabolomics analysis was performed at West Coast Metabolomics Center (UC Davis, Davis, CA) as we previously described (Spring et al., 2020a,b). Briefly, following sample preparations, plasma samples were analyzed by gas chromatography (GC) - mass spectrometry (MS) using a time-of-flight mass spectrometer (GC-TOF MS; Leco Pegasus IV). An Agilent 690 GC was equipped with an automatic linear exchange (ALEX; Gerstel corporation) and cold injection system (CIS; Gerstel corporation) for data acquisition.

Following data acquisition and processing, the values for quantified metabolites were reported as peak height. The metabolomics data analysis was performed using MetaboAnalyst 3.092 (available online at: http://www. metaboanalyst.ca/faces/ModuleView.xhtml). A principal component analysis (PCA), pathway impact analysis and hierarchical clustering analysis were performed.

Cecal Contents Microbiome

Cecal contents DNA isolation, amplicon sequencing, sequence data analysis, and taxonomic classification were performed following our previously published protocols (Shili et al., 2020). PCR amplification, microbial amplicon sequencing and bioinformatics were done at Novogene Corp. (Sacramento, CA, USA). Briefly, after DNA isolation from cacal contents, amplifying the16S rRNA V4 region by PCR and sequencing library preparation, the Illumina HiSeq 2500 platform (Illumina, Inc.) was used to sequence the library. Following sequence data analysis, taxonomic classification was performed, and rarefaction curves were generated. The beta diversity of bacterial populations was assessed by principal coordinate analysis (PCoA) and weighted and unweighted UniFrac methods. Further, linear discriminant analysis (LDA) with effect size measurements (LEfSe) was used for quantitative analysis of cecal microbiota composition within experimental groups.

Statistical Analysis

For assessing the rate of mortality associated with HS and the effect of diet on that, Log-Rank survival curve analysis was performed (IBM SPSS Statistics, Version 23, Armonk, NY, USA). Percent of birds alive was calculated on a per pen basis for each week of the experiment which was then analyzed using Mixed analysis procedure as previously described (Chapter 3). Diet, temperature, time, interaction of diet and temperature, diet and time, temperature and time, and diet, temperature and time were included in the model as fixed effects. The chicken was the random variable. The covariance structure of the repeated measurements for each variable was modeled as either first-order antedependence, autoregressive, heterogenous autoregressive, compound symmetry, heterogenous compound symmetry or Toeplitz (Chapter 3). Oxidative stress biomarkers and peak height of metabolites in plasma were analyzed using GLM procedure (IBM SPSS Statistics, Version 23, Armonk, NY, USA) with including diet, temperature, and interaction of diet and temperature in the model. Paired ttest was used for comparing diet treatments within TN and HS groups. Differences were considered significant at $P < 0.05$ and trend at $0.5 < P \leq 0.10$.

Results

Survival

Overall, the effect of temperature on survival of birds was significant with birds in HS group having a lower survival (%) than those assigned to TN (67.4% vs. 69.85%, respectively). Further, the effect diet × temperature on survival of birds was significant. This was due to significant diet effect on survival of birds within TN group, but not in HS group (Figure 1A and B). Although LPTN had a lower survival rate than NPTN (77.8% vs 82.6%, respectively), there was no difference in survival rate between LPHS and NPHS (57.1% vs. 57.1%) groups.

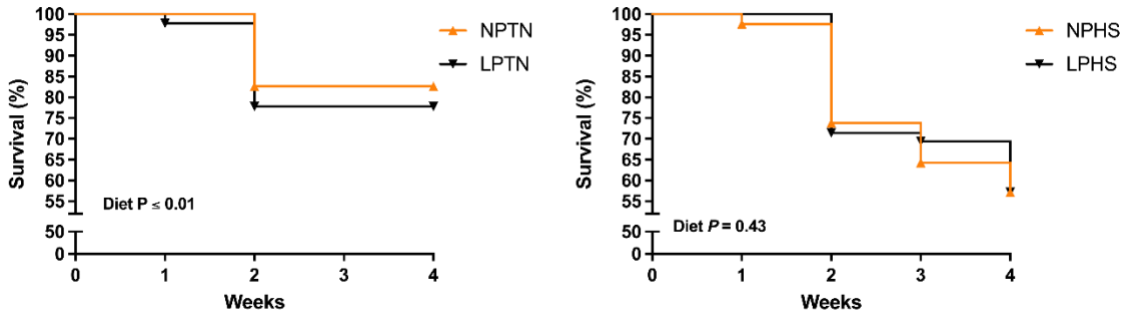


Figure 1. Survival curve of broilers fed with low protein diets during experimentally induced heat stress
Effect of low protein diets on survival (%) of broilers during thermoneutral (TN) (A) and heat stress (HS) (B). NPTN: normal protein diet under TN; LPTN: low protein diet under TN; NPHS: normal protein diet under HS; LPHS: low protein diet under HS. The P-values for the overall model effects for diet, temperature (temp), week, diet × temp, diet × week, temp × week and diet × week × temp were 0.27, ≤ 0.01, ≤ 0.01, 0.02, 0.14, ≤ 0.01, 0.38. n=9 per treatment.

Oxidative Stress Biomarkers in Plasma

The effect of diet × temperature on SOD activity was significant (P= 0.05). This was due to significant diet effect on SOD activity within TN group, but not in HS group (Figure 2A and B). Compared to NPTN, LPTN reduced SOD activity by ~14%. The effect of temperature on plasma MDA was significant with HS having higher values for MD than TN (88.0 vs 79.8μM, respectively). Relative to NPTN, LPTN reduced MDA concentrations by ~3% (Figure 2E). The effect of diet on LPO was significant with LP having a higher values for LPO compared to that in NP (normal protein) (11.8 vs. 9.2 μM, respectively). Compared to NPHS, LPHS increased the concentration of LPO by 51% (Figure 2H). Although NPTN and LPHS were not compared statistically, numerically when compared to NPTN, LPHS showed an increased MDA and LPO (~8% and 7%, respectively) but a decrease in SOD and GPx (~9% and 5%, respectively).

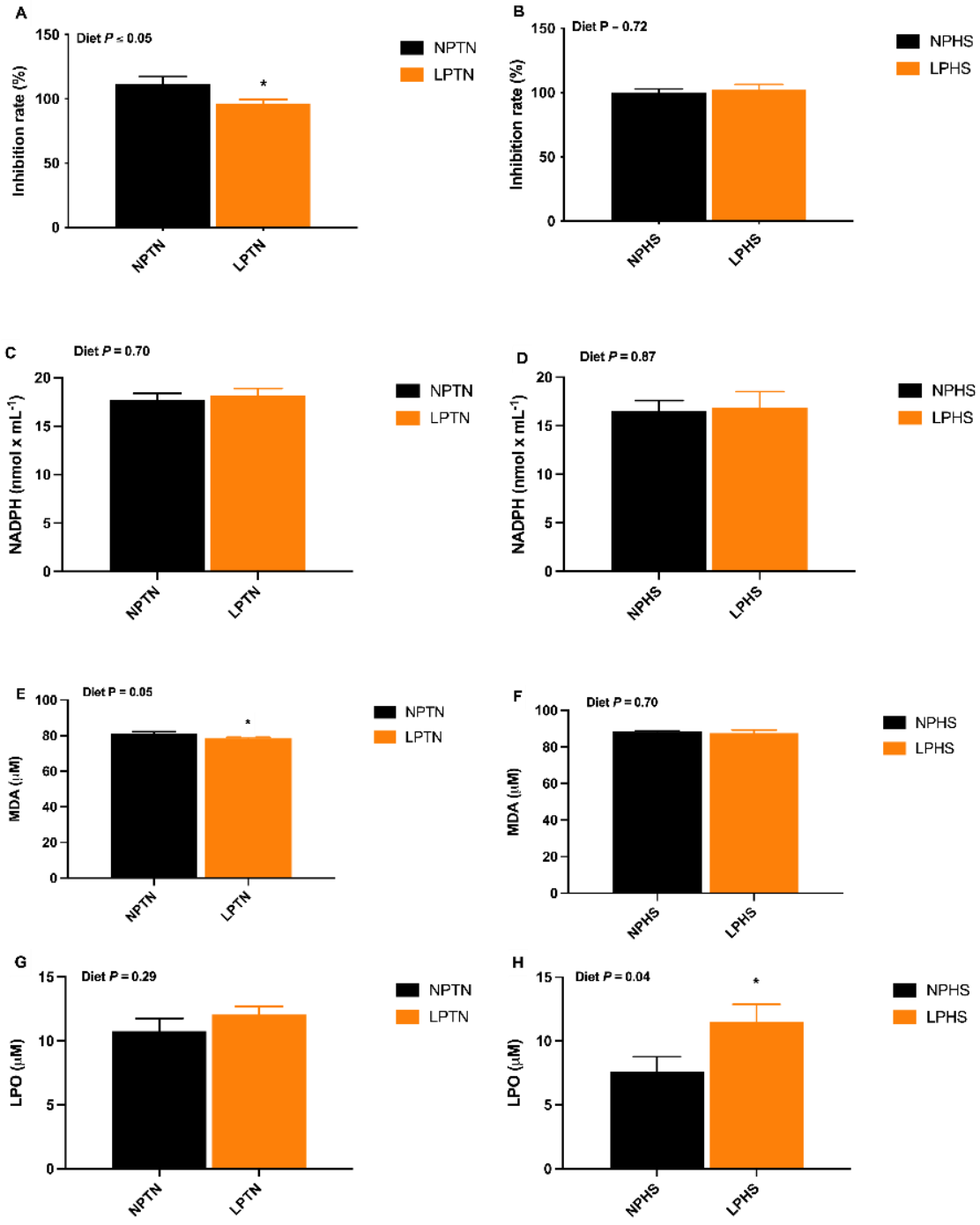

A
Diet P < 0.05
Inhibition rate (%)
150
100
50
0
*
NPTN
LPTN
NPTN
LPTN
B
Diet P = 0.72
Inhibition rate (%)
150
100
50
0
NPHS
LPHS
NPHS
LPHS
C
Diet P = 0.70
NADPH (nmol x mL-1)
20
15
10
5
0
NPTN
LPTN
NPTN
LPTN
D
Diet P = 0.87
NADPH (nmol x mL-1)
20
15
10
5
0
NPHS
LPHS
NPHS
LPHS
E
Diet P = 0.05
MDA (μM)
100
80
60
40
20
0
*
NPTN
LPTN
NPTN
LPTN
F
Diet P = 0.70
MDA (μM)
100
80
60
40
20
0
NPHS
LPHS
NPHS
LPHS
G
Diet P = 0.29
LPO (μM)
15
10
5
0
NPTN
LPTN
NPTN
LPTN
H
Diet P = 0.04
LPO (μM)
15
10
5
0
*
NPHS
LPHS
NPHS
LPHS

