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THE ROLE OF FEED ADDITIVES IN MITIGATING THE EFFECT OF STRESSORS ON GROWTH, DIGESTIBILITY, INTESTINAL MORPHOLOGY, PERMEABILITY, AND IMMUNE RESPONSE IN POULTRY

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

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ABSTRACT OF DISSERTATION

THE ROLE OF FEED ADDITIVES TO MITIGATE THE EFFECT OF STRESSORS ON GROWTH, DIGESTIBILITY, INTESTINAL MORPHOLOGY, PERMEABILITY, AND IMMUNE RESPONSE IN POULTRY

Three experiments were conducted to evaluate the interactive effects of dietary supplements in birds exposed to different stressors, on growth performance, nutrient, and energy digestibility, antioxidant status, intestinal permeability, and immune status. The first study was conducted to evaluate the effect of a commercially available algae-based antioxidant, containing Selenium yeast (EconomasE™, EcoE) and two inorganic sodium sources (NaCl and NaCl+$NaHCO_3$) on growth performance, nutrient and energy digestibility and utilization, antioxidant, and immune status of broilers challenged with oral gavage of dexamethasone (DEX). The inclusion of either of the sodium sources did not affect plasma corticosterone and antioxidant status levels, growth performance, and the relative weights of the lymphoid organs. Homeostasis was altered with DEX, evident in the increased ($P < 0.05$) levels of corticosterone in the plasma, reduced ($P < 0.05$) growth performance, and nutrient digestibility. The EcoE supplementation did not mitigate the performance parameters however, its supplementation in the diet increased ($P < 0.05$) nutrient and energy utilization and decreased corticosterone serum levels.

The objective of the second study was to evaluate the effect of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+$NaHCO_3$) in layers exposed to different environmental temperatures. Dietary treatment did not improve performance, egg quality, intestinal morphology, keel bone, bone-breaking strength, and HSP 70 and 90 during ET1 and ET2. Exceptions to this were the increase ($P < 0.05$) in the albumen height and Haugh unit with EcoE and NaCl+$NaHCO_3$ during TN2 and EcoE alone during the ET2 regimen, which suggests that the supplementation can improve the fresh appearance of the egg during ET conditions. Similarly, NaCl+$NaHCO_3$ as the sodium source helped limit the effects of respiratory alkalosis by reducing Cl^- levels and increasing HCO_3^- during the ET regimen. In normal temperature conditions, EcoE and NaCl+$NaHCO_3$ diet were able to improve ($P < 0.05$) villus height and villus height: crypt depth ratio.

The third study evaluated the effect of DEX and coccidia vaccine challenge in broiler chickens fed diets supplemented with or without Natustat™ (a natural plant-derived proprietary product composed of at least one yeast-derived MOS plus organic mineral nutrients and plant extracts) on growth performance, nutrient and energy digestibilities and utilization, intestinal barrier integrity, and immune response. Throughout the experiment, birds were fed a standard corn-SBM diet supplemented with or without Natustat™ at 1

g/kg. Within each diet group, the birds were randomly assigned to four treatments: CON (no-challenge), dexamethasone (DEX), coccidia vaccine (Cocci), and a combination of Cocci and DEX (CocciDex) challenge. The DEX and CocciDex groups received dexamethasone in the feed at 1.5mg/kg of diet for 7 days, while the Cocci and CocciDex groups were orally gavaged with 20x coccidia vaccine. The DEX and CocciDex-challenge were able to induce stress and reduce performance, digestibility, intestinal permeability, and immune response. The coccidia vaccine challenge did not affect performance. However, total tract nutrient and energy utilization and the jejunal mRNA expression of (TLR4) and pro-inflammatory cytokines 7-days post-challenge, were impaired ($P < 0.05$). Finally, Natustat™ supplementation did not mitigate the negative effect of the stressors on performance, nutrient and energy digestibility and utilization, and intestinal morphology and permeability. The supplementation had a tendency to increase the expression of anti-inflammatory cytokine (IL-10) 7-days post-challenge. It also increased IL-10 and decreased the mRNA expression of IL-6, 14-days post-challenge.

In conclusion, stress was induced in the birds especially with DEX and some with coccidia challenge and heat stress, and the supplements had a limited effect in mitigating the effect of the stressors.

KEYWORDS: Dexamethasone, coccidia challenge, intestinal permeability, immune response, poultry, tight junction.

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(Name of Student)

October 5th, 2020

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Date

DEDICATION

In loving memory of my grandpas Babs Steven Osunlalu and Paul Olojede both of whom saw greatness in me and aspired me to be one

In loving memory of my grandma Mary Olojede

and

to my only living grandparent Grace Osunlalu.

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LIST OF ABBREVIATIONS

α alpha

β beta

γ gamma

ACTH adrenocorticotropic hormone

AGP antibiotic growth promoters

AME apparent metabolizable energy

AMEn apparent metabolizable energy corrected for nitrogen

ANS autonomic nervous system

BGA brain-gut axis

BWG body weight gain

$CaCO_3$ calcium carbonate

CAT catalase

CBG corticosteroid-binding globulins

Cl^- chloride ion

CORT corticosterone

CRH corticotropic releasing hormone

CRF corticotropic releasing factor

CO_2 carbon dioxide

DE digestible energy

DEB dietary electrolyte balance

DEX dexamethasone

DM dry matter

EcoE EconomasE™

EP epinephrine

FCR feed conversion ratio

FE feed efficiency FOS fructooligosaccharides

FITC-d fluorescein isothiocyanate

GALT gut-associated lymphoid tissue

GAS general adaptation syndrome

GC glucocorticoids

GIT gastrointestinal tract

GLUT glucose transporter

GR glucocorticoid receptor

HCO_3^- bicarbonate ions

H_2CO_3 carbonic acid

H^+ hydrogen ion

HPA hypothalamic-pituitary-adrenocortical

HS heat stress

HSP heat shock proteins

IFN interferons

IgA immunoglobulin A

IL interleukins

K^+ potassium ion

KBD keel bone damage

MOS mannan-oligosaccharides

mTOR mechanistic target of rapamycin

N nitrogen

Na^+ sodium ion

NaCl sodium chloride

$NaHCO_3$ sodium bicarbonate

NE norepinephrine

PAMP pathogen-associated molecular patterns

PRR pattern recognition receptors

PVN paraventricular nucleus

ROS reactive oxygen species

SCFOS short chain fructooligosaccharides

Se selenium

SGK1 serine/threonine-protein kinase

SGLT1 sodium/glucose cotransporters 1

SNS sympathetic nervous system

SOD superoxide dismutase

TGF transforming growth factor

TLR toll-like receptor

TNF tumor necrosis factor

TTR total tract utilization

TOS transgalacto-oligosaccharides

UPS ubiquitin-proteasome system

VE vitamin E

VP vasopressin

CHAPTER 1. LITERATURE REVIEW

1.1 POULTRY PRODUCTION

The popular image of American farming with cowboys rounding up steers on wide-open ranches or countryside backyard farming has been replaced with intensive farming, where tens of thousands of animals are housed in a controlled environment. The poultry industry is a typical example of the transformation of farming into a vertically integrated enterprise. The vertical integration enterprise has allowed poultry to remain the largest sector of the meat industry in terms of count and pounds produced (The meat institute, 2017). The US has led the world in large-scale farming, progressively using intensive poultry farming to produce meat and egg for human consumption - representing the largest broiler chicken industry in the world, and the second-largest producer of egg products (FAO, 2019). In concert, all over the world, the livestock sector is rapidly becoming an organized market chain contributing 17% to kilocalorie consumption and 33% to protein consumption worldwide (Thornton, 2010). This is driven by the increasing population, greater purchasing power, and urbanization (Speedy, 2003; Thornton, 2010).

Accompanying this growth, the poultry industry is faced with an enormous challenge to maintain the health and well-being of the birds. Exposure to different sources of stress is detrimental to the health and well-being of the bird, which they are prone to in intensive farming. Even in the best controlled and state-of-the-art facility, birds can still be affected by environmental stress factors like extreme cold, heat, and humidity; management stress like light intensity, litter quality, beak trimming, stocking density, and poor ventilation; nutritional stress like nutrient excesses, nutrient inadequacies, digestive disorders, and feed intake problems; physical stress like catching and transportation; social

stress like overcrowding, lack of uniformity in body weight; and biological/pathological stress like viral, bacteria, fungi, and protozoa infections. (Virden et al., 2007). These stress factors are exacerbated in farms with poor biosecurity and management practices, predisposing the birds to excessive activation of their immune system and a compromised gut. As such, immune dysregulation, gut barrier dysfunction, cellular oxidative stress, malabsorption, nutrient digestibility maladies, decreasing productivity, and increased susceptibility to other infectious diseases ensue (Quinteiro-Filho et al., 2012).

Stress can impinge on animal productivity and diminish animal welfare. Producers, veterinarians, and production animal specialists can all cite specific economic endpoints that are directly related to the incidence and intensity of stress and disease-related factors in livestock and poultry. Consequently, much effort and expense are directed toward minimizing the incidence of disease in these animals. Considerable advances have been achieved over the years to control infections or exposure to unnecessary stressors in poultry production. A combination of vaccination programs, drug-therapy, development of disease-resistant bird strains, optimized nutrition, and adequate biosecurity (NRC, 1999) has made it possible to achieve high levels of productivity in the intensive production system. However, we are dealing with a multiplicity of stressors that coalesce over time to reduce the overall productivity of the animal. For infectious diseases, bolstering specific immunity against pathogens by vaccinations is helpful, but vaccines are expensive, not only to buy and administer but in terms of lost productivity due to the cost associated with the immune response (Cook, 1999). Moreover, for other stress factors that cause low morbidity but still affect animal productivity, vaccination is not an option. Thus, we rely on the bird's immune system to thwart challenges from most pathogens. Hence,

the need to understand the chicken's ability to cope with stress while maintaining a steady state of functioning is important.

Maintaining healthy flocks and strong immune systems should minimize losses associated with various diseases and other stressors. Healthy animals are generally characterized as having a well-functioning intestinal tract that is efficient at protection against insults in feed, water, and the bird's environment, transportation of feed and digesta, digestion and absorption of nutrients, as well as excretion (of undigested and unabsorbed components of the feed, and uric acid). Growth-promoting antibiotics have been a major tool in modulating host-pathogen interactions and limiting clinical and subclinical bacterial infection in confined animal production. However, regulatory pressures to limit antibiotic use in poultry production have limited the use of AGPs. Currently, it is unlikely that there is any single substance that could replace the function of feed antibiotics especially since the growth benefit found from feeding antibiotics is achieved through its many different effects on the GIT. A potential single substance intended to replace the role of antibiotics in farm animals will be subject to the intense scrutiny that antibiotics have been subjected to over the years and, would have to be accompanied by a combination of nutritional, management, husbandry, and sustainable practices. Hence, the appropriate strategy would be to increase the levels of certain nutrients in the diet to accommodate for nutritional strategies needed during diseased or stressed periods. The objective would be to target different goals including improving nutrient digestion and absorption, regulating the gut microbiota to more favorable bacterial species, and modulate the immune system to enhance disease resistance.

The focus of this review is to summarize the literature about the effect of stress on the animal's health and well-being. Special emphasis is placed on the effect of the stressors on performance, nutrient and energy digestibility and utilization, intestinal morphology, intestinal permeability, immune response. This dissertation will address the interrelationships of stress and nutrition in chickens, with a specific focus on the benefits of adding feed additives to the diet to ameliorate the negative effect of stress.

1.2 THE CONCEPT OF STRESS

Living organisms survive by maintaining a relatively stable equilibrium termed homeostasis that is constantly challenged by intrinsic or extrinsic forces, real or perceived insults, referred to as stressors. This steady-state is constantly maintained by a complex repertoire of the physiologic and behavioral central nervous system and peripheral adaptive responses, which attempt to counteract the effects of the stressors to maintain homeostasis (Chrousos and Gold, 1992). The concept of stress has been discussed in the scientific community for decades. In humans, stress is often described as a sequence of events associated with several perturbations such as infections, a variety of medical conditions, and emotional reactions (Tucker-Ladd, 2000; De Villers, 2003). These stress factors are mediated by brain perception that leads to biochemical, physiological, behavioral, and psychological changes. In animal husbandry, stress is conceived as a reaction impinged on animals from adverse environmental factors such as physical (handling, transport), psychological (fear, frustration), or biological stress which, may lead to many unfavorable consequences (Burkholder et al., 2008; Al-Aqil et al., 2009, Vicuña et al., 2015). These could range from discomfort, decrease in production, pathological losses, and eventually death. From an evolutionary standpoint, the ability of an organism to cope with stressors

gives a selective advantage, increasing their ability to cope with situations that require action or defense. Thus, adaptability and resistance to stress are fundamental prerequisites for life, and every vital organ and function participate in them (Selye, 1950).

1.2.1 Historical overview of stress concepts

Han Selye, in his 1973 paper, wrote, "everybody knows what stress is and nobody knows what it is." Its meaning and interpretation have eluded us over time because the concept is multidimensional and composite. Vaguely, it is often described as an agent, process, or a response (Le Moal, 2007). Implicitly, it implies a substantial imbalance within the physiological systems and the environment, and it seems to characterize a process leading to disease (Le Moal, 2007). Historically, investigations on stress response have always been categorized in terms of the physiological response to bodily injury. Bernard (1867) introduced the concept of a milieu - interieur as the ability of an organism to maintain a fluid matrix at optimal setpoints, independent of the external environment (Goldstein and Kopin, 2007; Antinori, 2017). Cannon, (1929) coined the term “homeostasis” to describe the maintenance of several physiological variables within acceptable ranges. Continuing in the tradition of Bernard and Cannon, Selye (1936) ventured on a quest to understand the concept of stress on the body. Selye redefined the word stress, from its meaning in physics as a force that puts pressure or strain on something to deform its shape and the opposing force that creates a resistance to restore the unstressed state. His definition of stress does represent “the nonspecific response of the body to any demand upon it” (Selye, 1973). His primitive experiments on the effect of stress conditions (e.g., fasting, extreme cold, operative injuries, and drug administration) on rats led to the publication of his historical article titled “A syndrome produced by diverse nocuous

agents". The characteristics manifestation of the response observed includes a rapid decrease in the size of lymphoid organs, enlargement of the adrenal gland, tissue catabolism, hypoglycemia, gastro-intestinal erosions, and discharge of secretory granules from the adrenal cortex, among others. He concluded that the effect of acute non-specific agents produces a response independent of the nature of the damaging agent, and these adaptive responses represent the animals' ability to alleviate physiological stress. This adaptive response, he later described as the "General Adaptation Syndrome" (GAS), develops in three stages: the alarm, resistance, and exhaustion stage (Selye, 1950).

The GAS theory describes the response of stress to the activation of adrenocorticoid and pituitary hormones, challenging the pre-existing theory of specific etiology, which states that a specific disease must arise from a specific pathogen. The alarm stage is the initial response to a stressor that animal exhibits. Similar to this phenomenon is the characteristic physiological response known as the fight/flight response, first described by Cannon (1929). These actions are mediated by the release of catecholamines, which stimulate immediate energy production that allows the animal to cope with emergencies. The downstream effect alters various homeostatic processes, including regulatory processes affecting blood pressure, circulating levels of glucose, electrolyte balance, distribution of blood flow, and membrane permeability (De Villers, 2003). If the stressor is of moderate intensity and the duration is short, reversible responses ensue where the animal's physiological state automatically returns to normal. As the name implies, the resistance stage serves as an adequate mechanism for adaptation, where the body continues to fight the stress long after the effects of the "fight or flight" response have worn off. At this point, the stress hormone, corticosterone, is released and inhibits glucose and fatty acid

storage to stimulate the mobilization of energy substrates such as glucose, amino acids, and free fatty acids from storage sites (muscles, fat tissue, and liver). A shift of energy substrates from the storage sites to the bloodstream, although short-term, helps to resist stress.