Figure 2. Superoxide dismutase activity, glutathione peroxidase activity and lipid peroxidation in plasma of broilers fed with low protein diets during experimentally induced heat stress
Effect of low protein diets on superoxide dismutase (SOD) activity (A and B), glutathione peroxidase (GPx) activity (C and D), lipid peroxidation of malondialdehyde (MDA) (E and F) and lipid hydroperoxide (LPO) (G and H) of broilers during thermoneutral (TN) (A, C and E) and heat stress (HS) (B, D and F). NPTN: normal protein diet under TN; LPTN: low protein diet under TN; NPHS: normal protein diet under HS; LPHS: low protein diet under HS. The P-values for the overall model effects for diet, temperature (temp), diet × temp, for SOD activity were 0.12, 0.49, 0.05, for GPx activity were 0.74, 0.28, 0.98, for MDA were 0.12, ≤ 0.01, 0.35 and for LPO were 0.02, 0.11 and 0.24. Asterisks (*) in the bar plots indicate significant difference ($P \leq 0.05$, t-test). The values are the mean ± standard errors of means. n=5-6 per treatment for SOD activity, n=9 per treatment for GPx activity and n=10 per treatment for lipid peroxidation and lipid hydroperoxide.

Plasma Metabolites

The principal component analysis (PCA) score plot displays a clear separation between NPTN and LPTN for plasma metabolites (Figure 3A). There was a positive loading for NPTN and a negative loading for LPTN on the PC1 axis. The PC1 explains 71.6% of variation of metabolite changes within samples and PC2 is indicative of 10.8% of the variation. The PCA score plot between NPHS and LPHS (Figure 3B) displays a separation between these two groups for plasma metabolites. There was a negative loading for NPHS and a positive loading for LPHS on the PC1 axis. The PC1 explains 59.1% of variation of metabolite changes within samples and PC2 is indicative of 20.3% of the variation. The PCA score plot between NPHS and NPTN (Figure 3C) displays a slight separations between the two groups. There was primarily a positive loading for NPHS and a negative loading for NPTN on the PC1 axis. The PC1 explains 53.4% of variation of metabolite changes within samples and PC2 is indicative of 17.3% of the variation. The PCA score plot comparing LPHS and LPTN (Figure 3D) displays a slight separation between the two

groups. There was a negative loading for LPHS and a positive loading for LPTN on the PC2 axis. The PC1 explains 35.3% of variation of metabolite changes within samples and PC2 is indicative of 32.6% of the variation.

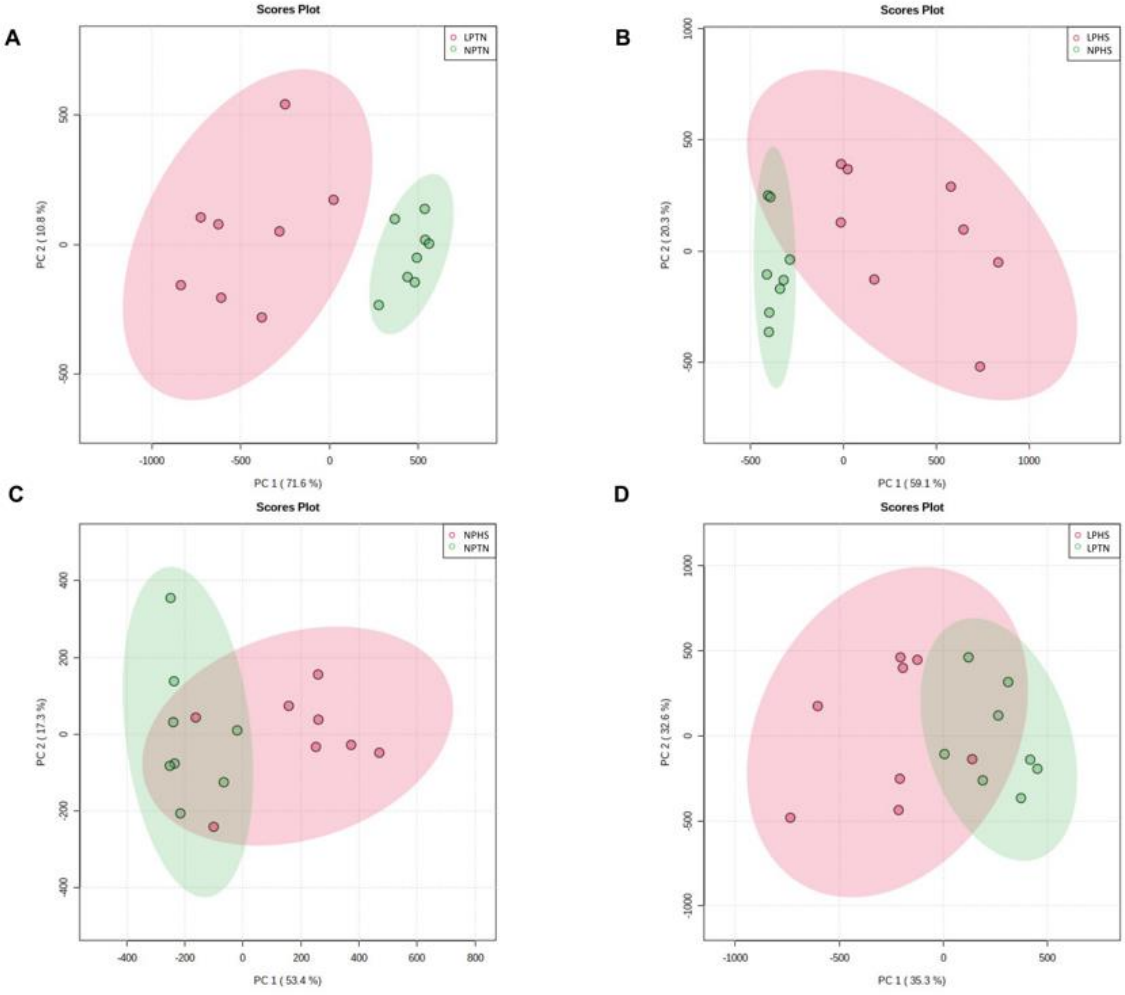


Figure 3. Principal component analysis (PCA) score plots of plasma metabolites in broilers fed with low protein diets during experimentally induced heat stress

PCA score plots of plasma metabolites for NPTN vs LPTN (A), NPHS vs LPHS (B), NPTN vs NPHS (C) and LPTN vs LPHS (D). NPTN: normal protein diet under TN; LPTN: low protein diet under TN; NPHS: normal protein diet under HS; LPHS: low protein diet under HS. Each node represents an individual bird. n=8

Hierarchical clustering heat map of significantly different plasma metabolites detected differentially expressed metabolites in plasma when data were analyzed for individual birds (Figure 4A) or as treatment group (Figure 4B). The metabolic pathway enrichment analysis showed that comparing NPTN and LPTN, the aminoacyl tRNA biosynthesis, glyoxylate and dicarboxylate metabolism, glycine, serine and threonine metabolism, alanine, aspartic acid and glutamate metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis, and linoleic acid metabolism pathways were influenced (Figure 5A). When NPHS was compared to LPHS, aminoacyl tRNA biosynthesis, glyoxylate and dicarboxylate metabolism, glycine, serine and threonine metabolism, alanine, aspartic acid and glutamate metabolism, phenylalanine metabolism, phenylalanine biosynthesis, and linoleic acid metabolism pathways were changes (Figure 5B). Comparing NPTN with NPHS, glyoxylate and dicarboxylate metabolism, alanine, aspartic acid and glutamate metabolism, D-glutamine and D-glutamate metabolism, glycine, serine and threonine metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis, starch and sucrose metabolism, and linoleic acid metabolism were affected (Figure 5C). When LPTN was compared with LPHS, phenylalanine metabolism, phenylalanine, tyrosine, and tryptophan

biosynthesis, linoleic acid metabolism, alanine, aspartate, and glutamate metabolism, starch and sucrose metabolism and D-glutamine and D-glutamate metabolism were changed (Figure 5D).

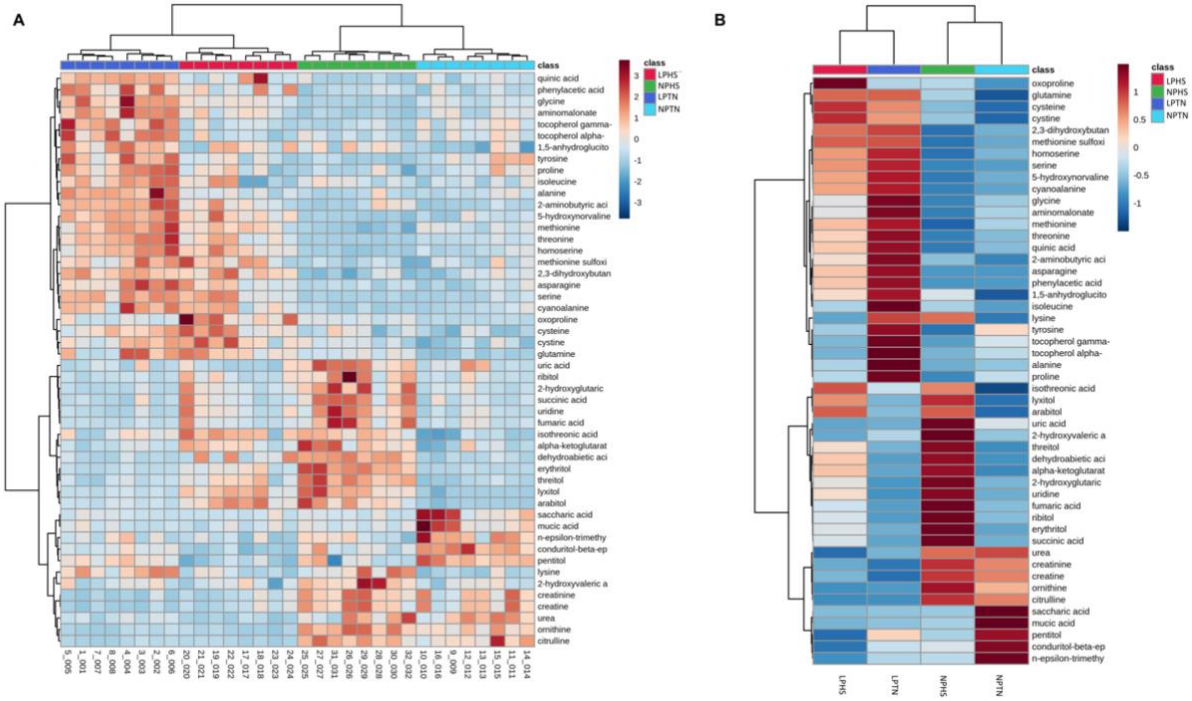


Figure 4. Heat map of plasma metabolites in broilers fed with low protein diets during experimentally induced heat stress

Hierarchical clustering of all significantly different plasma metabolites in birds used in the current study (A), and among birds received NPTN, LPTN, LPHS and HPHS (B). The rows display metabolites, and the columns represent the birds (Figure 4A) or experimental groups (Figure 4B). The dark red or blue the box is corresponding to the magnitude of difference when compared with the average value. The black dendrogram along the left side of the heatmap indicates both the similarity and the order that the clusters were formed. NPTN: normal protein diet under TN; LPTN: low protein diet under TN; NPHS: normal protein diet under HS; LPHS: low protein diet under HS. n=8

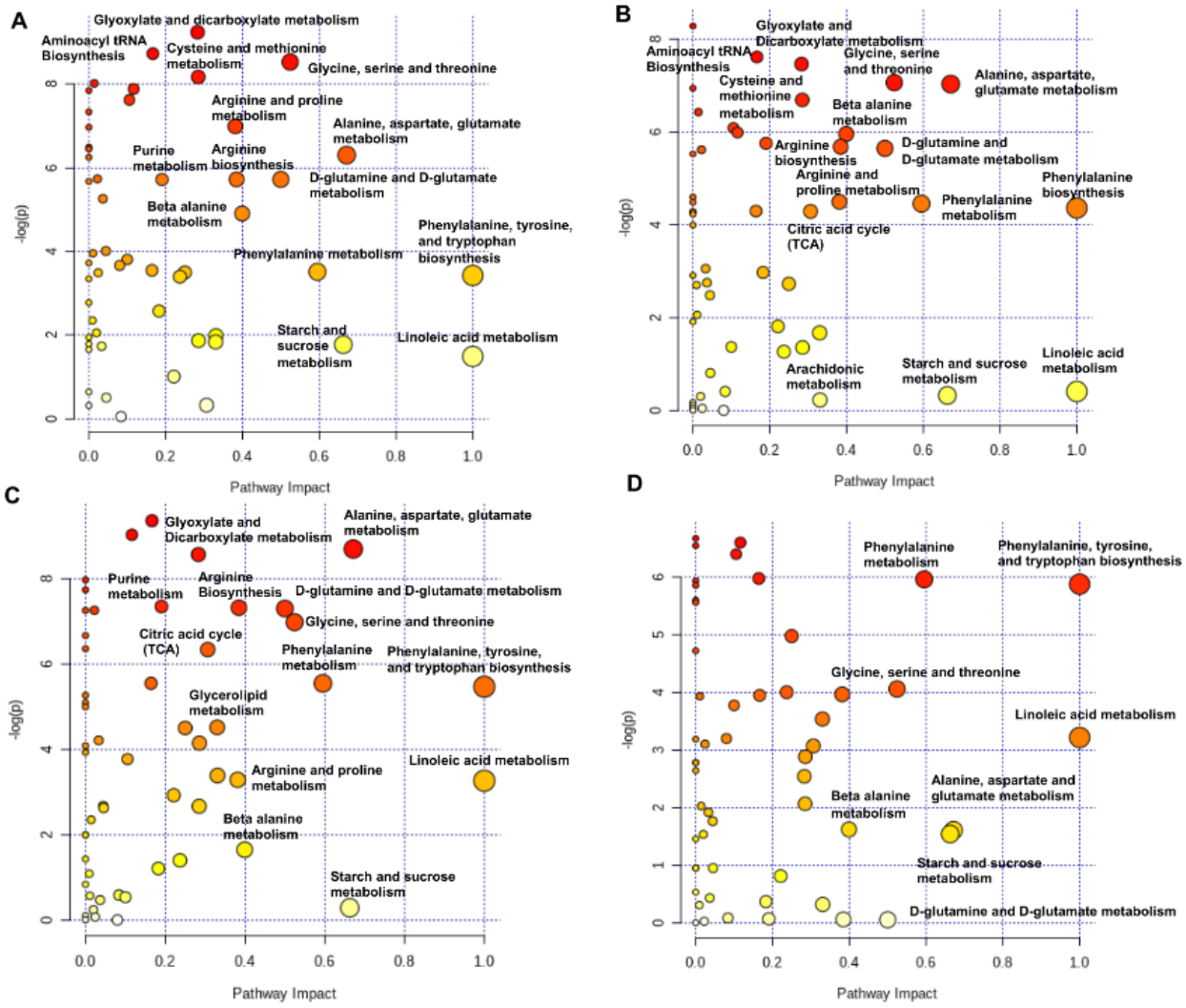


Figure 5. Pathways analysis map of plasma metabolites in broilers fed with low protein diets during experimentally induced heat stress

The map of pathway analysis for the metabolites detected in the blood serum of broilers fed with NPTN vs LPTN (A), NPHS vs LPHS (B), NPTN vs NPHS (C) and LPTN vs LPHS (D). Each circle shows a metabolic pathway. The scores were obtained from topology analysis with pathway impact (x axis) and the pathway enrichment analysis (y axis). The color of each circle is a function of its P value and pathway enrichment, while the size of each circle is based on its impact value. Therefore, darker colors circles are showing the metabolites with more significant changes and higher pathway enrichment and larger size circles are the ones with higher pathway impact. NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress. n=8

Overall, metabolic pathway enrichment analysis showed that metabolites involved in carbohydrates, lipids and protein and amino acids metabolism were affected with experimental treatments. All the plasma metabolites changed significantly among dietary groups are given in Table 1 while the non-significant plasma metabolites are presented in Supplementary Table 1.

Experimental groups affected the plasma metabolites related with amino acids and amino acids derivatives. Compared to NPTN, LPTN reduced aspartic acid, valine, tryptophan and tyrosine, but increased lysine, asparagine, isoleucine, alanine, cysteine, cysteine, glutamine, glycine, methionine, proline, serine and threonine. Compared to NPHS, LPHS tended to reduce aspartic acid, leucine reduced threonine, histidine but increased tyrosine, asparagine cysteine, cysteine, glutamine, glycine, methionine, serine, and tyrosine.