The long-term effect of the resistance phase affects metabolism and can result in bodyweight loss due to general tissue catabolism (Selye, 1978; Plytycz and Seljelid 2002). The stage of exhaustion occurs if the stressor persists, which triggers the depletion of energy stores. According to Selye (1950), the degree of adaptability of any animal depends on the amount of "adaptation energy" which is determined by the level of resources available to the animal. This depletion of adaptation energy is often accompanied by enhanced activity of the hypothalamic-pituitary-adrenocortical (HPA) axis and the onset of pathophysiological changes in the body, ultimately resulting in death. This concept not only emphasizes the inadequacy of any organ system under stress to succumb to the lethal stage of exhaustion but the failure of adaptations within these systems to maintain homeostasis over the long term. This inefficiency revolves around increased secretion of glucocorticoids by the adrenal cortex, to the response and adaptation towards the stress factors.

1.2.2 Physiological responses to stress

The central neurochemical circuitry responsible for activating the stress system has been studied extensively. Based on the literature, an animal confronted with a stressful situation, in this case, transportation, overcrowding, prolonged irritation, diseases, etc., responds by initiating what is called a “stress cascade.” (Asres and Amha , 2014) First, the stress system relays stressor-relevant information to the brain, which recruits both neural

(autonomic nervous system; ANS) and neuroendocrine (hypothalamic-pituitary-adrenal; HPA) systems to respond and minimize the cost to the organism while maintaining homeostasis (Charmandari et al., 2005; Ketchesin et al., 2017). As part of the ANS, the sympathetic nervous system (SNS) enables the body to mobilize resources necessary to meet the increased metabolic demand associated with fight and flight. In conjunction, the nervous signals are relayed to the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland, and the adrenal gland, all of which are described as the principal effectors of the stress response (Smith and Vale, 2006; Antinori, 2017). These collective structures are referred to as the HPA axis. The sequence of events starts with specific neurons in the PVN of the hypothalamus signaling the release of a corticotropin-releasing hormone (CRH) into the hypophyseal portal system. The CRH travels to the anterior pituitary and stimulates the pituitary corticotropes to release adrenocorticotropin hormone (ACTH) into the blood. The principal target for the circulating ACTH is the adrenal cortex, where it stimulates the synthesis and release of glucocorticoids (GC), which together with catecholamines produced by the SNS, are considered to be a major stress hormone (Figure 1.1, Matteri et al., 2000; Charmandari et al., 2005).

The GC - released as cortisol in humans and as corticosterone (CORT) in avian species, passes through the blood barriers, crosses plasma membranes, and connects with intracellular receptors to initiate the consequences of stress. This occurs through the brain and body via the neuroendocrine, autonomic, immune, and metabolic systems (McEwen, 2013). In turn, these systems are responsible for either the successful adaptation of the body or the development of pathologies depending on the severity. The association between stress and the endocrine function is the result of an evolutionary process that has shaped

the stress response. The released GCs have a pleiotropic effect, including anti-inflammatory or immunosuppressive effects, undesired metabolic effect, suppressing bone formation, and influencing glucose uptake (McEwen, 2013). These effects are exerted ubiquitously through widely distributed intracellular receptors. In circulation, GCs are in equilibrium between bound and unbound forms. According to Hammond et al. (1987), circulating GCs are reversibly bound to a specific serum glycoprotein called transcortin or corticosteroid-binding globulins (CBG), and to a lesser extent to albumin, a low-affinity nonspecific binding protein. The CBGs are produced primarily in the liver, and low levels have been detected in the thymus, lung, and kidney (Southwick, 1982; Hammond et al., 1987). Bound CORT is physiologically inactive and acts as the rate-limiting step of a stress response.

When the stress response is activated, the proportion of the free CORT increases, which enters into target cells via simple diffusion mechanisms (Puvadolpirod, 1998), bind with unoccupied cellular receptors and alter enzymatic and nucleic acid activity (Thompson and Lippman, 1974). Most, if not all, of the cellular and tissue responses to corticoids, may be mediated via intracellular GCs, which have been found in numerous tissues including liver, lung, kidney, gut, heart, muscle, brain, skin, fibroblasts, and peripheral leukocytes (Thompson and Lippman, 1974; Southwick, 1982). Corticosteroids bind to the receptor proteins in the cytoplasm and form steroid-receptor complexes (Southwick, 1982), which undergo a conformational change and moves into the nucleus. The steroid-receptor complex binds to target sites in DNA initiating gene transcription, where it directs the synthesis of specific mRNAs (Southwick, 1982; Odhiambo, 2004) leading to the synthesis of enzymes/protein that alter cell function.

As described earlier, a stimulus–cognition–response process is initiated by stress factors perceived to be excessively demanding, which disrupts homeostasis of the body. It elicits a coordinated physiological response within the body in an attempt to reestablish homeostasis. The biomedical sciences have long been concerned with how animals and humans alike, respond to, and tolerate environmental stress (Selye and Fortier, 1949). Of importance is the fact that the response to a stressor does not categorically increase or decrease with the level of stress. In acute stress responses, adaptation, and survival via biological responses are promoted. By maintaining homeostasis during the stress response, Sterling and Eyer (1988) referred to this adaptation as "allostasis" or "stability through change." The adaptive outcomes developed via biological responses are evident, presenting a combination of the four general biological defense responses: the behavioral response, the autonomic nervous system response, the neuroendocrine response, and the immune response (Moberg 2000) that help the animal to manage the situation.

On the other hand, the potential for a cumulative overload in chronic stress is evident when the allostatic systems are either overstimulated or underperforming. This leads to repeated and perhaps persistent activation of the biological response essential for adaptation but then transitions into maladaptation and dysfunction (McEwen, 1998; McVicar et al., 2014). McEwen (1998, 2007) identified this exaggerated activity as an imposition of excessive allostatic load on the systems that are creating stability during the stress, such as the autonomic nervous system and the hypothalamus-pituitary-adrenal axis. The consequence of this load exacerbates pathophysiology through the same systems that are dysregulated.

Stress response, whether caused by physical or neurogenic factors, is meant to be of short or limited duration. The time course for the response usually involves a wave of enhanced secretion of catecholamines (epinephrine and norepinephrine) from the SNS, the activation of the HPA axis, among other things. Subsequently, the secreted hormones exert most of their effects on targeted tissues, where they bring about the significant physiological changes in response to the stressor (Puvadolpirod, 2000a-d; Sapolsky, 2000). According to Sapolsky (2000), these changes include, decreasing feed intake, mobilizing stored energy and inhibiting subsequent storage, suppressing inflammatory mediators, and inhibiting reproductive physiology (Figure 1.2). Depending on the severity of the exposure, the biological cost might be negligible or minimal. For example, if an animal is stressed, the catecholamine secretion utilizes the glycogen stores to readily utilizable glucose for metabolic use. Once the stressor is alleviated, the glycogen stores are quickly replenished by gluconeogenesis to pre-stress levels, and the stress effect becomes inconsequential to the animal's welfare (Moberg, 2000). On the other hand, continuous exposure to the stressor disrupts biological functions and shifts necessary resources away from other critical functions. In response to the stress, the hormones secreted exert a catabolic effect on the muscle, adipose tissue, connective tissue, skin, and lymphoid tissue. This involves increased degradation and reduced synthesis of proteins, fat, DNA, and RNA and decreased uptake of glucose, amino, and nucleic acids (Baxter, 1976). The aftermath of these catabolic actions includes harmful consequences such as the inhibition of growth, osteoporosis, and enhanced susceptibility to infections.

1.3 STRESS IN CHICKENS

Stress is a broad term generally used to describe the cumulative detrimental effect of a variety of factors on the health and performance of animals. Under this condition, the animal becomes unable to maintain a normal physiological state because of the various factors adversely affecting its wellbeing. At which point, a set of physiological and behavioral changes are elicited in response to the aversive stimuli. Like all complex organisms, the bird's response to stressors depends on the integrative capacities of the nervous and endocrine systems. The incoming stress stimuli increase the hypothalamic production of CRH, which then stimulates the anterior pituitary gland to increase the synthesis of ACTH. The ACTH moves via the blood to stimulate the proliferation of adrenal cortical tissue cells and increases the synthesis and release of GC (Siegel, 1980; Smith and Vale, 2006; Antinori, 2017). This is the body's way of eliciting a coordinated physiological response in an attempt to reestablish homeostasis. Short-term stress can be expected and are typical of minimal concern. However, a combination of stressors over time that exceeds the host's coping mechanisms, can have far-reaching detrimental effects on poultry production (Siegel, 1980). Many aspects of daily life may not qualify as stress nevertheless may have an adverse effect on the body. Undoubtedly, one of the challenges the producer must overcome in the pursuit of increasing the production of poultry and poultry-byproducts to meet the increasing demand is to minimize the exposure of birds to potential stressors. Notwithstanding, birds are constantly confronted by various stressors during production, including environmental (thermal stress, high stocking density, transportation), management (lighting conditions, nutrient density, mycotoxin

contamination, vaccination programs), and various pathogenic (viruses, bacteria, and parasites) conditions (Siegel, 1980; Asres and Amha, 2014; Figure 1.3).

These changes in the environment make it difficult to determine which concurrent variations the animal is responding to or reacting to. Besides, animals seldom experience a single stressor that alone underwrites the overall impact of stress production or performance. As such, alteration from the optimal condition can cause an exaggerated or inappropriate immune response, disrupting the growth process, overall performance, and put considerable demands on intestinal health (Song et al., 2014). Morbidity and mortality are no longer the primary metrics monitored concerning disease and stress management in poultry production (Collett et al., 2019). Emphasis has now shifted to the economic output measured through the entire production chain, on performance, product quality, and animal welfare (Collett et al., 2019). Although husbandry practices in the poultry industry such as nutrition and management practices, maintenance of hygienic standards, disease prevention protocols, and animal welfare considerations, are implemented to prevent and control specific disease conditions, that is not enough. These practices are necessary with an intensive production system but do not entirely prevent exposure to stressors. Preventing and limiting the consequence of more complex multifactorial stress factors to maximize the productivity of the flock becomes paramount. Consequently, there is a need for continuous monitoring of the intestinal health status, particularly in intensively reared animals, where the intestinal function is often pushed to the limit.

1.3.1 Coccidiosis

Coccidiosis in domesticated animals has been studied for over a long time (Davies et al., 1963; Hammond and Long, 1973; Pellerdy, 1974; Long, 1982, 1990), and as the

world's poultry industry develops, it is one of the most widely reported diseases within the industry with significant economic importance (Biggs, 1982; Williams, 1999). The genus *Eimeria*, of the Coccidea class, within the phylum Apicomplexa, is arguably the most widespread and economically important protozoan parasites that affect the poultry industry (Cox, 1998). Indeed, has a protozoan species, they are ubiquitous in terrestrial and aquatics habitats as autotrophs or heterotrophs. However, they are also parasitic in nature and rely on host resources for their nutrition (Adlard and O'Donoghue, 1998). The complex life cycle of the *Eimeria sp* that causes coccidiosis has been extensively described (Cox, 1998; Lillehoj, 1998; Yun et al., 2000; Dalloul and Lillehoj, 2006; Kaiser, 2010). Basically, host-cell invasion by *Eimeria* involves the ingestion of sporulated oocysts, excystation into free sporozoites invading the epithelial cells, and one or more cycles of schizogony, gametogony, and sporogony. The final product, zygote, escapes to the lumen of the intestine and passes into the external environment via excreta. These oocysts containing sporocysts are infective and can persist for long periods before being ingested and starting the life cycle over again (Figure 1.4). Thus, continual recycling through a flock leads to a high number of oocysts in the litter within 3–4 weeks (Williams, 1994), and the risk for infection is further facilitated by the intensive rearing conditions in the poultry industry.

Through extensive research, seven species of *Eimeria- Eimeria acervulina, tenella, maxima, necatrix, brunetti, mitis*, and *praecox*, are recognized to colonize the gastrointestinal tract of domestic chickens (Lillehoj and Lillehoj, 2000; Chapman, 2014). Depending on the species, magnitude, and site of infection, they exhibit a characteristic degree of pathogenicity. The *E. acervulina* infects the cells of the duodenum, *E. maxima* infects the mid-gut, proximal as well as distal to Meckel's diverticulum, and *E. tenella*

infects the ceca (Williams, 2005; Chapman, 2014). The virulence-associated with these parasites is of varying level; a mild infection, causing no adverse effects, subclinical coccidiosis, resulting in slight but economically important reductions of growth and feed utilization, and full-blown clinical coccidiosis (Williams, 2005), which could result in significant economic losses as a result of high mortality and morbidity. The primary target of infection is the intestine, and defense against the parasite involves mobilizing the immune system mainly in the gut. One of the first observable effects of coccidiosis in poultry is diarrhea, dehydration, the reduction of weight gain, and a concomitant reduction in feed efficiency (Allen and Fetterer, 2002a; Dalloul et al., 2003; Amerah and Ravindran, 2015). Similarly, the invasion of the intestinal epithelial lining causes a variety of clinical manifestations including the destruction of the mucosal barrier and underlying tissue (necrotic gut lesions), increasing mucosal permeability, to atrophy of the villus, limiting absorption and digestibility of nutrients (Persia et al., 2006; Adedokun et al., 2016). Transit time of the digesta is increased resulting in decreased viscosity, and high permeability of plasma proteins (Williams, 2005). The intestinal damage from the parasites leaves the gut vulnerable to overgrowth by opportunistic bacteria. The subsequent secondary bacterial infections that follow coccidiosis infections lead to further reduced feed efficiency and nutrient digestibility. Moreover, the invasion of the host intestinal epithelial cells activates both the humoral and cell-mediated immune responses, where the cell-mediated component of the immune response plays a major role in disease resistance (Cox, 1998; Lillehoj and Lillehoj, 2000).

There are two means of preventing coccidiosis in the flock: chemoprophylaxis and vaccination. Much research effort has been focused on developing effective vaccination

strategies for coccidiosis with varying success (Lillehoj and Trout, 1993). These vaccination strategies include live oocyst vaccines, either unattenuated or attenuated, recombinant DNA or protein vaccine, and immunization using anti-idiotype antibodies (Lillehoj and Lillehoj, 2000; Dalloul and Lillehoj, 2005). Live unattenuated vaccines comprised of a mixture of Eimeria species have been successfully used in a commercial application to induce long-lasting protective immunity. One drawback of using live unattenuated vaccines is the possible introduction of new pathogenic species into a previously unexposed flock (Vermeulen et al., 2001). Since vaccination occurs within the first day of the chick's life when the adaptive immune system is not functionally immature, the risk of contracting coccidiosis or secondary infections caused by resident opportunistic bacteria is increased during this time, but decreases as immunity gradually develop over time (Lillehoj and Lillehoj, 2000; Vermeulen et al., 2001). Antibiotics and chemotherapeutics have remained the primary means of coccidiosis control (Lillehoj and Lillehoj, 2000). While these vaccines serve as the primary defense against the parasitic infection, AGPs are commonly used in addition to counteracting the growth problems occurring due to secondary bacterial infection.