Table 1. Significantly different plasma metabolites in broilers fed with low protein diets during experimentally induced heat stress

Metabolites	Treatments[1]					*P*-Values		
	NPTN[2]	LPTN[2]	NPHS[2]	LPHS[2]	SEM[2]	Diet	Temp[2]	Diet × Temp[2]
Microbiome Metabolism								
Daidzein	981	456	450	292[#]	77	0.11	≤ 0.01	0.57
Dehydroabietic acid	15535	11050	39862	22649[*]	2268	0.04	≤ 0.01	0.02
D-erythro-sphingosine	3241	1570[#]	4367	2602	274	0.05	≤ 0.01	0.57
Lanosterol	691	722[*]	437	615[*]	44	≤ 0.01	0.06	0.99
Lyxitol	67091	80787[*]	256403	180839	16853	0.99	≤ 0.01	0.02
Carbohydrate Derivatives								
Azelaic acid	4516	954[*]	1010	2492[*]	420	0.45	0.26	≤ 0.01
Conduritol-beta-epoxide	101941	35050[*]	47753	19999[*]	6070	≤ 0.01	≤ 0.01	0.09
Galactinol	27915	4352[#]	2314	4083	3215	0.08	0.02	0.03
Gluconic acid	13217	7666	16148	7308[*]	1067	≤ 0.01	0.25	0.05
Hypoxanthine	104405	74054	138897	146782	9351	0.23	≤ 0.01	0.45
Inosine	18380	12763	40730	49878	3913	0.16	≤ 0.01	0.22
Inulotriose	1236	382	236	313[#]	134	0.29	0.02	0.11
Maltotriose	1938	870	470	600	190	0.52	≤ 0.01	0.23
Melezitose	1386	413[#]	276	398	137	0.19	0.02	0.04
Mucic acid	6005	1306[*]	1860	1287	519	≤ 0.01	≤ 0.01	0.02
Pinitol	131195	14550[*]	25924	14371[*]	13666	≤ 0.01	0.02	0.03
Quinic acid	754	679[*]	970	708[*]	202	≤ 0.01	0.03	0.38
Ribonic acid	4072	2204[#]	4061	4129	279	0.82	0.03	0.24
Saccharic acid	45410	6313[*]	8514	6361	4327	≤ 0.01	≤ 0.01	≤ 0.01
Uridine	19823	10327	51224	23595[*]	3337	0.02	≤ 0.01	0.08
Xylitol	85529	35367[#]	146623	74144[#]	9576	0.02	≤ 0.01	0.33
Carbohydrates								
Alpha-ketoglutarate	72780	52059	109710	72904[#]	5242	0.25	≤ 0.01	0.08
Anhydroglucitol	17074	22049[*]	20792	18979	950	≤ 0.01	0.97	0.22

Arabitol	124635	137613*	342129	290263	23793	0.24	≤ 0.01	0.24
Ribose	79755	14043#	28413	14838*	8831	0.03	0.18	0.15
Erythritol	102640	76671	482304	169023*	32002	≤ 0.01	≤ 0.01	≤ 0.01
Erythrose	1857	940	1811	980*	115	≤ 0.01	0.50	0.42
Fructose-6-phosphate	2559	821*	1221	1088	169	0.02	0.16	≤ 0.01
Galactitol	68449	42934	135739	49422*	10523	0.02	0.02	0.02
Glucose-1-phosphate	7286	3336	9567	4894*	616	≤ 0.01	≤ 0.01	0.23
Glucose-6-phosphate	4009	1353*	3123	2191	262	≤ 0.01	0.34	0.16
Lactose	30940	11331	9422	10569	2704	0.21	0.02	0.06
Maltose	108960	27381#	14205	24420	11347	0.15	≤ 0.01	0.03
Raffinose	134815	11567*	2275	12737	16955	0.07	0.02	0.03
Ribitol	17360	8404	47434	16659*	3597	0.02	≤ 0.01	0.06
Sorbital	127390	69622	229553	105516*	15463	0.02	≤ 0.01	0.08
Sucrose	229381	19729#	2622	24149	31247	0.12	0.04	0.05
Tagatose	2062	1328	2371	1370*	111	0.04	0.08	0.04
Threitol	13480	10000	24489	14506*	1117	≤ 0.01	≤ 0.01	≤ 0.01
Xanthosine	805	518	1309	837	97	0.50	0.02	0.57
Xylose	35808	28893*	36246	31959	1386	0.05	0.12	0.25
Protein Metabolism								
Creatinine	164384	22548*	161980	47249*	14773	≤ 0.01	0.14	0.94
Ornithine	237847	52406*	295108	49267*	20748	≤ 0.01	≤ 0.01	≤ 0.01
Urea	269631	74397*	216172	45945*	20909	≤ 0.01	0.29	0.61
Uric Acid	448970	204713	698371	192455*	47905	≤ 0.01	0.02	≤ 0.01
Citrulline	77311	15647*	79028	16167*	6222	≤ 0.01	0.42	0.36
Xanthine	20784	11974	31289	21351	2161	0.48	≤ 0.01	0.76
Amino Acids								
Alanine	973266	2008637*	725231	788927	117331	≤ 0.01	≤ 0.01	≤ 0.01
Asparagine	68949	116533*	58051	87093*	5217	≤ 0.01	0.08	0.08
Aspartic Acid	332966	156243*	262639	169632#	15225	≤ 0.01	0.93	0.58
Cyanoalanine	1040	2143*	612	1741*	135	≤ 0.01	0.07	0.41
Cysteine	12932	21167*	15309	24415*	1342	≤ 0.01	0.12	0.83

Cystine	92902	102225*	101022	122176*	3516	≤ 0.01	0.06	0.81
Glutamine	1543054	4304606*	3263465	4764719*	294103	≤ 0.01	0.17	0.15
Glycine	2009209	3528145*	864965	1947990*	193982	≤ 0.01	≤ 0.01	≤ 0.01
Histidine	119164	76831	148762	52758*	8802	≤ 0.01	0.63	≤ 0.01
Homocystine	3018	2565	5747	2782#	395	0.27	0.04	0.03
Homoserine	1206	2145*	525	1785*	130	≤ 0.01	≤ 0.01	0.81
Isoleucine	952169	997787*	875481	709461	31524	≤ 0.01	0.05	≤ 0.01
Leucine	2153375	1601125	1888973	1308442#	69567	0.86	0.14	0.05
Lysine	49913	115908*	138591	49868*	9792	0.42	0.87	≤ 0.01
Methionine	268057	292729*	145867	248519*	14091	≤ 0.01	≤ 0.01	0.90
Oxoproline	1529819	1806087*	2423289	4983321*	292809	≤ 0.01	≤ 0.01	0.03
Proline	602393	622686*	403365	419135	24679	≤ 0.01	≤ 0.01	0.06
Serine	3638367	8429353*	2420920	6817181*	483269	≤ 0.01	0.06	0.30
Threonine	1363373	4080860*	278928	2330931*	281695	≤ 0.01	≤ 0.01	0.07
Tryptophan	632209	529272*	601717	472079	20849	0.20	0.41	0.02
Tyrosine	1687441	1592337*	760553	936369*	88889	≤ 0.01	≤ 0.01	0.33
Valine	839510	641914#	670200	567541	23844	0.13	0.02	0.12
Fatty Acid Metabolism								
2-aminobutyric acid	22894	51013*	28548	33046#	2581	≤ 0.01	0.05	≤ 0.01
2-deoxytetronic acid	6335	3268*	6897	5090	355	0.03	≤ 0.01	0.82
2-hydroxyglutaric acid	16007	7221	52619	19386*	3798	≤ 0.01	≤ 0.01	0.04
2-hydroxyvaleric acid	12376	84595	15595	8457*	739	≤ 0.01	0.03	≤ 0.01
3-hydroxybutyric acid	906092	783907	1457671	740685*	90306	0.72	≤ 0.01	0.92
Beta-sitosterol	10643	5324	5284	5139	592	0.41	≤ 0.01	0.10
Cholesterol	4343379	3459489#	3347360	3539811#	175985	≤ 0.01	0.39	0.72
Glycerol	1517631	714810	3270495	1716658	244585	0.11	≤ 0.01	0.37
Glycerol-alpha-phosphate	8362	3151*	10609	5407#	681	≤ 0.01	≤ 0.01	0.51
Lauric acid	27298	24131#	19406	17229	1231	0.08	≤ 0.01	0.09
Linoleic acid	5170	4769	3030	2728	391	0.21	≤ 0.01	0.37
Monostearin	1149	817	661	684	78	0.37	0.07	≤ 0.01
Palmitoleic acid	1475	1349*	2359	2237	204	0.18	≤ 0.01	0.93

Amino Acids Metabolism								
3-hydroxyanthralinic acid	755	1316*	989	580	97	0.06	0.11	≤ 0.01
Aminomalonate	173601	301913*	63777	164296*	18198	≤ 0.01	≤ 0.01	0.03
Fumaric acid	257967	98137	1051705	274893*	87753	≤ 0.01	≤ 0.01	0.03
Guanidinosuccinate	1975	1478	1359	1406*	71	≤ 0.01	≤ 0.01	0.60
Ketoisocaproic acid	35336	25286	29942	15621#	2043	0.18	0.06	0.05
Amino Acid Derivatives								
Cytosin	1575	920	2333	994*	179	0.04	0.08	0.10
Dihydroxypyrazine	12424	12135*	10803	12094#	359	≤ 0.01	0.98	0.56
5-hydroxynorvaline	6412	11442*	3516	9150*	627	≤ 0.01	≤ 0.01	0.36
Maleimide	12622	9896#	6841	8918*	584	≤ 0.01	≤ 0.01	0.40
Malic acid	443186	164881	1208719	421807#	100106	0.03	≤ 0.01	0.16
Malonic acid	1616	662	542	448	131	0.11	≤ 0.01	0.16
Phosphoethanolamine	195180	129800#	114912	147009*	376	0.01	0.22	0.67
Pimelic acid	7760	3957#	3794	7610*	614	0.11	0.70	≤ 0.01
Pipecolinic acid	1964	1603*	1041	1805*	123	≤ 0.01	0.16	0.11
Serotonin	38246	3197*	8308	7403	4485	0.04	0.15	0.04
Spermidine	1623	1769*	1350	2194*	125	≤ 0.01	0.40	0.44
Trans-4-hydroxyproline	581997	650934*	462090	587206*	30640	≤ 0.01	0.25	0.51
Vitamins								
Creatine	202876	24225*	194651	61177*	18256	≤ 0.01	0.12	0.80
Isothreonic acid	31145	28587*	44912	38834	1612	0.02	≤ 0.01	0.11
Methionine sulfoxide	46777	50248*	33994	54009*	2550	≤ 0.01	0.52	0.64
N-acetyl-D-galactosamine	4327	3112	9057	4913#	608	0.23	≤ 0.01	0.09
N-acetylglycine	2720	1839	916	1069	300	0.61	≤ 0.01	0.79
N-epsilon-trimethyllysine	5077	2024*	2594	1728	304	≤ 0.01	≤ 0.01	0.09
Nicotinamide	7677	13374*	10667	10662	655	≤ 0.01	0.91	0.02
Pentitol	1914	927*	954	587	110	≤ 0.01	≤ 0.01	0.54
Threonic acid	18822	12198	19678	10973*	851	≤ 0.01	0.50	≤ 0.01
Tocopherol alpha	38575	49087*	27368	22267	2628	≤ 0.01	≤ 0.01	≤ 0.01
Tocopherol gamma	2279	2942*	1236	1220	172	≤ 0.01	≤ 0.01	≤ 0.01