1.3.1.1 Performance, nutrient digestion, and absorption

Subclinical coccidiosis is often characterized by poor weight gain and a concomitant adverse effect on feed conversion efficiency (William, 1999; 2005). The industry reports a loss of more than $3 billion annually to coccidiosis (Lillehoj and Lillehoj, 2000). Using a compartmentalized model to quantify the monetary loss incurred by the poultry industry from coccidiosis control, William (1999) reported that in 1995, 17.5% was due to the cost of prophylaxis and therapy of commercial broilers, and 80.5% was due to

subclinical effects on their weight gain and feed conversion. Thus, because of its economic importance, coccidiosis has received much research interest, some of which have been directed toward understanding the effect of coccidia infections on nutrient digestion, absorption, and utilization. For research purposes, inoculating the birds with live oocyst of specific Eimeria sp (Isobe and Lillehoj, 1993; Laurent et al. 2001; Faber et al., 2012; Bortoluzzi et al., 2019), or sporulated oocyst of a combination of several Eimeria sp (Lee et al., 2011; Amerah and Ravindran, 2015), or an oral gavage using a vaccine with live oocyst at doses higher than the recommended dosage for day-old birds by the manufacturer (Persia et al., 2006; Adedokun et al., 2016), has been used to mimic coccidiosis in practical poultry production. These strategies often result in depressed feed consumption and BW gain with or without observable clinical symptoms. According to several published articles, the reduced broiler performance can be attributed to the reduced nutrient digestion and absorption caused by impaired absorptive capacity of the intestine (Preston-Mafham and Sykes, 1970; Russell Jr. and Ruff, 1978; Ruff and Wilkins, 1980; Adam et al., 1996). Coccidia challenge has the potential to influence the net movement of nutrients into the blood of the host by suppressing feed intake, digestion and absorption by the intestinal epithelial cells, and movement of nutrients through the absorptive cell into the bloodstream (Russell Jr. and Ruff, 1978).

Coccidiosis has been reported to decrease the digestion and or absorption of glucose, proteins, amino acids, lipids, metabolizable energy, minerals, and carotenoids (Turk et al., 1982; Willis and Baker, 1981; Adam et al., 1996; Persia et al., 2006). Ileal endogenous amino acid losses in broiler chickens have been extensively reported (Ravindran and Hendriks, 2004; Adedokun et al., 2007). A magnitude of reduction varying

from 8.5% (Parker et al., 2007), 15.2% (Adedokun et al., 2017), to 16% (Amerah et al., 2015) in mean ileal amino acid (AA) digestibility of broilers challenged with coccidia vaccine, has been reported. A suggested reason for the increase in endogenous losses of mucin-associated AA has been attributed to increased host intestinal mucogenic response and enterocyte turnover (Fernando and McCraw, 1973), as well as the activation and heightened immune response (Parker et al., 2007) during the infection. Similarly, since fat and carbohydrate are the major sources of energy supplied by commercial poultry diets, coccidiosis infection on caloric costs has been investigated. The reduction was observed in the digestibility of dry matter, nitrogen, energy, and fat in birds challenged with coccidia (Amerah et al., 2015). Eimeria infection reduced dietary apparent metabolizable energy corrected for nitrogen, apparent ileal digestibility of organic matter, nitrogen, and total AA (Rochell et al., 2017) with observed reductions in plasma concentrations of arginine, tyrosine, glutamine, and asparagine (Rochell et al., 2016). Adams et al. (1996) also reported a reduction of about 22% in lipid utilization when birds were infected with E. acervulina.

1.3.1.2 Changes in the intestinal epithelium during coccidia challenge

During coccidiosis, sporozoites infect the cells of the intestinal lining, causing tissue damage and trauma to the intestinal mucosa and submucosa. Reid and Johnson (1970) reported a downward trend in weight gains with increasing lesion scores with E. acervulina inoculation. Similarly, increasing lesions scores with *E. maxima* and *tenella* infection has been reported (Conway et al., 1990). In addition to these lesions are histopathological features that accentuate the damaged intestines (Lillehoj and Trout 1993). Villous atrophy and increased crypt depths have been observed with coccidia

infection (Amerah and Ravindran, 2015; Osho et al., 2019), contributing to malabsorption. Since the production of villus epithelial cells occurs in the crypt region, an increased crypt depth signifies an increase in crypt-cell production rate and overall stimulation of intestinal cellular turnover in the small intestine (Pluske et al., 1996; Montagne et al., 2003). Moreover, the infection also influences the villus height to crypt depth ratio, which suggests that the intestinal mucosa is not well-differentiated, more cells are exhibiting DNA fragmentation setting the cells up for programmed cell death (Montagne et al., 2003). From a production perspective, these changes are important because they are associated with a reduced digestive and absorptive capacity which plays a role in the reduced growth and performance observed in the infected chickens (Pluske et al., 1996). Furthermore, the activity of specific digestive enzymes on the brush border membranes of intestinal cells is often linked to morphological changes, especially in pathological states where the villus and crypt depth structure has been altered. Reductions in both pancreatic enzyme activity and expression of transporter proteins along the mucosal brush border have been reported during the damage to the intestinal epithelial cells (Adams et al., 1996). This is because as the turnover rate of the enterocytes increases, it reduces the maturity of digestive enzymes. Increased intestinal acidity can also impair digestive enzyme functions, as *Eimeria*-induced pH reductions can cause intestinal pH to fall below the optimal efficiency for digestive enzyme activity (Williams, 2005). Miska and Fetterer (2017) reported the downregulation of monosaccharides transporters sodium/glucose cotransporters 1 (SGLT1), Glucose transporter (GLUT) 2, and 5 at the height of coccidia infection.

1.3.1.3 The immune response of broilers during coccidia exposure

The immune system is mobilized, mainly in the gut mucosa, to produce complete resistance to reinfection (Davis, 1981; Powell, 1987). Since *Eimeria* parasites invade the intestine, immune responses are primarily coordinated by the gut-associated lymphoid tissue (**GALT**) (Yun et al., 2000). In general, the GALT serves as the host's defense against pathogenic infection by processing and presenting antigens, producing antibodies via the humoral immune system, and activating cell-mediated immunity (Dalloul and Lillehoj, 2006). Following a coccidia exposure, innate immunity is associated with the early phase of the initial infection. In contrast, adaptive immunity follows a secondary infection, as it takes longer to initiate due to a specific response to the invading pathogen (Lillehoj et al., 2007). During the early stage of infection, the innate immune system of the host can rapidly detect and respond to protozoan parasite infection via innate immune receptors (Dalloul and Lillehoj, 2006). The toll-like receptor (**TLR**) family are components of the innate immune system that sense conserved microbial patterns typically called pathogen-associated molecular patterns (**PAMPs**) and endogenous danger signals. The TLR, upon activation by recognition of PAMPs, recruits several different adaptor molecules, including MyD88 and TRIF-dependent pathways (Hong et al., 2006; Zhou et al., 2013). These signals trigger both the innate immune defenses such as inflammation, and the acquired immune defenses by secretion of cytokines such as interferons (**IFN**), tumor necrosis factor (**TNF**), and interleukins (**IL**).

Furthermore, the adaptive immune system is comprised of humoral and cell-mediated immunity, and after coccidia infection, both immune responses of the bird are activated. Cell-mediated immunity plays a major role in disease resistance. T cell-lymphocytes, NK cells, and macrophages are the three important cell types that are

involved in the cell-mediated immune response. There are two phenotypes of T cell-lymphocytes that recognize the antigen: cytotoxic T cell (**CD8+**) and T helper cell (**CD4+**). This involves the activation of cytotoxic T cells and various CD4+ T-helper cell subsets (T_H1 and T_H2)- the cytokines they ultimately produce to evoke the immune response (Cox, 1998; Lillehoj and Lillehoj, 2000; Laurent et al., 2001). The CD8+T cell recognizes the pathogen through the MHC class I molecules and kills the infected pathogen directly. Experimental evidence shows that chicken CD4+ T cell-lymphocytes mediate antigen-specific protective immunity in avian coccidiosis (Degen et al., 2005), recognizing pathogens through the MHC class II molecules, and subsequently activating B-cells and macrophages. This drives the counter-regulatory cell-mediated and humoral immune reactions interplay between several effector systems (Cox, 1998; Kaiser, 2010). Gadde et al. (2009) saw an increase in CD4+ and CD8+ T cells population, 11 d post-challenge with *Eimeria adenoeides* oocysts in turkey poults. In broiler chickens, a remarkable up-regulation of cytokine expression, such as IL-1β, IL-10, IL-6, TNF-α, transforming growth factor-beta (TGF-β4), IFN-γ, (Lillehoj, 1998; Dersjant-Li et al., 2016), as well chemokines IL-8 following coccidia infection, has been observed. In response to this infection, the cytokines depending on their biological process may function to induce or suppress inflammation. Anti-inflammatory cytokines like IL-4, IL-10, IL-13, and TGF-β4 are known to suppress the production of IL-1β, TNF-α, IFN- γ, and chemokine IL-8 in response to an infection (Dinarello, 2000). Therefore, following a coccidia challenge, a shift in the balance between the effects of pro- and anti-inflammatory cytokines can influence the outcome of the disease, whether in the short term or long term (Dinarello, 2000).

An aggregate of immune mediators in response to coccidia infection induces a state of inflammation in the gastrointestinal tract, compromising the integrity of the gut (Lu et al., 2013). This inflammation state threatens the normal continuity of the epithelium necessary to control both the paracellular and transcellular permeability of the intestine. As a sophisticated gatekeeper, the epithelium regulates the transport of solutes, large molecules, and cells across its barrier. This cellular function is controlled by a heterotopic association between inflammatory and mural cells, transcellular transport, and junctional complexes (Goddard and Iruela-Arispe, 2013). Because *Eimeria sp* has an affinity for the intestine and induces changes in intestinal permeability, its relevance to gut health can not be understated. Extensive animal experiments have shown that the gut communicates with the brain to regulate major epithelial and immune functions that are of importance to gut health and health in general (Groschwitz and Hogan, 2009; Vicuna et al., 2015). Thus, understanding the immune system-parasite interactions in the gut is crucial to understanding the consequences of the possible alterations associated with the absorptive, metabolic, and immunological functions of the gastrointestinal tract (**GIT).**

1.3.2 Heat stress

The earth's climate is changing at an unprecedented rate, and the impacts of global climate change are projected to intensify in the future. According to the Intergovernmental Panel on Climate Fifth Assessment Report, the planets' average surface temperature has risen about 1.62 °F (0.9 °C) since the mid-20th century and could increase between 1.4 and 5.8 °C by the year 2100 (IPCC, 2014). Among other things, heat waves (periods of abnormally hot weather lasting days to weeks) in different parts of the world are projected to become more intense, and cold waves less severe everywhere while summer

temperatures are projected to continue rising. The question most frequently raised in connection with climate change is concerning its impact on agriculture (crop and livestock production) and, from a broader perspective, on our food supply. Worldwide growth in the market for livestock and animal products continues a steady climb driven mostly by population growth, urbanization, and increasing per capita incomes in developing countries (Thornton, 2010). Besides, the potential for further growth is self-evident given the value of eggs and poultry meat as healthy alternatives to red meat and other protein sources. The poultry industry particularly assumes a leading role among agricultural sectors in many parts of the world. According to the Food and Agriculture Organization (FAO), about 103.5 million tons of annual global chicken meat produced contributed to about 34.3% of global meat production (Pawar et al., 2016; Nawab et al., 2018). On the other hand, world commercial egg production is setting record-breaking trends, where egg production shot up from 15 to 81 million tons between 1961 and 2016 (FAO, 2016). One of the potential deterrents to this growth is the aforementioned increase in extreme heat conditions, which can result in large economic losses for producers. Without the use of heat management strategies, U.S. livestock producers incur a loss of an average of $2.4 billion annually while accounting for the heat management strategies, incur an estimated loss of $1.7 billion. From this total, a $128 million loss comes from the poultry industry (St-Pierre et al., 2003). With this huge economic importance, the deleterious consequences of heat stress (HS) in livestock production are always under constant scrutiny.

The metabolic activities involved in regular metabolism, growth, egg production, and other physiological processes in birds lead to heat production. This can be influenced by the species, breed, body weight, level of production, level of feed intake, feed quality,

and environmental conditions (Pawar et al., 2016; Nawab et al., 2018). The thermoneutral zone (15 to 26 ℃) is the range in environmental temperature when the core temperature can be maintained with no change in metabolic heat production. Barring any underlying issue, the total heat production of the animal is at the minimum, and the energy from the various physiological processes is managed efficiently (Babinszky et al., 2011). At elevated temperatures, the total heat produced during those physiological processes can exceed the amount of energy flowing from the animal to its surrounding environment which, results in heat stress (Babinszky et al., 2011; Pawar et al., 2016; Nawab et al., 2018). The thermoregulation characteristics of poultry differ to some extent from those of mammals due to their high rate of metabolism associated with extensive genetic selection, more intensive heat production, and low heat dissipation capacity caused by their feathers and lack of sweat glands (Babinszky et al., 2011). Hence, to regulate thermoregulation and homeostasis in the absence of sweat glands, the bird dissipates body heat to the environment by radiation, conduction, convection, and evaporation. The first three routes work efficiently when the temperature difference between the bird and its environment is not dramatically different. As such, the normal behavioral patterns, feed intake, or metabolism is not drastically altered. When the environmental temperature reaches an upper critical limit, heat loss shifts to the evaporative mechanism (Lara and Rostagno, 2013; Bhadauria et al., 2014). Air sacs are also very important in this process, contributing to increasing gas exchanges and bringing air circulation toward the surface (Lara and Rostagno, 2013; Bhadauria et al., 2014; Nawab et al., 2018). The various signs indicating heat stress in poultry include gasping, panting, spreading of wings, lethargic and droopy acting, extremely pale cones and wattles, closed eyes, lying down, drop in egg production,

reduced egg size, egg weight, poor shell quality, increased thirst, decreased appetite, loss of body weight, and increased cannibalism (Lara and Rostagno, 2013; Pawar et al., 2016). This implicates a wide variety of physiological, behavioral, neuroendocrine, and molecular responses in broilers and layers causing unfavorable consequences like depressed growth performance, immune suppression, an endocrine disorder, and electrolyte imbalance which reduces productivity and profitability (Lara and Rostagno, 2013; Nawab et al., 2018; Figure 1.5).

1.3.2.1 Heat stress on growth and reproductive performance

Under high environmental temperature conditions, the HPA axis is activated, altering the neuroendocrine profile in poultry, which increases the secretion of GCs. In poultry, HS has been shown to cause elevated CORT concentrations (Garriga et al., 2006; Quinteiro-Filho et al., 2010). Consequently, the presence of GC reduces the activity of the appetite center in the medulla oblongata. Thus, exposure to high environmental temperature triggers a thermoregulatory response in the anterior hypothalamic area to depress feed intake, which in turn causes body weight loss (Miller and Heath, 1970; Lara and Rostagno, 2013). In addition to the limited feed intake, nutrient digestibility is impaired, such as decreasing the activity of trypsin, chymotrypsin, and amylase (Hai et al., 2000). Though the variation in intensity and duration of responses may vary depending on the intensity and duration of the heat stress, the birds, however, succumb to the heat stress (Lara and Rostagno, 2013). As documented by several researchers, broilers and layers subjected to heat stress experience poor growth and performance (Hsu et al., 1998; Mashaly et al., 2004; Harsini et al., 2012; Quinteiro-Filho et al., 2012; Sohail et al., 2012). Sohail et al. (2012) reported that broilers held at 35°C elevated temperature consumed 15.4% less

and gained 18.3% less on d 21 and consumed 25.4% less and gain 49.6% less on d 42 compared with the control group (26 ℃ temperature). Similarly, Harsini et al. (2012) observed a reduction in body weight, feed intake, and increased feed conversion ratio when broilers were exposed to a cycling temperature of 23.9℃ to 37℃ to induce heat stress. Similar to its effects on broilers, HS in laying hens resulted in decreased BW, feed efficiency, and other production parameters (Mashaly et al., 2004; Deng et al., 2012).