Lipids								
Zymosterol	9932	8408[#]	5406	8045[*]	619	≤ 0.01	0.07	0.37
Miscellaneous								
Butyrolactam	22155	17057	25627	16183[#]	1314	0.42	0.73	0.04
Dihydroxybutanoic acid 2-3	30697	39023[*]	19976	39404[*]	2054	≤ 0.01	0.13	0.56
Ethanolamine	159642	114369	185066	136941	8063	0.73	0.05	0.34
Phenylacetic acid	2038	3109[*]	1674	2521[*]	172	≤ 0.01	0.09	0.11
Pseudouridine	4679	2651	5355	3891	337	0.34	0.02	0.99
Succinic acid	907060	509673	2894350	982356[*]	201883	≤ 0.01	≤ 0.01	0.02

[1]The values are the mean peak height; n=8 per treatment

[2]NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress; SEM: standard error of means; Temp: temperature.

[*]Within rows, NPTN vs LPTN and NPHS vs LPHS $P \leq 0.05$

[#] Within rows, NPTN vs LPTN and NPHS vs LPHS $0.05 \leq P \leq 0.10$

Compared to NPTN, LPTN reduced dihydroxypyrazine, pipecolinic acid and serotonin and tended to decrease maleimide, phosphoethanolamine and pimelic acid, but increased, 5-hydroxynorvaline, spermidine, and trans-4-hydroxyproline. Compared to NPHS, LPHS decreased cytosine and tended to decrease malic acid but tended to increase dihydroxypyrazine, and increased 5-hydroxynorvaline, maleimide, phosphoethanolamine, pimelic acid, pipecolinic acid, spermidine, and trans-4-hydroxyproline.

For carbohydrate compared to NPTN, LPTN decreased fructose-6-phosphate, glucose-6-phosphate, raffinose, xylose and tended to decrease ribose, maltose, and sucrose but increased anhydroglucitol and arabitol. When compared to NPHS, LPTN decreased ribose, erythritol, erythrose, galactitol, glucose-1-phosphate, ribitol, sorbital, tagatose, threitol, and tended to decrease alpha-ketoglutarate.

For carbohydrate derivatives, relative to NPTN, LPTN reduced azelaic acid, conduritol-beta-epoxide, quinic acid and tended to decrease melezitose, ribonic acid and xylitol but increased mucic acid, pinitol and saccharic acid. Compared to NPHS, LPHS decreased conduritol-beta-epoxide, gluconic acid, pinitol, quinic acid, uridine and tended to decrease xylitol but increased azelaic acid and tended to increase inulotriose.

For protein metabolism, compared to NPTN, LPTN decreased creatinine, ornithine, urea, and citrulline. Compared to NPHS, LPHS decreased creatinine, ornithine, urea, uric acid and citrulline. For amino acids metabolism, relative to NPTN, LPTN increased 3-hydroxyanthralinic acid and aminomalonate. Compared to NPHS, LPHS

decreased fumaric acid and tended to decrease ketoisocaproic acid but increased aminomalonate and guanidinosuccinate.

For fatty acid metabolism, relative to NPTN, LPTN decreased 2-deoxytetronic acid, glycerol-alpha-phosphate, lauric acid, palmitoleic acid and tended to decrease cholesterol but increased 2-aminobutyric acid. Compared to NPHS, LPHS decreased 2-hydroxyglutaric acid, 2-hydroxyvaleric acid, 3-hydroxybutyric acid, glycerol-alpha-phosphate, but tended to increased 2-aminobutyric acid and cholesterol.

Cecal Microbiota

The rarefaction curve analysis showed that all samples analyzed reached a stable plateau at 40,000 reads for each sample suggesting that the sequencing depth was sufficient to saturate the bacterial communities in cecal samples (Supplementary Figure 1).

The beta diversity of cecal bacterial community was analyzed by principal coordinates analysis (PCoA) of unweighted UniFrac distances representing the diversity of cecal bacterial populations across all birds assigned to four treatments (Figure 6A), unweighted UniFrac distances shown across dietary groups (Figure 6B), PCoA of weighted UniFrac distances representing the diversity of cecal bacterial populations across all birds assigned to four treatments (Figure 6C), and weighted UniFrac distances shown across dietary groups (Figure 6D). PCoA showed a tendency for separation and clustering when

NPTN and NPHS were compared using unweighted UniFrac (P = 0.07; Figure 6A) suggestive of the differences in cecal microbiota composition among birds assigned to these groups (Figure 6B). Cecal microbial composition showed no clustering among groups when weighted UniFrac was considered (Figure 6C and 6D).

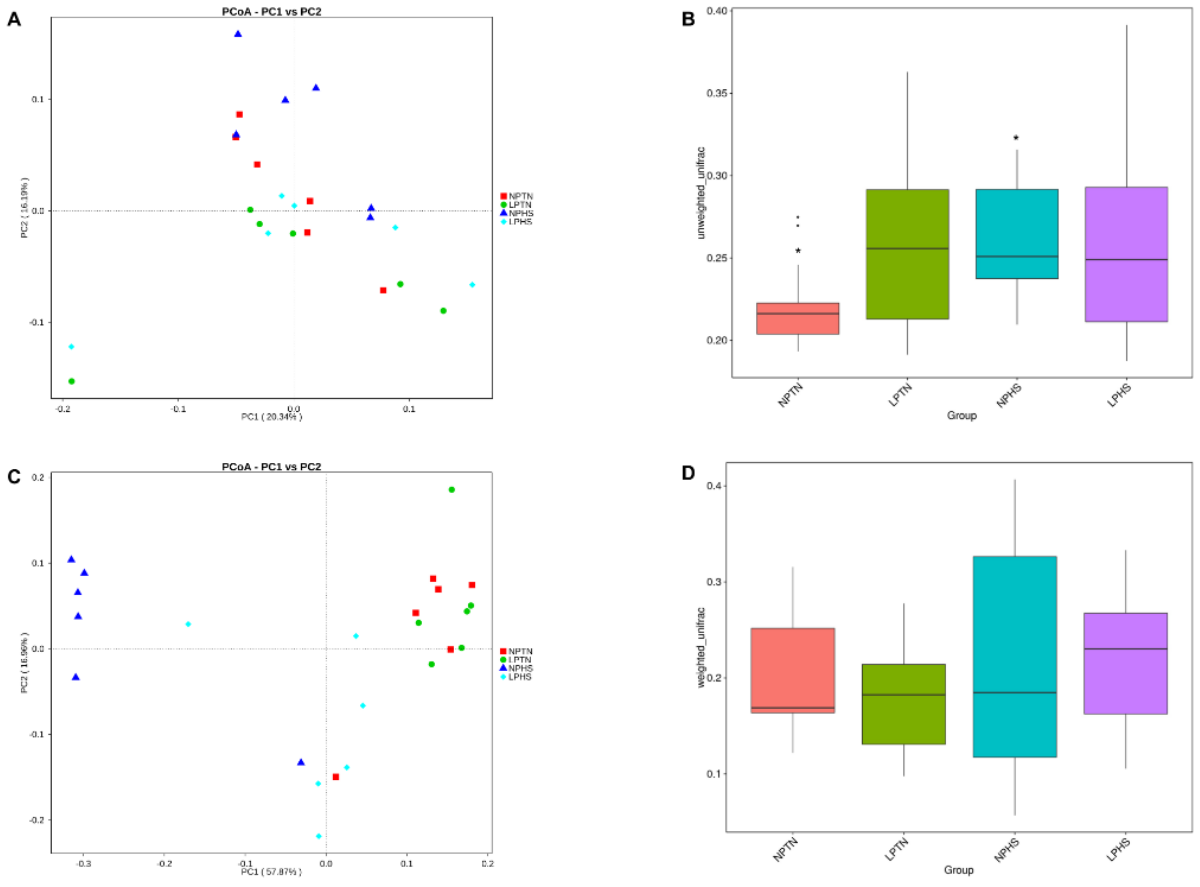


Figure 6. Beta diversity of cecal bacterial community in broilers fed with low protein diets during experimentally induced heat stress

Principal Coordinates Analysis (PCoA) of unweighted UniFrac distances representing the diversity of cecal bacterial populations across all birds assigned to 4 treatments with each node being indicative of an individual bird (A), unweighted UniFrac distances shown across dietary groups (B), PCoA of weighted UniFrac

distances representing the diversity of cecal bacterial populations across all birds assigned to 4 treatments with each node being indicative of an individual bird (C), and weighted UniFrac distances shown across dietary groups (D). NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress. Common symbols in the box plots indicate a tendency for significance ($0.05 < P \leq 0.1$, Tukey HSD). n=6 per treatment.