The deleterious effect of heat stress has far-reaching consequences in laying hens not only because of the reduced feed intake but the direct effect on the reproductive hormones. The decrease in the size of reproductive organs (follicular and oocyte development) in response to the decreased secretion of gonadotrophin-releasing hormone (GnRH) and the subsequent decrease in the release of follicle-stimulating and luteinizing hormones has been observed (Donoghue et al., 1989; Nawab et al., 2018). Moreover, in males, semen volume, sperm concentration, the number of live sperm cells, and motility decreased with exposure to elevated temperature (McDaniel et al., 1995). Experimental results showed that increasing environmental temperature significantly decreased egg production, egg weight (Hsu et al., 1998; Deng et al., 2012) and caused inferior egg quality, including eggshell thickness, shell breaking strength, specific gravity, and eggshell weight in laying hens (Hsu et al., 1998; Mashaly et al., 2004; Bozkurt et al., 2012). Moreover, the hyperventilation at elevated temperature decreases CO2 blood levels, which reduces eggshell thickness by approximately 12% (Bhadauria et al., 2014). Additionally, a reduction in feed conversion, egg production, and egg weight was identified in laying hens subjected to HS (Star et al., 2009). Ebeid et al. (2012) reported HS caused a 1% reduction in egg weight, eggshell thickness, eggshell weight, and eggshell percent. Ajakaiye et al.

(2011) also showed a reduction in eggshell weight and thickness. Corroborating these results, Mack et al. (2013) and Franco-Jimenez et al. (2007), observed decreased egg production, egg weight, eggshell thickness, and Haugh units in laying hens exposed to HS.

1.3.2.2 Acid-base balance and electrolytes during heat stress

At high environmental temperature, the bird dissipates heat by the evaporation of moisture from the respiratory tract through increased panting. At ambient temperature, the kidney and lungs work together to balance the acid-base ratio by combining hydrogen ion (H^+) with bicarbonate ions (HCO_3^-) to form carbonic acid (H_2CO_3). The H_2CO_3 is converted to carbon dioxide (CO_2) and water (H_2O) by the action of carbonic anhydrase. The resulting CO_2 from this reaction is removed by the lungs, and the H^+ ions with the HCO_3^- are excreted via the kidneys. However, at elevated temperature, the bird's respiratory rate increases, CO_2 is lost from the lungs, which leads to a reduction in the partial pressure of CO_2 in the blood. In turn, the bicarbonate buffer system lowers the concentration of H^+ and H_2CO_3, excretion of HCO_3^- increases, and H^+ decreases via the kidney which, causes a rise in plasma pH and plasma bicarbonate levels. This condition is known as respiratory alkalosis (Borges et al., 2004; Ahmad and Sarwar, 2006; Borges et al., 2007; Figure 1.6). On the other hand, the reduction in serum HCO_3^- from excretion via the kidney affects eggshell formation because in laying hens, HCO_3^- plays an important role in the formation of calcium carbonate ($CaCO_3$) by the shell gland to form eggshell (Borges et al., 2007; Allahverdi et al., 2013). According to Frank and Burger (1965) and Balnave et al. (1989), reduced bicarbonate concentration in the lumen of the shell gland adversely affects eggshell quality. Similarly, Harrison and Biellier (1967) reported that when the air temperature rises abruptly, eggshell quality declines within one oviposition

cycle which illustrates that as the hen experiences respiratory alkalosis, blood ionized calcium level drops dramatically within a short period (Odom, et al., 1986).

Other variables that change simultaneously with an acid-base balance are the electrolyte status. Especially the monovalent minerals - Na^+, K^+, Cl^-, which are essential for, among other things, the maintenance of intracellular and extracellular homeostasis and acid-base balance (Gezen et al., 2005). To maintain osmoregulation, the homeostasis of intracellular ions like K^+ and extracellular ions like Na^+ and Cl^- contents are kept within narrow limits. According to Mongin (1981), to keep the acid-base homeostasis as close as possible to normal levels in broiler chickens, an optimal dietary electrolyte balance (DEB; a total of $Na^+ + K^+ - Cl^-$, mEq/kg) of around 250 mEq/kg of feed should be provided. This however changes, depending on the ambient temperature. For the body to maintain the homeostasis of electrolytes in body fluids, the level of K^+ and Na^+ excreted in the feces and urine increases, while the Cl^- concentration of the blood rises. Borges et al. (2004) reported a decrease in plasma Na^+, K^+, and pCO_2, due to heat stress, probably as a result of hemodilution following increased water consumption. It stands to reason that there would be a progressive alteration to the acid-base balance in response to HS especially since some results have not been able to report any changes. Similarly, plasma levels of calcium, sodium, inorganic phosphorus, and magnesium were depressed in birds subjected to elevated temperatures (Koelkebeck and Odom, 1995).

1.3.2.3 Intestinal morphology and immune response in birds during heat stress

Gut health plays a significant role in the efficient digestion of feed and absorption of nutrient, water, electrolyte balance as well as immune system development. Hall et al.

(1999) found evidence of hypoxia in the intestinal villi of heat-stressed rodents. Lambert et al. (2002) reported that hyperthermia can induce changes to the morphological structure of the intestine. Rats subjected to a core body temperature above 41 ℃ exhibited damages to the intestinal epithelial cells, intestinal lesions, epithelial cells vacuolization, increased villus tips sloughing rate, and damage to the luminal membrane with loss of microvilli. Liu et al. (2009), reported that temperature at 40 ℃ resulted in epithelium shedding at the tips of the intestinal villi, a short villus height, and a shallow crypt depth in pigs. Similarly, in poultry, decreased villus height, villus surface area, and increased crypt depth have been observed at elevated temperatures (Hu et al., 2010; Quinteiro-Filho et al., 2010; Deng et al., 2012; Song et al., 2014; Abdelqader and Al-Fataftah, 2016). Thus, heat stress induces morphological changes to the small intestine, which inevitably leads to inefficient intestinal digestion and absorption capacity and can in part explain the reduced body weight gain.

A healthy morphology and integrity of the small intestine are important to prevent bacteria translocation from the intestinal tract to the body as well as for efficient digestion and absorption of nutrients. Indeed, Geraert et al. (1996) suggested that growth reduction can partly be explained by the direct effect of high temperature and partly by decreased metabolic utilization of nutrients, increased heat production, reduced protein retention, and enhanced lipid deposition. Since the GIT is highly sensitive to hyperthermia, a compromised mucosa is pivotal to the pathobiology of HS. Pearce et al. (2013) reported that HS reduces blood and nutrient flow to the GIT, causing hypoxia. Hypoxia is said to deplete ATP stores and potentially cause intracellular acidosis and changes ion pump activity (Hall et al., 1999). Also, the depletion of ATP and acidosis can jeopardize tight junctions of the intestinal epithelium which, ultimately compromises intestinal integrity

and function. Intestinal tight junctions, which are made up of a complex of integral membrane proteins, are known to play an important role as a paracellular barrier of the permeation of harmful agents from the lumen to the animals' body (Lambert, 2009). Expressions of occludin, claudin-1, zona occludin 1, and JAM2 all part of a tight junction protein network, were decreased with elevated temperatures (Shin et al., 2018; Goo et al., 2019). Burkholder et al. (2008) and Song et al. (2014) reported that heat stress induces intestinal barrier dysfunctions in broilers by damaging the intestinal epithelium integrity, which causes an imbalance of the normal intestinal microflora, and increases intestinal colonization of pathogens. Heat stress can also cause oxidative stress which, increases tight junction permeability and membrane damage, which can result in bacterial translocation (Lambert, 2009).

The morphology and characteristics of intestinal mucosa together with intestinal immune cells are constantly changing depending on the situation the animal is exposed to. Compromised intestinal epithelial integrity and a compromised mucosal immune response during stress may facilitate the secretion of endotoxins from gut microbes. This stimulates an immune response involving the production of pro-inflammatory cytokines from cells such as monocytes and macrophages. This response likely causes further inflammatory damage to the intestinal epithelium and the initiation of a vicious cycle of events (Lambert, 2009). Several authors have reported the reduction in thymus, spleen, bursa of Fabricius, liver, and lymphoid organ weights in chicken exposed to elevated temperature (Quinteiro-Filho et al., 2010; Ghazi et al., 2012). Also, increased levels of circulating antibodies, specifically IgM and IgG, and subsequent reduction of lymphocytes and IgA secretion in the gastrointestinal tract were observed (Deng et al., 2012). Although, Mashaly et al. (2004)

reported a decrease in total white blood cell (WBC) counts and antibody production, T- and B-lymphocyte activities were not affected in hens in the HS group. On the other hand, Pearce et al. (2013) reported that with acute HS in pigs, the expression of IL-8, IL-1β, and TNF-α was reduced in circulation and remained unchanged in the ileum epithelium. Similarly, B- and T-cell proliferation were not significantly affected by HS. While these observations are not consistent, the immune response activated in response to HS may depend on the length and intensity of the exposure.

1.3.3 Glucocorticoid action to induce stress

Glucocorticoid hormones influence many metabolic functions in several tissues which, bring about major physiological changes in response to stress. One of the classical studies done to understand the effect of stress observed the role of the adrenal gland in rats on the activation of a stress response. It was reported that adrenalectomy in rats resulted in the inability to regulate carbohydrate stores and electrolyte balance (Addison, 1855). The adrenals, which are embedded in the fatty tissue directly next to the kidneys, are composed of two separate endocrine glands, an outer layer of cells (the adrenal cortex) and an inner adrenal medulla. These glands are responsible for producing a large number of steroids (mineralocorticoids, GCs, and the androgen dehydroepiandrosterone), and catecholamines (epinephrine and norepinephrine), respectively (Southwick, 1982). The mineralocorticoids are essential for maintaining sodium balance and extracellular fluid volume, and the GCs exerts their effect on hepatic gluconeogenesis, inhibit glucose uptake by peripheral tissues, suppress inflammation, suppress numerous immune reactions, and inhibit the secretion of several hormones and neuropeptides (Munck et al., 1984). While GCs themselves are not directly responsible for the metabolic processes, they maintain a normal sensitivity to the

hormones which activate them via a permissive action. For example, Exton and Park (1967) observed that in fasted adrenalectomized rats, the normal stimulatory effect of glucagon on gluconeogenesis was lost in the liver and only restored with GCs treatment. An increase in the circulating catecholamines and GC causes changes in intermediary metabolism in several tissues including skin, fat, lymphoid cells, muscle, connective tissues, brain, blood cells, heart, liver, and kidney (Baxter and Rousseau, 1979; Southwick, 1982). However, these hormones generally express inhibitory actions on skin, fat, lymphoid cells, muscle, and connective tissues, which result in catabolism and anti-anabolism. On the other hand, the steroid hormones enhance anabolic effects and may utilize glucose diverted from other tissues to tissues which are perhaps more 'essential' than others (brain, blood cells, heart, liver, and kidney) (Munck, 1971, Baxter, 1976; Trout, 1993).

The major metabolic effects of GCs and catecholamines hormones involves providing energy to resist stress via glycogenolysis, gluconeogenesis, and lipolysis activities (Granner, 1979; Berne and Levy, 1993). Similarly, during a stress response, a crosstalk between the endocrine and immune systems is activated which triggers the signaling cascades of both arms of the immune system. Inherent in any discussion on livestock production, or particular poultry production, is the effect of stress on metabolism. While this review has reported the impact of stresses from infection and environmental conditions, the effector and regulatory mechanisms affected and activated transcend several stressors. To induce controlled stress at the adrenal level, several researchers have opted for the administration of CORT or ACTH (Siegel and Van Kampen 1984; Davison et al., 1985; Puvadolpirod and Thaxton, 2000a, b; Virden et al., 2007). Others have suggested that glucocorticoid analogs like dexamethasone (DEX) can be used to study

hormone-mediated stress in chicks (Huff et al., 1998; Wideman Jr. and Pevzner, 2012; Vicuna et al., 2015). The administration of CORT or analogs of CORT is a promising tool in the research of adaptation to stress by broiler chickens. It can be used to delineate specific outcomes associated with inducing physiological stress in broilers.

1.3.3.1 Glucocorticoid actions on carbohydrate metabolism

There are at least three distinct transmitting pathways through which the CNS regulates hepatic glucose output to peripheral organs. This can be through epinephrine (EP) secretion, glucagon secretion, and inhibition of insulin secretion, and a direct innervation of the liver (Southwick, 1982). The breakdown of glycogen to glucose in the liver is initiated when EP interacts with β-adrenergic receptors on the cell membrane to activate a well-known sequence that produces cAMP, which subsequently phosphorylates a series of protein kinases that regulates glycogenolysis and gluconeogenesis (Berne and Levy, 1993). Various factors affect the relative contributions of α- and β-adrenergic receptor mechanisms to hepatic glucose production, which vary among species (Young and Landsberg, 1998; Odhiambo, 2004). Under normal conditions, an increase in the uptake and oxidative catabolism of glucose in the liver, muscle, and adipose tissue is triggered by the pancreatic β-cell hormone insulin, which simultaneously inhibits glycogenolysis and gluconeogenesis in the liver. In response to stress, along with drop-in insulin levels, both EP and norepinephrine (NE) undergo several functional adaptations that favor glucose homeostasis disruption.

By the early 1940s, the actions of GCs on energy metabolism have been well established. In two different studies by Evans (1936) and Long et al. (1940), evidence shows that injecting adrenalectomized fasted rats with GCs increased the total carbohydrate

in the liver, followed by a concomitant increase in production of urea, the principal by-product in the conversion of amino acids. They concluded that it is unlikely amino acid degradation was solely from liver proteins but must also have been derived from catabolic processes in extrahepatic tissues. Similarly, early studies using domestic fowl to understand the effect of chronic stressors by repeatedly injecting ACTH or GCs observed an increase in plasma concentrations of CORT, glucose, uric acid, and a concomitant decrease in weight gain, and increase in the relative weights of the liver (Freeman and Manning, 1975; Siegel, 1980; Davison et al., 1985).

There are two general hypotheses proposed regarding the source of these effects. First, the hypothesis recognized that GCs are gluconeogenic- increasing the hepatic production of glucose from non-carbohydrate precursors (Exton, 1979). The precursors include primarily endogenous amino acids from extrahepatic tissues such as skeletal muscle (Munck, 1971; Exton, 1979), which are degraded to provide carbon skeletons that can be converted to glucose (Leeson and Summers, 2001) and stored in the liver. This occurs in conjunction with glucagon and catecholamines, were they initiate substrate supply and modulate enzymes to stimulate glycogenolysis, hepatic gluconeogenesis, and glycogen deposition (Southwick, 1982). Which means under stressful conditions where the circulating GCs level is high, there is a diversion of energy to exercising muscle, in part by mobilizing stored energy, inhibiting subsequent energy storage, and stimulating gluconeogenesis (Southwick, 1982; Siegel and Van Kampen 1984; Davison et al., 1985; Puvadolpirod and Thaxton, 2000a; Virden et al., 2007). The consequence includes unfavorable actions on peripheral tissues, such as skeletal muscle, bone, and adipose tissue.

The second hypothesis explained in the review paper by Munck (1971) outlined that alternatively, GCs decrease glucose uptake and utilization in various peripheral tissues by desensitizing the peripheral tissues to insulin, thus inhibiting glucose uptake. Evidence shows that blood glucose increased by 80 to 100 minutes after adrenalectomized rats were injected with cortisol along with an increase in glucose uptake by muscle but incorporation into adipose tissue was decreased (Munck, 1968). Similarly, in broilers, an increase in the concentration of plasma glucose with DEX-challenge has been reported (Lin et al., 2006a; Li et al., 2009). This means the two hypotheses are not mutually exclusive alternatives and can best be considered as complementary. Moreover, the transport of glucose is regarded as a crucial "chemical message" carried by hormones to initiate general metabolic pathways of the cell. If glucose transport is inhibited, the general metabolic characteristics of the cell dictate the response and eventually becomes manifested in the whole organism. This is probably most recognizable from the administration of CORT or ACTH or DEX treatment in chickens on live performance. A sharp reduction in BW gain is observed (Siegel and Van Kampen, 1984; Klasing et al., 1987; Puvadolpirod and Thaxton, 2000a,b), and a concomitant increase in abdominal fat deposition and reduction in muscle accretion (Siegel and Van Kampen, 1984; Virden and Kidd, 2009). These effects most likely occur because of the action of GCs on glucose metabolism. This is because a decrease in BW and muscle growth is independent of increased feed intake (Bartov et al., 1980; Siegel and van Kampen, 1984; Puvadolpirod and Thaxton, 2000d; Lin et al. 2004a, b; Dong et al. 2007; Wang et al. 2010). Since the goal of a poultry operation is to convert feed into food as economically as possible, managing both the risk and consequence of a stress challenge is critical. While the biological potential for feed conversion is governed primarily by

intrinsic or genetic determinants, in an intensive production system it is the extrinsic determinants, including nutrition, minimization of stress by management, and disease that ultimately decide the efficiency of the operation in both biological and financial terms.

1.3.3.2 Glucocorticoid action on protein metabolism

The main effect of GCs on protein metabolism is to decrease the rate of protein synthesis and stimulate protein degradation (Dong et al., 2007). This increases amino acid export, which ultimately increases net protein degradation in skeletal muscle (Hasselgren, 1999; Vegiopoulos and Herzig, 2007). Experiments have shown that increased circulating GC levels, whether due to pathophysiological conditions or exogenous GC treatment, is associated with muscle atrophy (Hasselgren, 1999; Vegiopoulos and Herzig, 2007; Wang et al., 2015). The biological significance of this effect lies in the altered regulation of metabolism under conditions of stress. As such, a balance between hypertrophy and atrophy in skeletal muscle development is controlled by the action of counter-regulatory anabolic and catabolic signals. The inhibitory action of GCs can limit the transport of amino acids into the muscle affecting protein synthesis (Kostyo and Redmond, 1966). Skeletal muscle formation, myogenesis, is controlled by insulin-like growth factors (**IGFs**). Studies have shown that in vitro, IGF promotes muscle differentiation (Florini et al., 1991) and in vivo regulates the formation, maintenance, and regeneration of skeletal muscle (Liu et al., 1993).

To promote muscle differentiation, IGFs activates multiple intracellular signal transduction cascade, including phosphatidylinositol 3-kinase (**PI3K**)-Akt, targeting the molecules that are both upstream and downstream of myogenin. Glucocorticoids are said to blunt IGF-1 signaling, inhibiting the protein synthesis machinery, and controlling the

initiation step of mRNA translation (Schakman et al., 2008). Kim et al. (2016) reported that DEX affected the IGF/PI3K/Akt pathway resulting in increased proteolytic protein for muscle degradation and decreased ribosomal S6 phosphorylation. Similarly, the mechanistic target of rapamycin (mTOR) signaling pathway has emerged as a central mediator of metabolism and growth. The mTOR acts as the central regulator of protein metabolism (Deng et al., 2009) and ribosome biogenesis by sensing and integrating signals from several stress factors. The Akt activates the mTOR pathway, which increases protein synthesis via activation of ribosomal protein S6 kinase (**S6K**) and eukaryotic translational initiation factor 4B (eIF4B) (Kim et al., 2016). Wang et al. (2015) reported that in vivo, muscle protein synthesis is depressed by DEX administration as evidenced by the decreased phosphorylation of mTOR and ribosomal protein S6K.

Moreover, mTOR activation inhibits glucocorticoid receptor (GR) transcription function and efficiently counteracts the catabolic processes in muscle provoked by GCs (Shimizu et al., 2011). Another inhibitory action occurs via the inhibition of myogenesis through the downregulation of myogenin, a transcription factor mandatory for the differentiation of satellite cells into muscle fibers (Schakman et al., 2008). On the other hand, the enhancement of skeletal muscle protein degradation in response to GC treatment has been attributed to these major cellular proteolytic systems, the ubiquitin-proteasome system (UPS) and the lysosomal system (cathepsins) (Hasselgren 1999; Song et al., 2011), while calcium-dependent proteolysis plays a minor role in protein degradation (Hasselgren 1999). Therefore, both decreased amino acid uptake, as well as increased intracellular breakdown, contribute substrates that circulate and ultimately feed into the gluconeogenesis pathway which occurs in the liver and kidney (Baxter, 1976; Kobayashi

et al., 1989). This is consistent with the observation by Song et al. (2011), where there was a consistent increase in circulating concentrations of urate/uric acid with DEX administration; reflecting increased protein/amino-acid catabolism. Considering the many effects of physiological stress on protein metabolism, Puvadolpirod, and Thaxton (2000d) examined the effect on AA digestion and utilization. Using ACTH-dispensing implants in broilers to induce stress, they reported that challenged broilers treated had a significantly lower protein digestibility than broilers in the nonstressed control group. Percentage nitrogen in excreta was increased in ACTH-treated chicks compared to the control.

1.3.3.3 Glucocorticoid action on mineral metabolism

Another consequence of stress and the release of corticoids is the effect on mineral metabolism. These actions mediated by the GR and mineralocorticoid receptor are integrally involved in the acid-base balance in several species as such can alter mineral and water metabolism (Selye, 1950; Selye, 1976). Glucocorticoids can influence fluid balance by increasing the glomerular filtration rate and renal plasma flow or by antagonizing the release of the antidiuretic hormone which explains the findings that cortisol tends to promote the excretion of 'free-water' (Baxter, 1976). Enhanced water diuresis due to GCs may also result in actual loss of sodium from the body (Dickson, 1984) even though GCs possess considerable sodium retention activity. Furthermore, through the action of catecholamines, CORT can affect mineral metabolism both directly and indirectly (Selye, 1950; Hulter et al., 1980; Lupien et al., 2007) causing an increase in urinary excretion of calcium, magnesium, and phosphorus. Specifically, GCs have a significant impact on bone cells and continued exposure of the skeletal tissue to these steroids results in indirect effects on osteoporosis. Although the pathogenesis of GC mediated bone loss is not completely

understood, evidence suggests both direct effects upon bone cells and indirect effects via calcium homeostatic regulation (Gennari et al., 1984).

Histologic bone studies in patients chronically treated with GCs demonstrate an increase in bone resorption presumably due to inhibition of the modulation of osteoblastic activity (Rasmussen, 1974) or at least in part, to an increased parathyroid hormone activity (Canalis and Delany, 2002). This action decreases calcium absorption in the gastrointestinal system and increases the urinary excretion of calcium. Also, a decrease in both the synthesis of collagen by pre-existing osteoblasts and the recruitment of progenitor cells to functioning osteoblasts is observed (Hahn et al., 1979). Other possible mechanisms involved in bone resorption in GC-induced osteoporosis include decreased gonadotropin production, which may result in increased bone resorption due to estrogen deficiency. The estrogen-deficient state increases the levels of tumor necrosis factor (TNF)-α secreted by T cells which play a central role in bone resorption (Canalis and Delany, 2002). Whereas the effect on bone formation is probably the result of a direct inhibitory action of glucocorticoids on bone cells decreasing cell replication and preventing the terminal differentiation of cells into mature functioning osteoblasts (Reid, 1997; Canalis and Delany, 2002). Besides, the synthesis of type I collagen, a major component of the bone extracellular matrix, can also be hindered which alters the function of the osteoblast with a consequent decrease in the bone matrix available for mineralization (Reid, 1997; Canalis and Delany, 2002). Other effects include increased urinary calcium excretion, reduction in tubular reabsorption of phosphate, and decreasing serum phosphorus, all of which lead to a negative calcium balance resulting in decreased bone formation (Canalis and Delany, 2002).

1.3.3.4 Glucocorticoid action on immunity and inflammation

The biological effects of GCs are usually adaptive except in circumstances that trigger an inadequate or excessive activation of the HPA axis, which may contribute to the development of pathologies. In addition to the general metabolic effects ascribed to stress above, excessive activation of the hypothalamus-hypophysis system gears the body for defense. To understand the actions of HPA axis-related factors on the immune system, we refer back to Selye's 1936 paper "A syndrome produced by diverse nocuous agents" which demonstrates that one of the first responses to stress is the involution of lymphoid organs (thymus, spleen, and lymph glands) in the rat. This probably represents one of the first reported evidence of immune neuroendocrine interaction. As we know, the systemic defense measures against both general and localized stress factors are coordinated through the HPA axis and the autonomic nerves to peripheral systems. Organs of the immune system including the thymus, bone marrow, spleen, lymph nodes, and the GALT, receive sympathetic noradrenergic innervations (Felten and Felten, 1991). Similarly, some immune cells of both the innate and cell-mediated aspects of the immune system also contain β-adrenergic receptors (Odhiambo, 2004). Hence, the immune system, once thought of as an autonomous entity, was soon realized to interact with the neuroendocrine system in response to a foreign substance.

Like other hormones of the steroid-thyroid family, GCs initiate a molecular interaction in their target cells through binding to their nuclear receptors, activating the neural, endocrine, and immune system. The development of lymphoid cells is influenced by the endocrine function of the bursa and thymus, which is coordinated with the neuroendocrine system through hormones (Marsh and Scanes, 1994). Likewise, during an

inflammatory stress response, several cytokines –IL-1β, TNF-α, IL-2, IL-6, and IFN-γ stimulate the secretion of CRH via the HPA axis (Sapolsky et al., 2000; Elenkov et al., 1999). However, the products released from activated immunological cells during immune responses induce both autonomic and endocrine mechanisms that suppress the immune response (Elenkov et al., 1999). The fact that GCs suppress the inflammatory and immunological responses is not surprising considering our understanding of the catabolic and anti-anabolic actions of GCs on lymphoid cells, mast cells, macrophages, other blood elements, endothelial cells, and fibroblastic tissues. (Baxter, 1976; Elenkov et al., 1999). Reports have stated that GCs not only inhibit most aspects of the immune response (Baxter, 1976; Munck et al., 1984), they also cause substantial changes in both acute and chronic immunocompetence and the exaggerated responsiveness of certain components of the immune reaction (Baxter, 1976; Elenkov et al., 1999). Indeed, GCs affect antigen processing and presentation to T-cells; reduce the accumulation of monocytes and granulocytes at inflammatory sites; suppress the activity of humoral and cell-mediated immunological components; suppress the numbers of circulating leukocytes and their migration to extravascular fluid spaces for inflammatory reactions; and affect macrophage function (including phagocytosis) (Baxter, 1976; Siegel, 1980; Munck et al., 1984; Elenkov et al., 1999; Odhiambo, 2004).

1.4 GASTROINTESTINAL HEALTH

The GIT is an open-ended, epithelium-lined tube that runs from the beak to the cloaca in birds. The entire length of the GIT (beak to cloaca) is lined with a mucous membrane or mucosa which allows it to interact continuously with dietary antigens and diverse microorganisms (DeSesso and Jacobson, 2001). Other layers of the GIT include

the submucosa, muscularis propria, and serosa. Within the intestinal mucosa is the muscularis mucosae, lamina propria, and the epithelium, all of which work together to protect the complex multicellular organisms from the external environment (DeSesso and Jacobson, 2001). The intestinal epithelium, which is the innermost layer, constitutes the largest interface and most important barrier that separates the intestinal lumen from the host's internal milieu (Groschwitz and Hagan, 2009). This single layer of intestinal epithelial cells acts as a selectively permeable barrier, critical for fluid and electrolyte secretion and nutrient absorption while also maintaining an effective defense against intraluminal toxins, antigens, and enteric flora, thereby shaping and guiding mucosal immune responses (Santos and Perdue, 2000; Matter and Balda, 2007; Groschwitz and Hagan, 2009). To exert these functions, a variety of cells including enterocytes, goblet cells, endocrine cells, Paneth cells, M cells, stem cells (crypt), and intraepithelial lymphocytes cooperatively form a physical and immunological network for the creation and maintenance of homeostasis between the environments inside and outside the intestine (Santos and Perdue, 2000; Goto and Kiyono, 2012).

Similarly, these sophisticated gatekeepers allow epithelial permeation through two major mechanisms that regulate the transport of solutes, large molecules, and cells across the vessel wall - transcellular and paracellular pathways. In general terms, the transcellular pathway allows the transport of molecules across the epithelium. Through predominantly energy-dependent trafficking, it regulates selective transport of amino acids, electrolytes, short-chain fatty acids, and sugars from the luminal space to the interstitial space (Goddard and Iruela-Arispe, 2013; Adedokun and Olojede, 2019). The paracellular pathway is regulated by junctional complexes or intercellular junctions – desmosomes, tight junction

proteins, and adherens junctions localized at the apical-lateral membrane junction and along the lateral membrane (Groschwitz and Hagan, 2009; Turner, 2009). The paracellular pathway mediates the passive movement of luminal fluid and solutes in the spaces between epithelial cells. Because this barrier is not absolute, it serves as a fence that selectively allows the passage of proteins and lipids between the apical and basolateral plasma membrane domain (Laukoetter et al., 2006; Matter and Balda, 2007). Overall, these intercellular junctional complexes recruit signaling molecules that participate in the regulation of cell proliferation and they restrict diffusion across the epithelial surface (Laukoetter et al., 2006; Matter and Balda, 2007).

Intestinal barrier function is a critical aspect of gut health. In addition to its barrier function, the GIT is also important for the digestion and utilization of nutrients. In this case, fingerlike structures (villi) covered by a thin layer of epithelial cells within the intestinal mucosa of the small intestine, are responsible for the digestion of food and absorption of nutrients (Murphy and Weaver, 2016). Its efficiency is maintained through continuous cellular turnover of the epithelial cells, derived from stem cells in the crypts of Lieberkühn of the epithelium, and the presence of various enzymes on the apical surface and within epithelial cells (Pluske et al., 1996; Santos and Perdue, 2000). Within the crypt unit, epithelial stem cells divide during migration along with the villus structure or in the base of the crypt into enterocytes. The enterocytes migrate along with the villus structure in a spiral path to the tip of the villi and depending on the length of the villus and age, survive between 48 and 96 hours before being lost by apoptosis (Smith et al., 2014). One of the most important gut-specific features is its ability to not respond inappropriately to food derivatives. Under normal circumstances, digested feed (macronutrients; proteins,

carbohydrates and fats, and micronutrients; vitamins and minerals) reaches the epithelium where they are effectively absorbed through several physical and biochemical processes. While these processes are quite honed, the intestine is frequently exposed to an unlimited number of antigens including dietary components, toxins, commensal and pathogenic microorganisms which can predispose it to gastrointestinal inflammation. Hence, in diseased or stressful conditions, excessive penetration of antigens through the epithelial layer promotes a cascade of reactions in the GIT including impaired intestinal barrier function, increased risk of bacterial translocation, and unnecessary inflammatory responses (Vicuña, 2015). This may result in a decrease in digestive efficiency and reduced absorption of both micros- and macronutrients (Celi et al., 2017).