Alpha diversity of cecal bacterial community in each sample is shown in Figures 7A-D. Chao, Observed_species, Shannon and Simpson diversity of cecal bacterial community were significantly different between NPTN and NPHS (Figures 7A-D). Further, Shannon and Simpson diversity were significantly different between NPHS and LPHS (Figure 7C and 7D). Overall, the three main phyla present in all four dietary treatments were Clostridia, Mollicutes, and Bacteroidia (Figure 8A and Supplementary Figure 2). At genus level, *Ruminococcacea_UCG-014*, *Bacteroides* and *Akkermansia* were the most abundant communities across all dietary treatments (Figure 8B and Supplementary Figure 2)

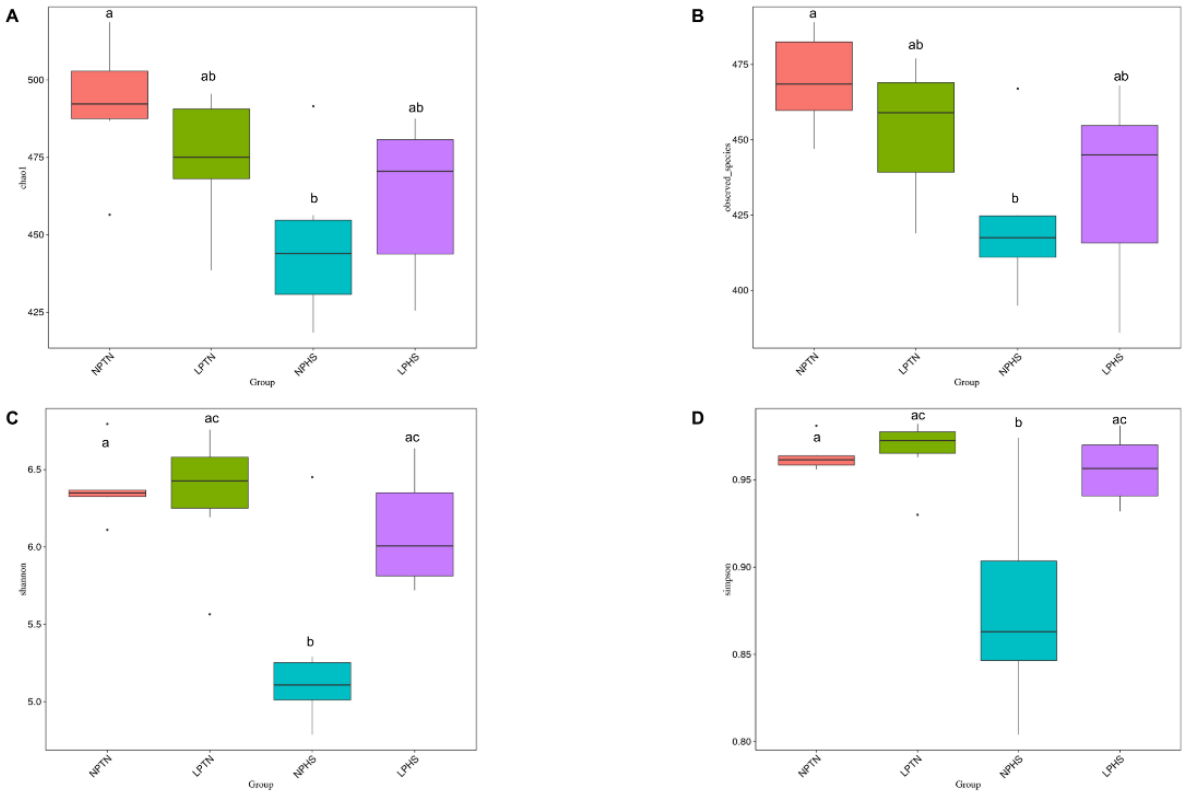


Figure 7. Alpha diversity of cecal bacterial community in broilers fed with low protein diets during experimentally induced heat stress

Chao1 (A), Observed (B), Shannon (C) and Simpson (D). NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress. Different letters in the box plots indicate significant differences ($P \leq 0.05$, Tukey HSD). n=6 per treatment.

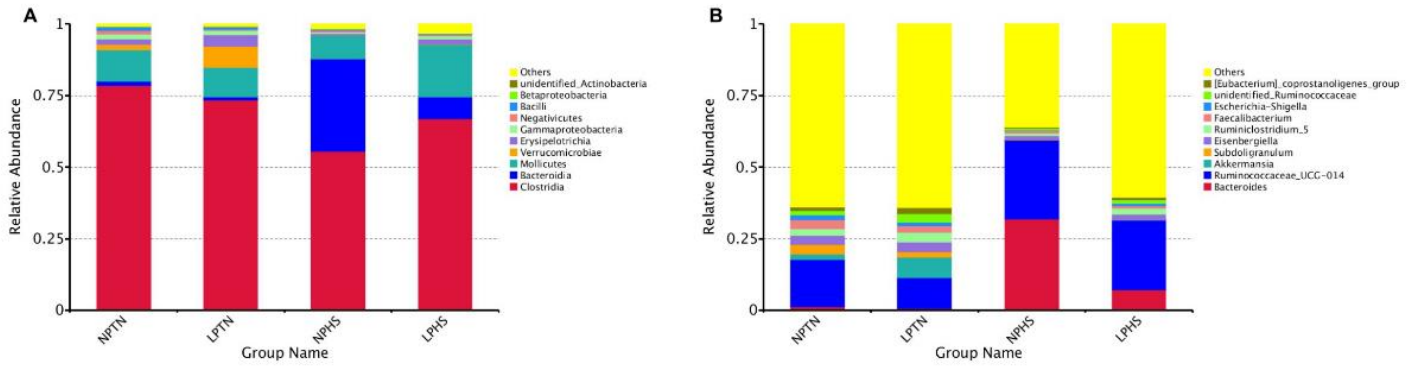


Figure 8. The composition of cecal bacterial populations in broilers fed with low protein diets during experimentally induced heat stress

The relative abundance of bacterial community composition at phylum level (A) and at genus level (B). NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress. For clarity reasons, only the top 10 phyla and genera are shown. n=6 for each treatment.

Linear discriminant analysis (LDA) with effect size measurements (LEfSe) was used to identify organisms that are different among dietary treatments. Compared to NPTN, cacal contents of LPTN had higher proportions of c*_Erysipelotrichia*, *o_Erysipelotrichales*, and *f_Erysipelotrichaceae* (LDA [log10] score> 2.0; Fig. 9A). Cecal contents of birds in LPHS group were more enriched in *p_Tenericutes, c_Mollicutes, c_Mollicutes_RF9, and f_tachnospiraceae* compared to those in the NPHS treatment, while NPHS had higher abundance of bacteria under phylum Bacteroidetes (Fig. 9B). Relative to NPTN group, birds subjected to NPHS treatment had higher proportions of *c_Bacteroidia*, *o_Bacteroidales*, *p_Bacteroidetes*, *f_Bacteroidaceae*, and g*_Bacteroides*, while NPTN group had higher proposition of *o_Verrucomicrobiales, c_Verrucomicrobiae, f_Verrucomicrobiaceae,* and *g_Akkermansia* (Figure 9C). Lastly,

compared to LPTN, cecal contents of birds in LPHS treatment had higher proportions of *g_Ruminococcaceae_UCG_014*, *c_Bacteroidia*, *o_Bacteroidales*, *p_Bacteroidetes*, *f_Bacteroidaceae, and g_Bacteroides* while cecal contents of LPTN birds were more enriched in *c_Erysipelotrichia*, *o_Erysipelotrichales*, *f_Erysipelotrichaceae*, *o_Verrucomicrobiales*, *c_Verrucomicrobiae*, *f_Verrucomicrobiaceae* and *g_Akkermansia* (Figure 9D).