Like mammalians species, chickens also have a separate mucosal immune system within the intestinal epithelium that plays an active role in immunological and inflammatory events. The mucosal immune system is said to be an early evolutionary step necessary to deal with the vast populations of commensal bacteria that co-evolved with vertebrates (Murphy and Weaver, 2016). About 70% of the cells of the immune system are in the GIT (Vighi et al., 2008; Celi et al., 2017), which emphasizes the importance of immune function relating to GIT functionality. This function is carried out through the GALT - a compartmentalized structure comprised of organized lymphoid structures such as the bursa of Fabricius, ceca tonsils, Peyer's patches, Meckel's diverticulum, and small lymphoid aggregates scattered along the intraepithelial and lamina propria of the GIT (Lillehoj and Trout, 1996). Accordingly, antigen-presenting cells, immunoregulatory cells, and effector cell types are derived from these distinct structures. Considering that intestinal inflammation can negatively impact gastrointestinal function by disrupting the structure

and function of the intestinal mucosa, these defense mechanisms are essential to protect them from foreign invasion. The determination of the presence of intestinal inflammatory activity is crucial for the assessment of the gastrointestinal barrier and prevent the invasion or adherence of luminal pathogens to the cell surface of the epithelial cell.

1.4.1 Stress and brain-gut axis

The process of stress combines three major conceptual domains: the sources of stress, mediators of the stress response, and the manifestations of the stress pathologies (Pearlin et al., 1981). In recent years it has been recognized that diseased conditions or stressful events can interfere with the functionality of the GIT. Not only does it have to maintain the elaborate luminal bacteria within its periphery, but it also has divergent functions in terms of nutrient absorption and host local defense. This means the GIT plays an important role in health and well-being in so many ways. As previously discussed, poultry is faced with several stress factors including environmental, biological, or physiological stressors that lead to the activation of the stress response. Biological (Lillehoj and Trout, 1993; Lillehoj and Lillehoj, 2000; Yun et al., 2000; Laurent et al., 2001; Amerah and Ravidran, 2015; Adedokun et al., 2017), environmental (Scott and Balnave, 1988; Mashaly et al., 2004; Babinszky et al. 2011; Quinteiro-Filho et al., 2012; Sohail et al., 2012), or chemically-induced (Binder, 1978; Spitz et al., 1994; Puvadolpirod and Thaxton, 2000a-d; Li et al., 2009; Zhao et al., 2012; Vicuna et al., 2015), stressors can predispose the birds to gut health issues. The impact of the central nervous system sometimes called the “brain-gut axis,” is not fully elucidated. The stress response is comprised of complex interactions between components of the CNS and peripheral systems - the endocrine, immune, and commensal bacteria in the GIT. Indeed, there is ample evidence that suggests

the entities of the GIT microbiome, GIT barrier, and the enteric nervous system (ENS) function to confer epithelial defense, improve metabolic function, and maintain overall gut health in stressful conditions (Santos and Perdue, 2000; Söderholm and Perdue, 2001; Bischoff, 2011)

The first link between stress and gastrointestinal diseases was from the observation of a wounded soldier which showed that fear or anger can significantly influence gastric physiology, especially acid secretion (Beaumont and Beaumont, 1847). In the later years, the crucial role the ENS (also known as "little brain") play in the interactions between the CNS and the gut was elucidated (Konturek et al., 2011). The brain communicates with the gut through multiple parallel pathways including the ANS, the HPA, and other connections which were termed the brain-gut-axis (BGA) to interact with the immune system (Konturek et al., 2011; Foster et al., 2017). This synergy regulates the physiological gut functions including secretion, motility, the release of various neuropeptides and hormones, and creating a defensive barrier between externally derived pathogens and the internal biological environment (Söderholm and Perdue, 2001; Konturek et al., 2011; Foster et al., 2017). While we would not focus much on the microbiota in this review, it is important to state the existence of a three-dimensional interaction. The brain-gut axis, gut microbiota, and the immune system play an important role in the modulation of the stress response of the gut to develop different gut disorders. Through different mechanisms, the microbiota communicates with the BGA: either by direct interaction with mucosal cells (endocrine message), via immune cells (immune message), and or via contact to neural endings (neuronal message) (Konturek et al., 2011).

To investigate the consequence of chronic stress on gut mucosa and pathology, adult rats were exposed to water avoidance stress (WAS, standing on a platform to avoid surrounding water) or no stress for 10 days. The WAS group exhibited an increase in the levels of circulating corticosterone and failed to gain weight during the experimental period. Mucosal abnormalities, physiological changes of ion secretion, and barrier dysfunction, but also morphological changes were observed (Tache and Perdue, 2004). Maejima et al. (1984) reported that rats that received thermal injury exhibited viable *Escherichia coli, Proteus mirabilis, Staphylococcus sp., and Clostridium sp*. in their mesenteric lymph nodes two days after exposure. This supported the concept that indigenous bacteria do not pass through the GI mucosa unless there is an overgrowth of certain bacteria populating the GI tract or when the host immune defense mechanisms are compromised. Similarly, in response to an acute stressor, colonic paracellular permeability increases and has been associated with the development of visceral hypersensitivity (Ait-Belgnaoui et al., 2005). Moreover, immune cells have receptors for the catecholamines epinephrine and norepinephrine, which can respond to signals from the HPA axis and become either activated or downregulated.

1.5 NUTRITIONAL MANAGEMENT

1.5.1 Sub-therapeutic use of antibiotics

The efficiency of antibiotic use has been unparalleled for the prevention, control, and treatment of infectious diseases in humans (McEwen, 2007). The same is true for its use in the livestock industry. The in-feed administration of non-therapeutic doses of antimicrobial growth promoters (AGP), has been successfully used to promote animal growth for more than 60 years. In 1946, Moore et al. reported that adding antibiotics

(streptomycin) to the diet, improved growth responses in chicks. Similarly, another study reported the beneficial effects of adding antimicrobials to poultry diets while in search of an inexpensive source of vitamin B_{12} (Stokstad et al., 1949). Vitamin B_{12} is considered an essential nutrient and is suggested to be produced by some bacteria species. Stokstad et al. (1949) reported that adding *Streptomyces aureofaciens* metabolites (known to produce vitamin B_{12}) in the diet, improved BW gain in the chickens, with a markedly decrease feed intake. Later studies revealed that the metabolites included not only vitamin B_{12} but also the antibiotic, chlortetracycline (Stockstad and Jukes, 1950). This indicated that the beneficial effects were not entirely the effect of Vitamin B_{12} but rather the advantageous effect of the AGP added (Stockstad and Jukes, 1950). Further studies showed that the supplementation of chlortetracycline in diets improved BW gain of turkey poults (Stokstad and Jukes, 1950), and chickens (Whitehill, 1950). Similar production advantages were observed in swine and cattle research (Bartley et al., 1950; Loosli and Wallace, 1950; Gaskins et al., 2002).

A combination of beneficial effects is derived from AGP use – growth promotion, feed efficiency, therapy for clinical bacterial infections, and prophylactic against clinical infections in a large group of animals. Hence the administration of AGPs became an effective tool for ensuring the development of intensive and large-scale farming (Gustafson and Bowen, 1997; McEwen, 2007). It became a common practice in the poultry industry that low levels of certain antibiotics be added to the feed, for an extended period, to dampen potential systemic infections, stabilize the gut microflora and, diminish the effects of subclinical infections (Mateos et al., 2002). The growth benefits observed with antibiotics use is suggested to be from its modification of the microbial population in the gut (Coates

et al., 1955; Coates et al., 1963). However, further observations demonstrated that the growth improvement caused by AGPs is a subject of their antimicrobial activity, as antibiotics do not have growth-promoting effects in germ-free animals. (Coates et al., 1963; Coates, 1980). Gaskins et al. (2002) summarised that AGPs enhance growth through, decrease microbial competition for nutrients, reduce the production of microbial metabolites, inhibit sub-clinical infections, and increase nutrient assimilation through a thinner intestinal wall. By inhibiting the growth of bacteria, AGPs limit the growth of gut microbiota and environmental pathogens. This reduces energy and nutrient competition among bacteria, thereby allowing more energy and nutrients to be allocated to the growth of the host animal.

A multitude of AGPs is used in the broiler industry. The information obtained from the 2002 feed additive compendium reported that over 33 antimicrobial compounds were approved by the United States Food and Drug Administration (FDA), to be used in broiler feed without veterinary prescription (Miller, 2001). Fifteen of those compounds were used for the treatment of coccidiosis, 11 used as AGPs (for growth promotion and to alleviate the effects of bacterial infections), and 7 were used for the control of other diseases (Jones and Ricke, 2003). Currently, there is some level of resistance to therapeutic levels of all of these compounds due to their continued use as anticoccidials. hence, alternative methods are sought out to substitute for the growth permitting properties and additional antimicrobial activity characteristic of AGPs. After so many years of antibiotic use in human and veterinary medicine, concerns about the development of antibiotic resistance in the human population surfaced (Wierup, 2001). Recommendations were made to ban sub-therapeutic use of AGPs in animal feeds, particularly the European Union. in the

1980s. Concerns that the emergence of pathogenic bacteria resistant to several antimicrobial agents might pose a risk to human and animal health was compelling (World Health Organization, 2000; Dibner and Richards, 2005). The WHO recommended that the prophylactic use of antimicrobials in livestock be reduced, and those in the same class as used for humans be terminated or rapidly phased out (WHO, 2000; Dibner and Richards, 2005). The United States Food and Drug Administration further issued restrictions on the use of AGPs (Costa et al., 2011).

In addition to this, consumer concerns on the subject have impacted legislation. The trend for "natural" or "organically grown" animal products continues its upward trajectory in recent years among consumers. Whether for health or environmental reasons, consumers are making conscious decisions about what they eat or buy. This is especially true in western countries. The bans on AGPs have caused major problems for animal health and producer profitability due to dysbacteriosis, or imbalance of microflora, and other intestinal issues caused by bacterial overgrowth. So, to maintain productivity, producers are considering efficient alternatives to AGPs (Costa et al., 2011). A renewed interest in the immunologic and growth-regulating functions of the GIT as well as the immunomodulatory effect of certain nutrients are being investigated. Emphasis is placed on finding alternatives to improve the birds' health, through nutritional regimens to modulate the immune system of chickens (Khan et al., 2012). Dietary modulations using products like antibacterial vaccines, immunomodulatory agents, bacteriophages and their lysins, antimicrobial peptides (AMPs), pro-, pre-, and sym-biotic, plant extracts, inhibitors for bacterial quorum sensing (QS), biofilm and virulence, and feed enzymes, vitamins and minerals are some of the alternatives for AGPs. (Cheng et al., 2014).

For any livestock operation, nutrition is important; however, with the removal of AGPs, nutrition should be placed at a higher priority because poor nutrition will predispose birds to opportunistic pathogens. Almost all nutrients in the diet play a crucial role in maintaining an "optimal" immune response such that deficient and excessive intakes can have negative consequences on immune status and susceptibility to a variety of pathogens. This means in a diseased or stressed state, increasing the intake of specific nutrients becomes paramount. Nutritionists formulate diets that supply adequate amounts of specific nutrients including carbohydrate, protein (amino acids), fat, minerals, and vitamins. Hence, the concentration of the nutrient in the feed is dependent on the physiological and health status of the animal. For maintenance, nutrient requirements are fairly low, but they are increased for growth or egg production (National Research Council, 1984) or in a diseased state. More research into nutrient-nutrient interactions and immune function in avian species is needed. Currently, the best dietary advice to enhance the immune function is to ensure variety, balance, and moderation of the nutrients. We can only achieve this if there is a set standard to how much of these additives can be added to the diet, especially under challenging conditions.

1.5.2 Alternatives to antibiotics

The biotechnology industry has offered the animal industry numerous potential applications including, the development of innovative products, advanced husbandry techniques, and clarity of understanding of the digestive physiology. This helps to improve the animal's performance, enhance production potential, and improve health status. Because of this knowledge and technological advancement, different classes of nutrients (carbohydrates, protein, fat, etc) can be modified or protected, resulting in improved diet

formulation that more accurately meet the specific needs of the animal. Pre- and probiotics are viable alternatives to AGPs explored to improve nutrient digestion, disease resistance, and good health. Pre- and pro-biotics or immune supplements can inhibit pathogenic gut microorganisms or make the animal more resistant to them. Exogenous enzymes can be used to improve nutrient availability from feedstuffs, lower feed costs, and reduce nutrient excretion into the environment. Vitamins and minerals, a group of complex inorganic compounds present in minute quantities in natural food, are required for normal growth and maintenance (Lloyd et al., 1977). Substantial information available in the literature indicates that certain dietary components can modulate a bird's susceptibility to infectious challenges, and subtle changes in the level of nutrients or the types of ingredients may at times, be of critical importance.

1.5.2.1 Probiotics

Probiotics are defined as live, naturally occurring microorganisms that beneficially affect the host animals and can improve their intestinal microbial balance (Fuller, 1989), or the properties of the local microflora (Song et al., 2014). Through competitive exclusion, these microorganisms inhibit the growth of pathogenic microorganisms and compete with them for a place in the intestinal epithelium. They can also modulate the immune system to increase the production of immunoglobulins and produce antimicrobial metabolites that inhibit the growth of pathogens. Studies have shown that the ability of probiotics to modulate the diversity and composition of the gut microbiota, may reduce the growth of pathogenic bacteria such as *E. coli* (La Ragione et al., 2004; Arreguin-Nava et al., 2019), *Salmonella sp* (Grimes et al. 2008), *Clostridium perfrigens* (Rahimi et al., 2019), *Campylobacter jejuni* (Ghareeb et al., 2012; Smialek et al., 2018), and *Eimeria sp* (Dalloul

et al., 2005; Lee et al., 2007; Wael-Abderlrahman et al., 2014; Sokale et al., 2019). The effectiveness of probiotics on performance is an important factor considered in the poultry industry. Although, some studies have demonstrated the beneficial effect of probiotics on BW, BWG, and lowered feed conversion ratio (Awad et al., 2009), others have reported no effect (Murry et al., 2006; Liu et al., 2007).

There are three major categories of probiotics approved by the FDA. Live apathogenic bacterial strains belonging to genus lactic-acid producing bacteria (*Bifidobacterium, Lactobacillus*, and *Streptococcus*), *Bacillus sp*, and yeast (*Saccharomyces*), are used in livestock and poultry (Dharma et al., 2011). Lactic acid bacteria-based probiotic has been reported to significantly reduce the incidence of *Salmonella enteritidis* by 60 to 70 % or *Salmonella typhimurium* by 89 to 95 % in the cecal tonsils of 1-day old broilers (Higgins et al., 2008). Broilers fed diets supplemented with B. subtilis exhibited a higher body weight, lower mortality, and lower intestinal necrotic enteric (NE) lesion score in birds challenged with NE (Sokale et al., 2019). Similarly, probiotics can also modulate either the innate or adaptive immune system (Dugas et al., 1999). Pinto et al. (2009) found that pretreating the gut epithelium cells with *Lactobacilli* significantly increased IL-8 expression compared to the media treated group in vitro. These authors (Matsuzaki et al., 1997; Calcinaro et al., 2005; von Boehmer, 2005) also reported that probiotics could induce regulatory T cells or Th2 cells that could produce anti-inflammatory cytokines, IL-10 and IL-4, and reduce pro-inflammatory cytokines such as interferon IFN-γ. Li et al. (2015) reported that *Bacillus amyloliquefaciens* decreased IL-1β, increased IL-10, and elevated the expression of jejunal TLR4 in broilers challenged with lipopolysaccharide (LPS).

1.5.2.2 Prebiotics

Prebiotics, on the other hand, is defined as dietary components that are not digested in the gastrointestinal tract until they reach the hindgut where they are acted upon by bacteria. They selectively stimulate the growth or activity of one or a limited number of bacterial species already resident in the colon and thus, attempt to improve the host health. Prebiotics are not hydrolyzed or absorbed in the upper regions of the digestive tract. In other words, prebiotics is not a microorganism but rather, a substrate meant to support the growth of beneficial gastrointestinal microbes. Only recently have oligosaccharide prebiotics being of interest in the area of animal nutrition and health. Oligosaccharides compounds have several classifications based on the type of monosaccharides (glucose, fructose, galactose, mannose, etc.), and consist of groups of monosaccharides. The groups of monosaccharides range in length from 2 to 60 and are linked by β-(2,1) bonds which, prevent hydrolytic digestion in the upper gastrointestinal tract of monogastric animals (Briggs et al., 2011).