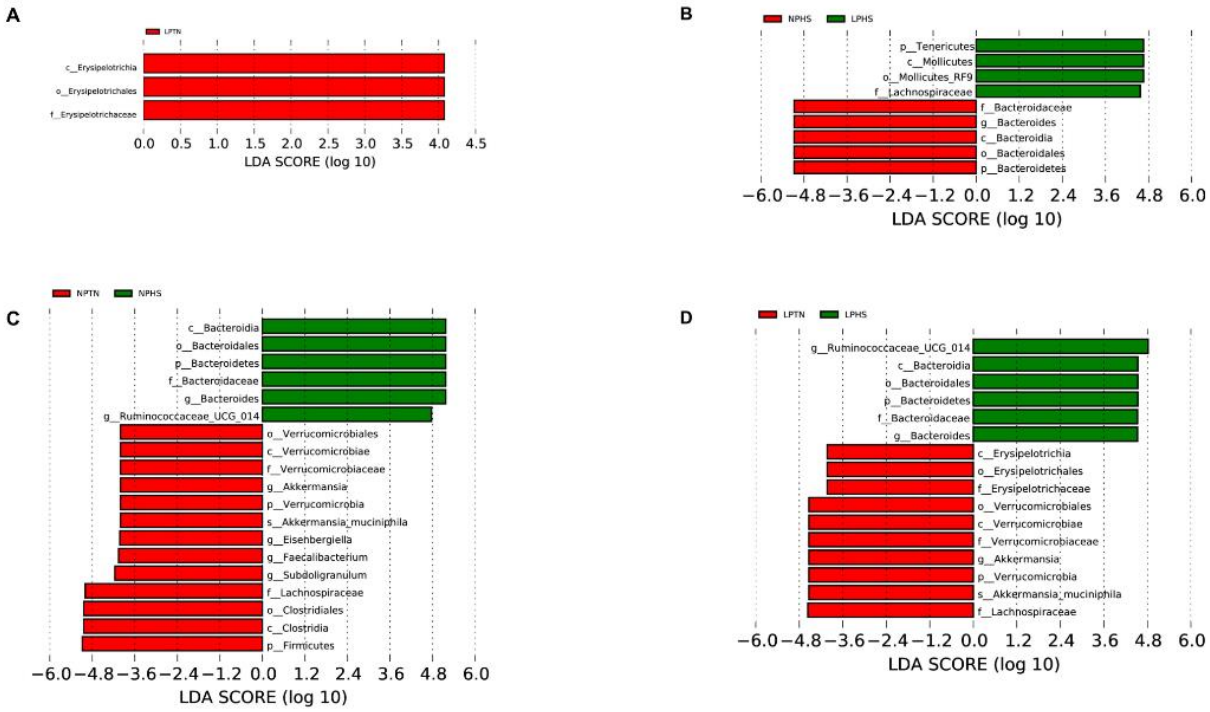


Figure 9. Histograms of cecal microbiota composition at phylum level in broilers fed with low protein diets during experimentally induced heat stress

Histograms of cecal microbiota composition using linear discriminant analysis (LDA) with effect size (LEfSe) for NPTN vs LPTN (A), NPHS vs LPHS (B), NPTN vs NPHS (C) and LPTN vs LPHS (D). NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress. n=6 per treatment.

Discussion

Heat stress is a major environmental concern which adversely effects the birds' immunity, metabolism, microbiota composition and growth performance (Nawab et al., 2018, Kumar et al., 2021). Low-protein diets have been shown to improve the survivability of birds under HS by reducing heat production (low heat increment) and feed intake (Ghasemi et al., 2020, Daghir et al., 2009, Awad et al., 2017, Swennen et al., 2007, Syafwan et al., 2011; Musharaf et al., 1999). Little is known whether beneficial effects of LP diets on survivability of birds under HS are associated with changes in the gut microbiota composition and blood metabolites profile. Therefore, the objective of this study was to assess the effects of LP diets on cecal microbiota composition, plasma metabolomics profile and oxidative stress in broiler chickens under experimentally induced HS. Overall, our study revealed several key findings: (1) metabolites involved in carbohydrates, lipids and protein and amino acids metabolism were affected with experimental treatments, (2) compared to NPHS, chickens in LPHS group had higher abundances of *p_Tenericutes*, *c_Mollicutes*, *c_Mollicutes_RF9*, and *f_tachnospiraceae* compared to those in the NPHS treatment, while NPHS had higher abundance of bacteria under phylum Bacteroidetes, (3) cecal contents of birds in LPHS treatment had higher proportions of *g_Ruminococcaceae_UCG_014, c_Bacteroidia and o_Bacteroidales,* while cecal contents of LPTN birds were more enriched in c*_Erysipelotrichia*, *o_Erysipelotrichales*, *f_Erysipelotrichaceae, o_Verrucomicrobiales, and g_Akkermansia*, (4) under TN

conditions, birds fed with LP had a lower plasma SOD activity and LPO compared to NP, but no differences on these measurements were detected during HS. In summary dietary protein content influences plasma metabolites with similar patterns during both TN and HS conditions but had differential effects on cecal microbiota composition under TN and HS conditions.

Three main phyla found in cecal contents were Clostridia, Bacteroidia, and Mollicutes. At genus level, *Ruminococcacea_UCG-014, Bacteroides* and *Akkermansia* were the most abundant communities. Other studies show that Clostridia numbers were lower in birds under (Awad et al., 2018). In a study by Song et al., (2014), Lactobacillus was found to be the most abundant in birds under TN and HS conditions followed by Bifidobacterium. It is believed that changes in stressors including internal and external stressors such as environmental temperature, pH, and water availability cause bacteria to alter their response and may also cause a challenge for survival if the stressors are too much for the specific bacteria (Traub-Dargatz et al., 2006). Clostridium and *Bacteroides* have been shown to have polysaccharide-degrading activity against nonstarch polysaccharides (NSP) which can be found in cereal grains such as corn which make up large component of poultry diets (Singh et al., 2012, Beckmann et al., 2005, Knudsen et al., 2014). Mollicutes, another major bacterium found at higher abundance in LPHS compared to NPHS, has been associated with increased body fat and upregulation of metabolic pathways involved with the import and fermentation of simple sugars and host glycans (Fujimura et al., 2010). It has been suggested that Mollicutes metabolize carbohydrates to

short chain fatty acids which are readily absorbed by the host (Turnbaugh et al., 2008). Relative to LPTN and NPHS treatments, LPHS had higher population of Bacteroidia and Mollicutes which is suggestive of an increased metabolism and digestibility of NSPs and uptake of simple sugars by LPHS. Therefore, it appears that LP diets increase the population of bacteria that digest carbohydrates under HS conditions.

Dietary protein content impacted plasma metabolites related with metabolism and biosynthesis of alanine, aspartate, glutamate and phenylalanine during TN and HS. Lu et al., 2018 also found that amino acids such as proline, cysteine, methionine, and cysteine are increased in birds under HS conditions. It has been suggested that chronic HS decreases muscle protein deposition in birds by specifically reducing protein synthesis (Zuo et al., 2014) which might explain the increased levels of amino acids found in the plasma. In addition to protein amino acids, an increase in non-protein amino acids such as ornithine, creatine and citrulline have been reported in birds during HS (Lu et al., 2018).

Under TN conditions, birds fed with LP diets had a lower plasma SOD and LPO compared to NP, but no differences on these measurements were detected during HS. Also, birds fed with LP diet had a higher lipid hydroperoxides than NP under HS. During HS, reactive oxygen species (ROS) levels exceed the level of antioxidants which can damage lipids, proteins, carbohydrates, and DNA which could lead to aging, loss of protein function, reduced enzyme activity, and development of pale, soft and exudative meat often referred to as PSE or ‘atypical poultry meat’ conditions (Estevez et al., 2015; Solomon et

al, 1998; Zaboli et al., 2019). SOD and GPx play vital roles in protecting cell membrane from ROS damage. Others found that birds in HS conditions had an increased MDA which is the result of an increased production of ROS (Altan et al., 2010). In our study, we found that that under TN conditions, LP had a lower MDA plasma concentration when compared to NP and in HS, LP had a numerically lower MDA plasma concentration compared to NP. This may suggest that LP fed birds had a lower oxidative stress when compared to NP fed birds. It has previously been reported that birds under HS conditions have increased levels of oxidative stress which in return will cause physiologic changes such as muscle pH drop (Zaboli et al., 2019). MDA results are indicative of decreased oxidative stress in LP fed birds in both TN and HS conditions.

Conclusion

Dietary protein content impacted plasma metabolites related with metabolism and biosynthesis of alanine, aspartate, glutamate and phenylalanine during TN and HS. Compared to NP, chickens fed with LP diet had higher abundances of *p_Tenericutes*, *c_Mollicutes*, *c_Mollicutes_RF9*, and *f_tachnospiraceae* under HS. Our data show that dietary protein content influences the plasma metabolites with similar pattern during TN and HS, but that had differential effects on cecal microbiota composition under TN and HS.

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Supplementary Tables

Supplementary Table 1. Non-significant plasma metabolites in broilers fed with low protein diets during experimentally induced heat stress

Metabolites	Treatments[1]					*P*-Values		
	NPTN[2]	LPTN[2]	NPHS[2]	LPHS[2]	SEM[2]	Diet	Temp[2]	Diet × Temp[2]
Aconitic acid	10961	7194	10977	6944*	641	0.24	0.71	0.15
Adenosine	554	457	1513	749	175	0.38	0.06	0.19
Adenosine-5-monophosphate	1836	1506	1951	1420	158	0.81	0.77	0.24
Alloxanoic acid	1102	685	739	737	66	0.80	0.51	0.49
Aminovaleric acid	27753	10081	6239	9171*	4320	0.72	0.18	0.29
Anhydro-D-galactose	2743	1977	1864	2013	115	0.12	0.18	0.41
Arachidonic acid	8050	4361	4972	4339	416	0.32	0.07	0.19
Beta alanine	30291	24373	25862	22923	1952	0.59	0.73	0.60
Cellobiose	6968	4463	5279	4829	372	0.73	0.60	0.69
Chlorogenic acid	387	286	539	332	55	0.59	0.38	0.36
Cholesterone	929	489	589	533	50	0.54	0.32	0.32
Citric acid	1365683	953893	1149390	819214	68579	0.75	0.17	0.19
Cysteine glycine	2208	1692	2049	1597	80	0.57	0.88	0.23
Deoxy-5-methylthioadenosine	1019	674	959	883	53	0.67	0.25	0.93
Deoxypentitiol	4888	3695	4574	3885	206	0.45	0.60	0.57
Fructose	121412	19170	12677	18955	20703	0.29	0.17	0.20
Fucose	40990	24241	37045	28479	1662	0.17	0.18	0.95
Glucose	6775063	3551424	5890083	4807472	309293	0.22	0.32	0.39
Glutamic acid	661273	430980	798974	514513	46183	0.34	0.08	0.32
Glyceric acid	57758	49405*	55774	46473	2486	0.24	0.66	0.09

Glycolic acid	25366	19062	20280	16428	879	0.54	0.07	0.22
Guanosine	1433	774	989	1412	133	0.31	0.36	0.12
Hydroxybutanoic acid	171810	114226	149515	95181	12021	0.44	0.46	0.49
Hydroxycarbamate	3143	2602	2155	2434	161	0.07	0.17	0.97
Hydroxy-3-methylglutaric acid	1608	986	1040	993	74	0.82	0.08	0.30
Hydroxyphenylacetic acid	1652	1031	1011	982	81	0.74	0.06	0.47
Hydroxyphenyl propionic acid	3994	1855	1916	1291	362	0.25	0.06	0.58
Hydroxypropionic acid	41671	25135	30334	27577	1791	0.91	0.43	0.52
Indole-3-acetate	5263	8257*	4962	9756	1094	≤ 0.01	0.78	0.96
Indole-3-lactate	3608	3801	3209	2540	294	0.28	0.22	0.12
Isocitric acid	49960	29311	37732	29183	2566	0.31	0.24	0.89
Inosine-5-monophosphate	1964	883#	1721	1702	158	0.70	0.10	0.16
Lactic acid	4913087	3014734	4598820	3418810	322820	0.64	0.58	0.81
Maleic acid	1982	1379	2136	1345	110	0.45	0.36	0.21
Mannose	175608	84619	155980	117915	11786	0.28	0.34	0.54
Methyl-O-D-galactopyranoside	8834	5384	8560	7829	490	0.97	0.09	0.51
Myoinositol	435208	435208*	723105	608941	38954	0.20	0.22	0.17
N-acetylaspartic acid	7102	4839	6317	5656	448	0.78	0.63	1.00
N-acetylornithine	14447	9827	14836	14101	887	0.68	0.10	0.55
Nicotinic acid	1966	5647*	5577	5213	2336	0.23	0.10	0.26
Oxalic acid	103175	68793	64218	61229	6617	0.78	0.16	0.79
Pantothenic acid	5718	3054	4274	3734	333	0.56	0.96	0.32
Pentose	7030	5112	6071	5563	395	0.47	0.82	0.87
Phenylalanine	604111	375028	549289	392191	22022	0.14	0.68	0.50
Phenylethylamine	195180	129800	114912	147009*	11349	0.24	0.26	0.23
Phenyllactic acid	4071	1404	2752	1404*	354	0.19	0.09	0.54
Phosphate	2673412	1835797	1980977	1838620	80122	0.28	0.08	0.67
Phosphoglycerate	2218	1445	1229	1548	153	0.34	0.26	0.30
Picolinic acid	1964	1602#	1041	1805*	214	0.53	0.06	0.86
Putrescine	16802	8579	27303	19258	3801	0.56	0.12	0.88
Quinolinic acid	754	679	970	708	42	0.55	0.18	0.15
Shikimic acid	17727	7809*	17286	14991	1344	0.29	0.06	0.28
Thymine	1573	923	1052	1127	73	0.71	0.78	0.08
Xanthosine	805	518	1309	837	97	0.71	0.78	0.08

Xylonic acid	1559	918	1359	1013	84	0.24	0.72	0.81
Xylulose	19029	4141[#]	6905	5324	500	0.08	0.61	0.32

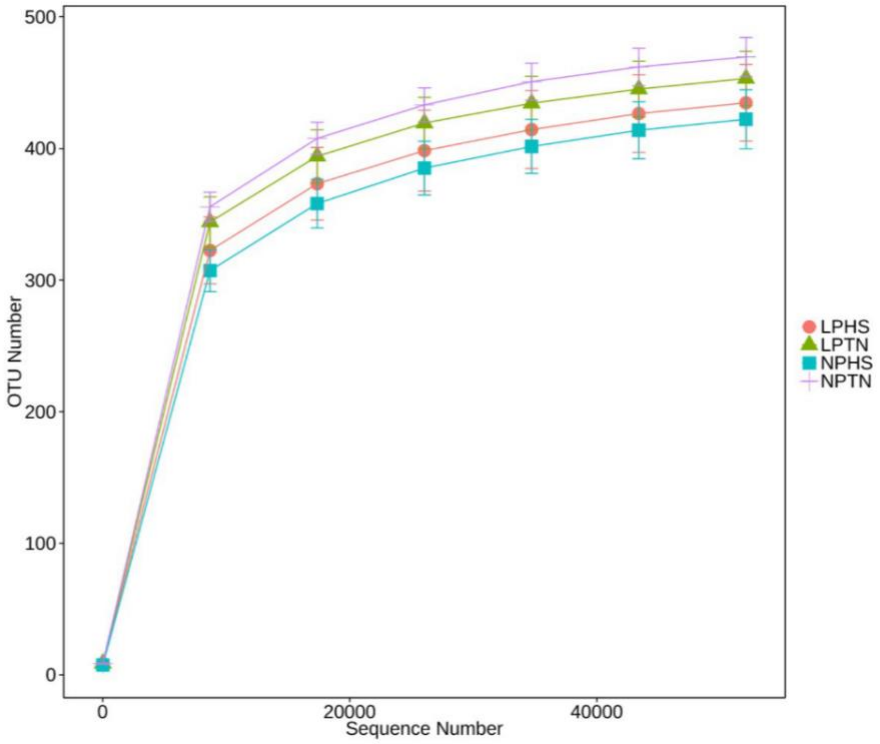
[1]The values are the mean peak height; n=8 per treatment

[2]NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress; SEM: standard error of means; Temp: temperature.

[*]Within rows, NPTN vs LPTN and NPHS vs LPHS $P \leq 0.05$

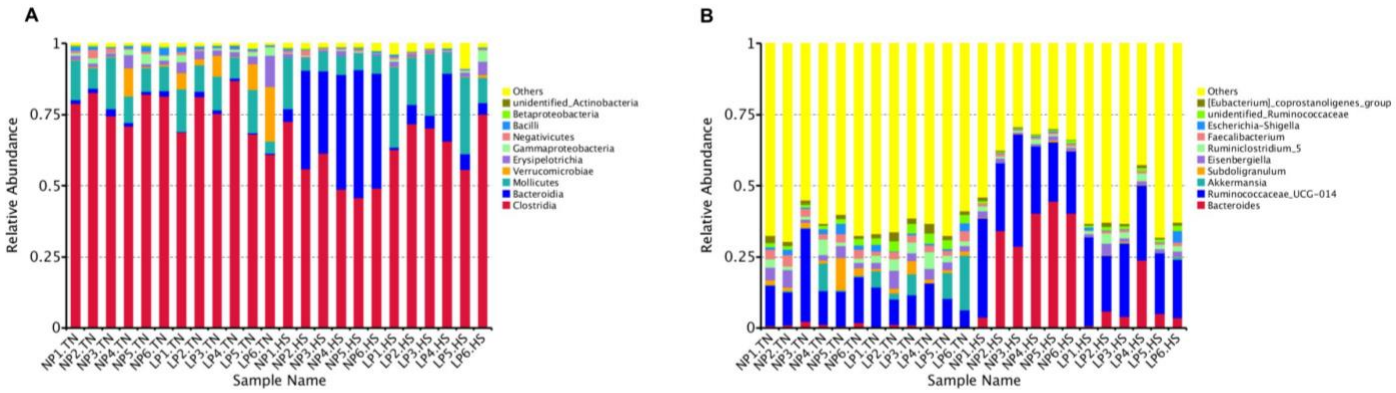
[#] Within rows, NPTN vs LPTN and NPHS vs LPHS $0.05 < P \leq 0.10$

Supplementary Figures



Supplementary Figure 1. Cecal rarefaction curve analysis for broilers fed with low protein diets during experimentally induced heat stress

The rarefaction curves from cecal samples represent the number of operational taxonomic units (OTU) as a function of the number of reads sampled. NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress. n=6 for each treatment.



Supplementary Figure 2. The composition of cecal bacterial populations in broilers fed with low protein diets during experimentally induced heat stress

The relative abundance of bacterial community composition in cecal samples of individual broilers at phylum level (A) and at genus level (B). NPTN: normal protein diet under thermoneutral; LPTN: low protein diet under thermoneutral; NPHS: normal protein diet under heat stress; LPHS: low protein diet under heat stress. For clarity reasons, only the top 10 phyla and genera are shown. n=6 for each treatment.

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Awards: First place in oral presentation at FAPC/IFT-OK Research Symposium

Third Place for excellent presentation, comprehension, and engagement College of Agriculture Sciences and Natural Resources 3 MT Competition

Publications:

1. Shili, Cedrick N et al., "Effect of a Phytogenic Water Additive on Growth Performance, Blood Metabolites and Gene Expression of Amino Acid Transporters in Nursery Pigs Fed with Low-Protein/High-Carbohydrate Diets." Animals, vol. 11, no. 2, 2021, p. 555., doi:10.3390/ani11020555

2. Parniyan Goodarzi et al., "PSIV-7 Effect of dietary tryptophan supplementation on growth, energy balance and blood metabolites in milk-fed low birth weight pigs, *Journal of Animal Science*, Volume 98, Issue Supplement_4, November 2020, Page 280, doi:10.1093/jas/skaa278.505

3. Mohammad Habibi et al., "The Combination of Dietary Branched-Chain and Limiting Amino Acids Modulates Feed Intake Through Both Peripheral and Central Mechanisms in Pigs Fed with Low Protein Diets", *Current Developments in Nutrition*, Volume 4, Issue Supplement_2, June 2020, Page 686, doi:10.1093/cdn/nzaa050_009