The common prebiotics used in poultry production includes fructooligosaccharide (FOS) products [oligofructose, inulin, and short-chain fructooligosaccharides (SCFOS)], mannan-oligosaccharides (MOS), gluco-oligosaccharides, galactooligosaccharides, stachyose, transgalacto-oligosaccharides (TOS), oligochitosan, and lactulose (Patterson and Burkholder, 2003; Zhang et al., 2003; Jiang et al., 2006; Huang et al., 2007). They are specific non-digestible materials that can selectively increase the proliferation of bacteria types like bifidogenic bacteria (*Bifidobacterium sp.*) and Lactic acid bacteria (Lactobacillus sp.) which, are beneficial to the host (Patterson and Burkholder, 2003; Xu et al., 2003; Yusrizal and Chen, 2003). Broiler chickens fed diets containing different levels (2.0 or 4.0

g/kg) of fructooligosaccharides had significantly lower feed conversion ratios compared to the controls. The addition of 4.0 g/kg FOS increased the average daily gain of broilers and enhanced the growth of *Bifidobacterium* and *Lactobacillus* but inhibited *Escherichia coli* in the small intestinal (Xu et al. 2003).

In another study, the effect of different oligosaccharides - inulin, oligofructose, SCFOS, MOS, and TOS supplemented in a basal corn-isolated soy protein diet, in intact and cecectomized roosters were examined (cecectomized roosters were used to eliminate any confounding effect of cecal microbes to allow for more accurate measurement of amino acid digestibility). Although the prebiotic additions had a little overall effect on amino acid digestibilities in cecectomized and intact roosters, improvements in isoleucine, lysine, methionine, and valine digestibilities were observed primarily in cecectomized birds fed diets supplemented with MOS or TOS. However, inulin supplementation depressed methionine digestibility in intact roosters (Biggs et al., 2007). Also, dietary supplementation of fructooligosaccharide (0.3% dose) or oligochitosan (0.1% dose) showed growth-promoting effects similar to antibiotic flavomycin (Huang et al., 2005) or aureomycin (Li et al., 2008). Wang et al. (2003) reported that dietary supplementation with 0.1% of chitosan reduced the number of E. coli in the GIT of chicks while improving the small intestinal microvilli density, serum antibody titer against the Newcastle disease virus. Similarly, positive changes in digestive enzymes, gut morphology, and increased serum concentrations of IgG, IgA, and IgM were noticed in birds given prebiotic-supplemented feed (Xu et al., 2003; Zhang et al., 2003; Huang et al., 2007).

1.5.2.3 Enzymes

Enzymes are naturally occurring and are produced by all living organisms for catalyzing chemical reactions. In the later part of the 19th century, enzymes were discovered and have been used in industry and food processes since the early 1900s (Clarkson et al., 2001). As special proteins, enzymes catalyze or accelerate the rate of specific chemical reactions in which the enzyme activity may be dependent on the substrate in a random manner or it may be through very specific sites on substrates such as fat, protein, or carbohydrates (Ferket, 1993). In the livestock industry, the addition of microbial enzymes to improve feed quality has dramatically increased. The benefits of enzyme use manifest by reducing the effect of anti-nutrients that cannot be hydrolyzed by endogenous enzymes and the efficient digestion and utilization of energy and nutrients from feed. This increases the amount of nutrients the animal could extract from their diet, as such microbial populations are reduced and so is the competition for intestinal nutrients by the hindgut microflora. Much of the performance response is thought to relate to changes in the microfloral population changes rather than the direct effect of the enzyme per se on diet digestibility. As a result, the response to enzymes is likely to be more pronounced in the absence of growth promoters than in their presence, although absolute performance is optimized in the presence of both products (Bedford, 2000).

In addition to improving nutrient and energy digestion, they enhance the degradation of anti-nutritional factors and toxins. (Campbell and Bedford, 1992; Bedford, 1996). However, enzymes cannot be applied broadly but rather used specifically to target appropriate substrates such as fiber, phytate, protein, carbohydrate, etc. White Pekin ducks fed diets supplemented with an enzyme mixture of amylase, protease and xylanase had increased BWG, feed efficiency, and ileal nitrogen digestibility however, ileal energy

digestibility was not affected (Hong et al., 2002). Starch digestibility has been reported to account for up to 35% of the improvement in available metabolizable energy as a result of xylanase supplementation, whilst fat accounts for 35% and protein for 30% (Bedford and Morgan, 1996). An enzyme blend (xylanase, amylase, and protease) in combination with a Bacillus-based direct-fed microbial, improved BWG, reduced FCR, increased energy efficiency, and reduced inflammatory responses compared to the non-supplemented groups challenged with coccidia vaccine in broilers (Dersjant-Li et al., 2016).

1.5.2.4 Vitamins and minerals

One of the many approaches proposed to enhance immune response is to supplement the diet with vitamins, which can modulate immune responses due to their extensive involvement in structural components and molecular mechanisms (Lloyd et al., 1977). According to Weber (2009), the feed industry recognizes that the minimum dietary vitamin levels required to prevent clinical deficiencies may not support the optimum health and performance of poultry. This is because the productivity of poultry farming has continued to grow through genetic improvement, enhanced nutrition, management, and husbandry systems, which might increase the demand for vitamins. Vitamin E (VE), a fat-soluble vitamin of plant origin, is essential for the integrity of the reproductive, muscular, circulatory, nervous, and immune systems (Leshchinsky and Klasing, 2001). It is most likely that VE, like other nutrients, affects the development and maintenance of immunocompetence through multiple functions. This can be by acting directly on immune cells or indirectly affecting metabolic and endocrine parameters, which in turn influences the immune system (Gershwin et al., 1985; Leshchinsky and Klasing, 2001).

Vitamin E is primarily known for its role as an antioxidant, reducing cellular free radical damage, but its deficiency causes a severe reduction in immune responses (Cook, 1991). Niu et al. (2009) found that abdominal exudate cells and the percentage of macrophages increased in heat-stressed broilers fed diets supplemented with 200 mg/kg VE. Qureshi et al. (2000) suggested that the process of phagocytosis by macrophages is a membrane-mediated phenomenon, maintained by the availability of higher levels of VE needed for the integrity of phagocytosis. Also, VE protects cell membranes from damage caused by reactive oxygen species by acting as an oxidant scavenger (Field et al., 2002). The results reported from supplementing VE in the diet varies, depending on the level and duration of the feeding program, assessment criteria, and stress conditions. Allen and Fetterer (2002b) reported that increased dietary levels of VE-acetate had no significant effects on weight gain and feed conversion in Eimeria maxima infected chicks. Eid et al. (2008) reported that VE supplementation in the diet (200mg/kg) decreased the levels of plasma triglycerides and TBARs in laying hens challenged with DEX but had no effect on BW.

Immune cells are particularly susceptible to oxidative damage and produce large amounts of free radicals during inflammation. Moriguchi and Muraga (2000), observed that VE improves the immune system by activating T-cells which, enhances host antiviral activity and increases the production of IFN-γ. Similarly, Erf and Bottje (1996) reported that broilers fed diets containing VE (100,000 IU/ton of feed) had a higher antibody response to S.pullorum, a T-cell independent antigen, than broilers receiving no supplemental VE suggesting that VE improves the response of B-cells to S. pullorum. As an antioxidant, VE also protects the phospholipids of cellular and sub-cellular membranes

from destruction by lipid oxidation and accordingly to maintain the morphological integrity and functionality of cells and tissues of the organism. As an essential micronutrient, VE optimizes the performance and reproduction of farm animals. In poultry, VE protects the ovarian follicles from oxidative damage and there is evidence that it also facilitates the release of vitellogenin as a precursor of the yolk from the liver and thus has an important function in egg production. Based on these effects, VE supplementation is recommended in broiler breeders and laying hen diets. In an HS study, laying hens fed diets supplemented with VE had increased egg production (Bollinger-Lee et al., 1999).

Zinc (Zn) and Se are some of the trace minerals that have been associated with an improvement in immunity or functions that support immunity. Zinc, an essential nutrient for nearly all organisms, is an essential cofactor in hundreds of proteins (enzymes) within the avian body. The immune system is dependent on the functions of cellular metabolism with Zn being central in cellular metabolism and functions both structurally and catalytically in important biochemical pathways. It has been hypothesized that the antimicrobial effect of Zn leads to growth promotion where gut microbiota is altered to reduce fermentation loss of nutrients and to suppress gut pathogens, a mechanism similar to antibiotic-based growth promoters (Yazdankhah et al., 2014). Similarly, other evidence suggests that pathogens can have a competitive advantage over the commensal microbiota under Zn limiting conditions, thereby being promoted under an inflamed state (Gielda and DiRita, 2012).

Recently, it was shown (Gielda and DiRita, 2012) that Zn competition exists in Campylobacter. jejuni and other bacterial species in the host-microbiota of conventionally raised versus germ-free broiler chickens. Under conditions of Zn deficiency, this might

lead to the preferential growth of bacteria able to survive at low-Zn levels. Furthermore, many recent studies have shown that prophylactic doses of Zn (as Zn oxide, ZnO) in various animal models increased the presence of Gram-negative facultative anaerobic bacterial groups, the colonic concentration of short-chain fatty acids (SCFAs), as well as overall bacteria species richness and diversity. Zinc supports the metabolism of numerous cells by functioning structurally and catalytically as metalloenzymes. Zinc's role in immunity has been reviewed (Kidd et al., 1996). Zinc is also essential for normal intestinal barrier function and the regeneration of damaged gut epithelium (Alam et al., 1994). Zinc supplementation has been reported to prevent tight junction opening in a rat colitis model (Sturniolo et al., 2002) and reduced intestinal permeability while increasing the concentration and expression of tight junction proteins in weaning piglets (Zhang and Guo, 2009). Dietary zinc also effectively prevents or improves the loss of intestinal integrity during malnutrition (Rodriguez et al., 1996), ethanol-induced intestinal damage (Lambert et al., 2003), chronic inflammatory bowel diseases (Sturniolo et al., 2001), and infectious diarrhea (Alam et al., 1994).

1.5.2.5 Other supplement strategies

The supplementation of sodium, potassium, and chlorides in the diet have been clearly defined to achieve a balance between cation and anion supply. Sodium (Na^+) and Cl^- are low-cost nutrients, and their manipulation has little influence on the diet cost. However, they are involved in several metabolic activities such as the nerve cells, acid-base balance, osmotic pressure regulation, and monosaccharide, and amino acid absorption, which requires that they are supplemented at precise levels quality (Murakami et al., 2001). Electrolyte imbalance is quite rare since the body's buffering system provides

maintenance of normal physiological pH value, however, adequate balance ensures optimum growth, bone development, good litter, and egg quality (Murakami et al., 2001; Baloš et al., 2016). Physiological stress is associated with changes in electrolyte imbalance (Borges, 1997; Sandercock et al., 2001; Murakami et al., 2001; Olanrewaju et al., 2006, and 2007). Thus, the maintenance of this value is determined by three major factors – balance and ratio of electrolytes in feed, endogenous acid production, and level of renal activity (Baloš et al., 2016).

To maintain acid-base homeostasis in poultry, the combined intakes of Na^+, K^+, and Cl^-, are important to determine dietary electrolyte balance (DEB) (Mongin,1981). Most commonly, the electrolyte balance is described by a simple formula $Na^+ + K^+ - Cl^-$ and expressed as mEq/kg in the diet (Mongin, 1981). Salts such as sodium bicarbonate ($NaHCO_3$), potassium chloride (KCl), calcium chloride ($CaCl_2$), and ammonia chloride (NH_4Cl) in water and/or feed proved beneficial for broilers under different heat stress regimens (Borges, 2003; Ahmad et al., 2005). Some researchers have reported the beneficial effect of substituting a part of the dietary inorganic Na provided in the diet as NaCl with $NaHCO_3$ (Frank and Burger, 1965; Makled and El- Gammal, 1977; Makled and Charles, 1987; Balnave and Muheereza, 1997; Yörük et al., 2004), others have reported no benefits (Cox and Balloun, 1968; Ernst et al., 1975; Grizzle et al., 1992).

The supplementation of Na^+ (without Cl^-) in feed leads to an increase in the concentration of HCO_3^- ions and elevated blood pH, whereas supplementation of Cl^- (without Na) decreases the concentration of HCO_3^- ions and pH value. This means to correct for the decrease in blood bicarbonate levels during high temperatures decreases, an electrolyte source with less Cl^- will be more efficient. Sodium bicarbonate in the diet may

help to maintain proper pH balance, eliminate acidosis, and ensure maximum growth and productivity (Ahmad et al., 2005; Naseem et al., 2005). Similarly, to correct for the reduced HCO_3 concentration in the lumen of the shell gland that negatively affects eggshell quality in high temperatures, (Hall and Helbacka, 1959; Wideman Jr. and Buss, 1985). Ghorbani and Fayazi, (2009) observed an increase in the egg weight, egg production, and feed intake in laying hens subjected to heat stress (36 – 42 ℃) and fed a diet containing $NaHCO_3$. Feeding corticosterone-treated broilers with supplemental water electrolytes improved body weight gain (Virden et al., 2009). The addition of 0.20 % dietary $NaHCO_3$ with monensin significantly improved body weight, uniformity, feed efficiency, and breast meat yield in coccidia challenged birds (Hooges et al., 1999).

1.6 OBJECTIVES

1.6.1 Scope of the dissertation

Unfavorable environmental stimuli that create a condition of stress can influence an animals' susceptibility to disease. Whether host resistance to disease is increased or decreased may depend on many factors, such as the type of stressor, the duration of the stress episode, the immune status of the animal, and the particular pathogen involved. In animal production, the consequences of stress-related factors are exacerbated by consumer demands to find economically viable strategies to the conventional use of sub-therapeutic antibiotics in poultry diets, without affecting production parameters. In summary, this literature review extensively outlined the negative impact of stress in poultry production. Specifically, focusing on stress factors like coccidiosis and heat stress conditions commonly affecting poultry, as well as using a glucocorticoid derivative to understand the stressful situations the birds might be predisposed to on the farm. From the background

information and studies presented in this review, the importance of intestinal health is paramount to the overall health and performance of poultry. Nutritional strategies play an important role in maintaining optimal gastrointestinal tract conditions to aid production Hence, the focus is to fully understand the potential of using feed additives during periods of stress conditions, and formulating diets to account for the malabsorption of nutrients to support poultry performance during the critical stages of development.

This dissertation hypothesized that the stress agents (DEX, coccidia-vaccine, and heat stress) used in the studies presented would induce negative effects on the birds. Some, or all the measured parameters such as the BW, FE, digestibility, and utilization of nutrients, intestinal morphology, permeability, and immune response (in broilers), and egg production, egg quality, blood profile, and structural integrity (laying hens) will be impaired or altered. However, the addition of the feed additives will mitigate the negative effect and confer protection by eliminating ROS, balancing the expression of inflammatory mediators, maintaining the intestinal integrity to achieve maximum digestion and utilization of nutrients and growth.

The overall aims of the research presented in this dissertation were to evaluate the effect of stress factors in poultry production on performance indices, intestinal barrier permeability, digestibility and utilization of nutrients, skeletal integrity, and immune response. With the limitation of antibiotics used in poultry production, understanding the effect of feed additives on specific cellular and molecular mechanisms is important for optimizing bird health and productivity. As such, any product that alters the balance of continuous antigenic challenge and epithelial, or mucosal defense is a potential target for

therapeutic strategies to modulate gut inflammation initiated or perpetuated by stress (Figure 1.7).

1.6.2 Specific objectives

1.6.2.1 First research objective

To determine the effect of dexamethasone-induced stress on performance, intestinal morphology, nutrient and energy digestibility and utilization, bone integrity, and immune response of broiler chickens, and the interplay of supplementing two different inorganic sodium sources (NaCl or a combination of NaCl and $NaHCO_3$) and EconomasE™.

1.6.2.2 Second research objective

To determine the effects of supplemental EconomasE™ (a blend of ingredients that maximizes and maintains the antioxidant status of the animal) and two inorganic sodium sources (NaCl or a combination of NaCl and $NaHCO_3$) in laying hens on their production parameters, egg quality, blood metabolites intestinal morphology, keel bone, bone-breaking strength, and ash at different environmental temperatures.

1.6.2.3 Third research objective

To outline the dysbiosis resulting from a well-established enteric broiler coccidia vaccine and DEX-challenge model with or without Natustat™ (a natural plant-derived proprietary product composed of at least 1 yeast-derived MOS plus organic mineral nutrients and plant extracts) supplementation. The analysis adopted a multifaceted approach that considered the effect of the challenge on performance, ileal nutrient and energy digestibility, intestinal morphology, gut permeability, and immune response. Furthermore, to understand how these stress factors alter intestinal inflammation-

associated permeability, uptake of fluorescein isothiocyanate (FITC-d) a 3-5 kDa, a marker used to measure tight junction permeability in enteric inflammation models, was evaluated.

1.7 FIGURES

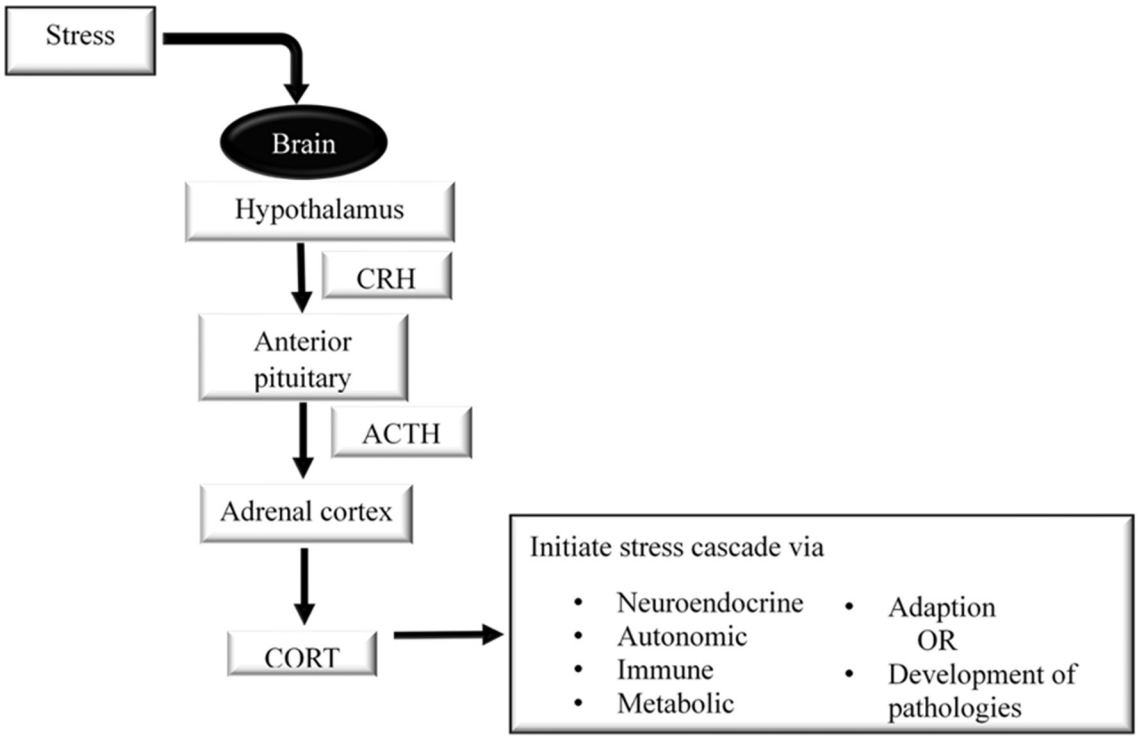

Figure 1.1 Schematic diagram of the major components of the hypothalamic-pituitary-adrenal (HPA) Axis. The activation of the HPA axis in response to stress initiates a cascade of events through the paraventricular nucleus (PVN) of the hypothalamus to secrete corticotropin-releasing hormone (CRH). Activation of the sympathetic pathway initiates the release of catecholamines from the adrenal medulla which act on various target organs and tissues. The CRH travels to the anterior pituitary. There, CRH binds CRF receptors to release adrenocorticotropic hormone (ACTH) into circulation. The ACTH binds to receptors in the zona fasiculata of the adrenal cortex and causes the release of glucocorticoids (GC). The GC secreted as corticosterone (CORT) in birds act on a variety of target tissues and organs to maintain homeostasis (Adapted from Matteri et al., 2000).

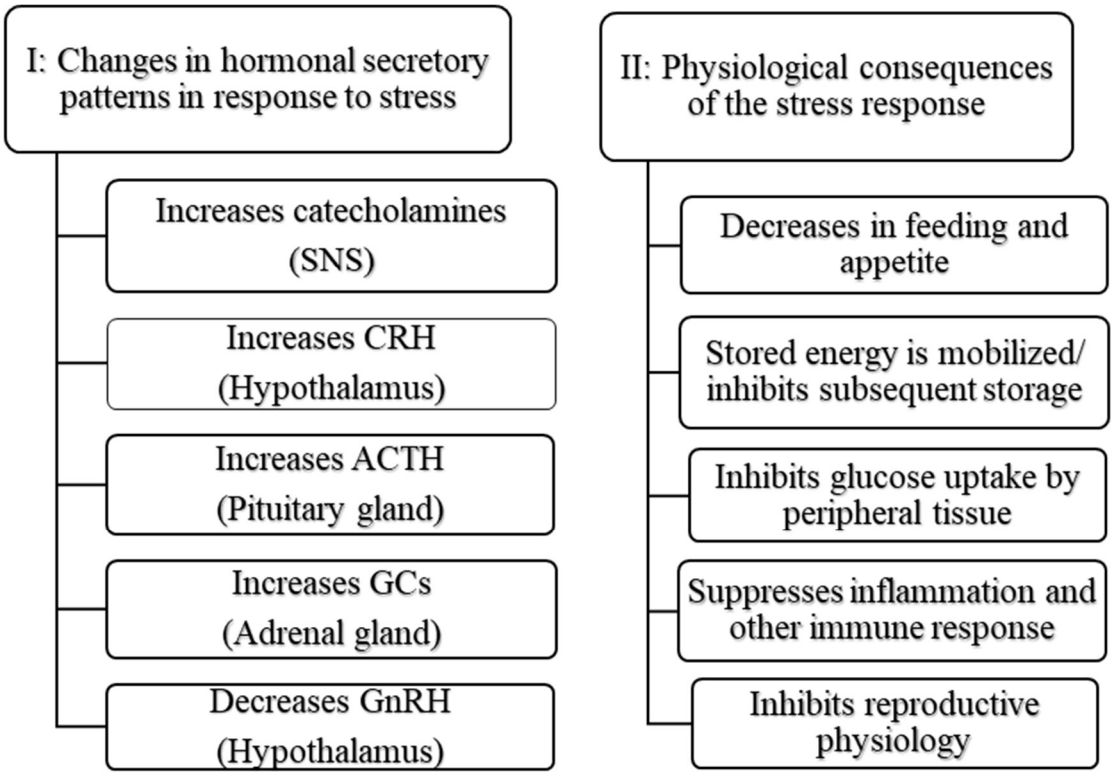

Figure 1.2 Schematic overview of the typical endocrine stress response. I. Changes in hormone-secretory patterns in response to a stressor. II. Physiological consequences of the stress response (Sapolsky et al., 2000). Abbreviations: SNS, sympathetic nervous system; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropin releasing hormone; GCs, glucocorticoids; GnRH, gonadotropin-releasing hormone

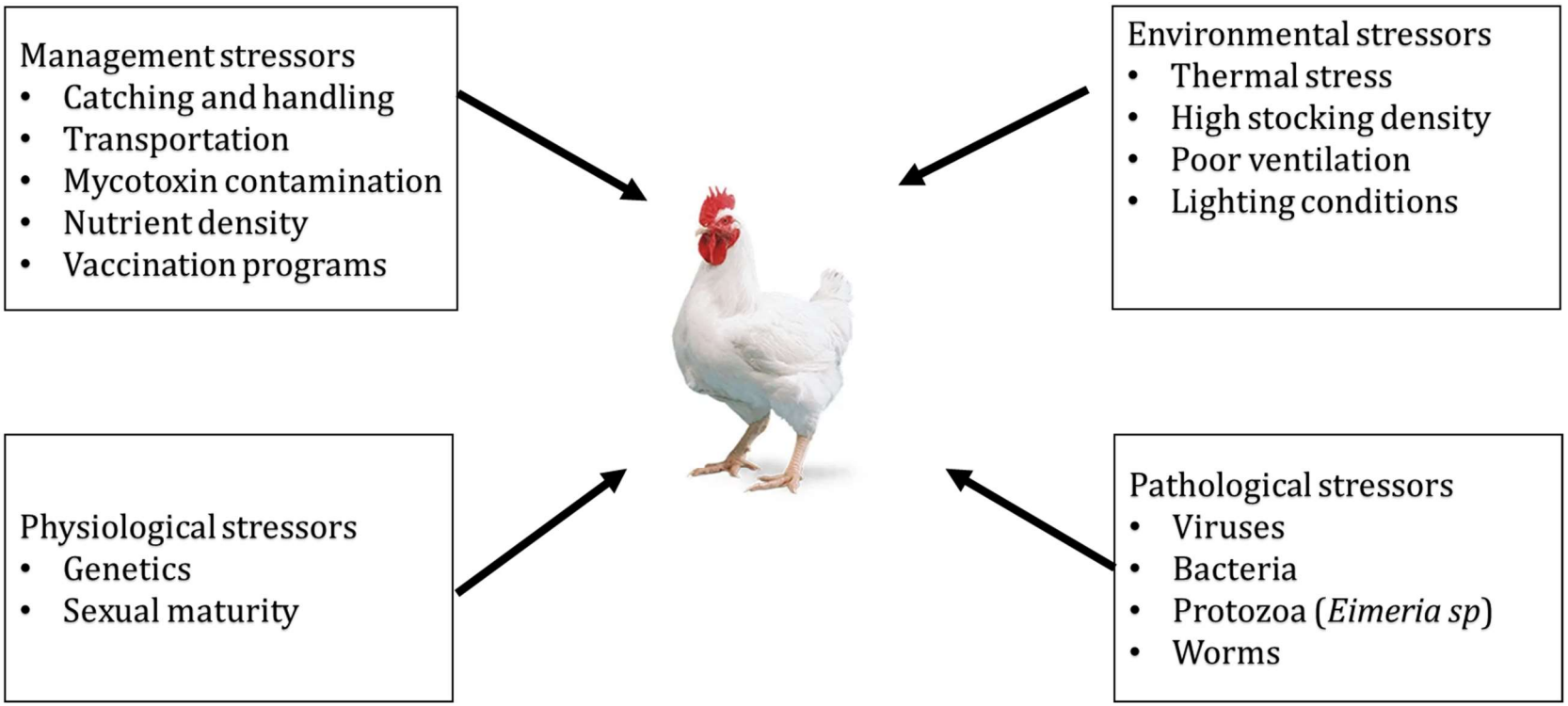

Figure 1.3 Some stressors poultry are exposed to during production.

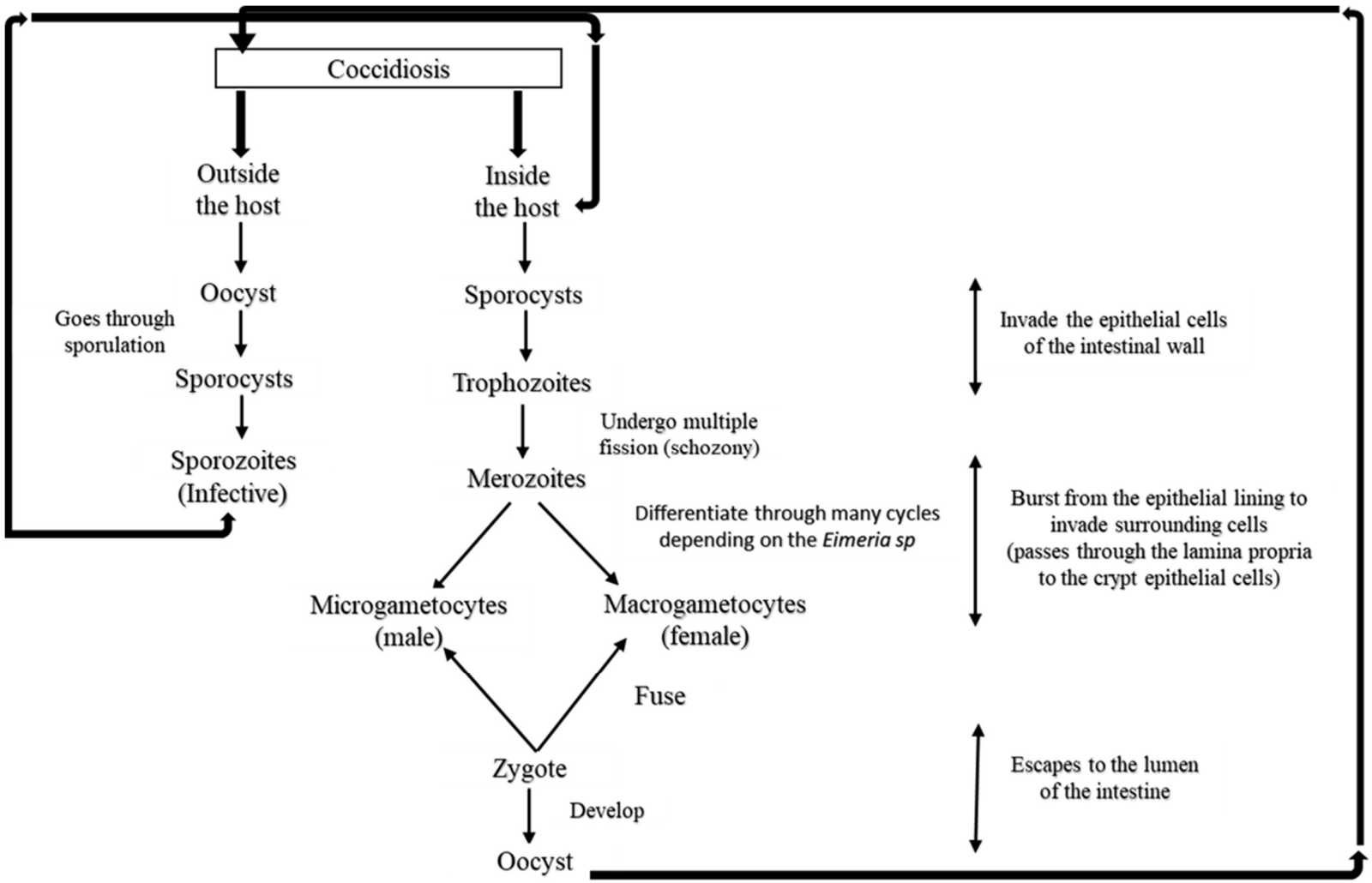

Figure 1.4 Schematic representation of the life cycle of Eimeria sp (Lillehoj and Lillehoj, 2000; Allen and Fetterer, 2002a).

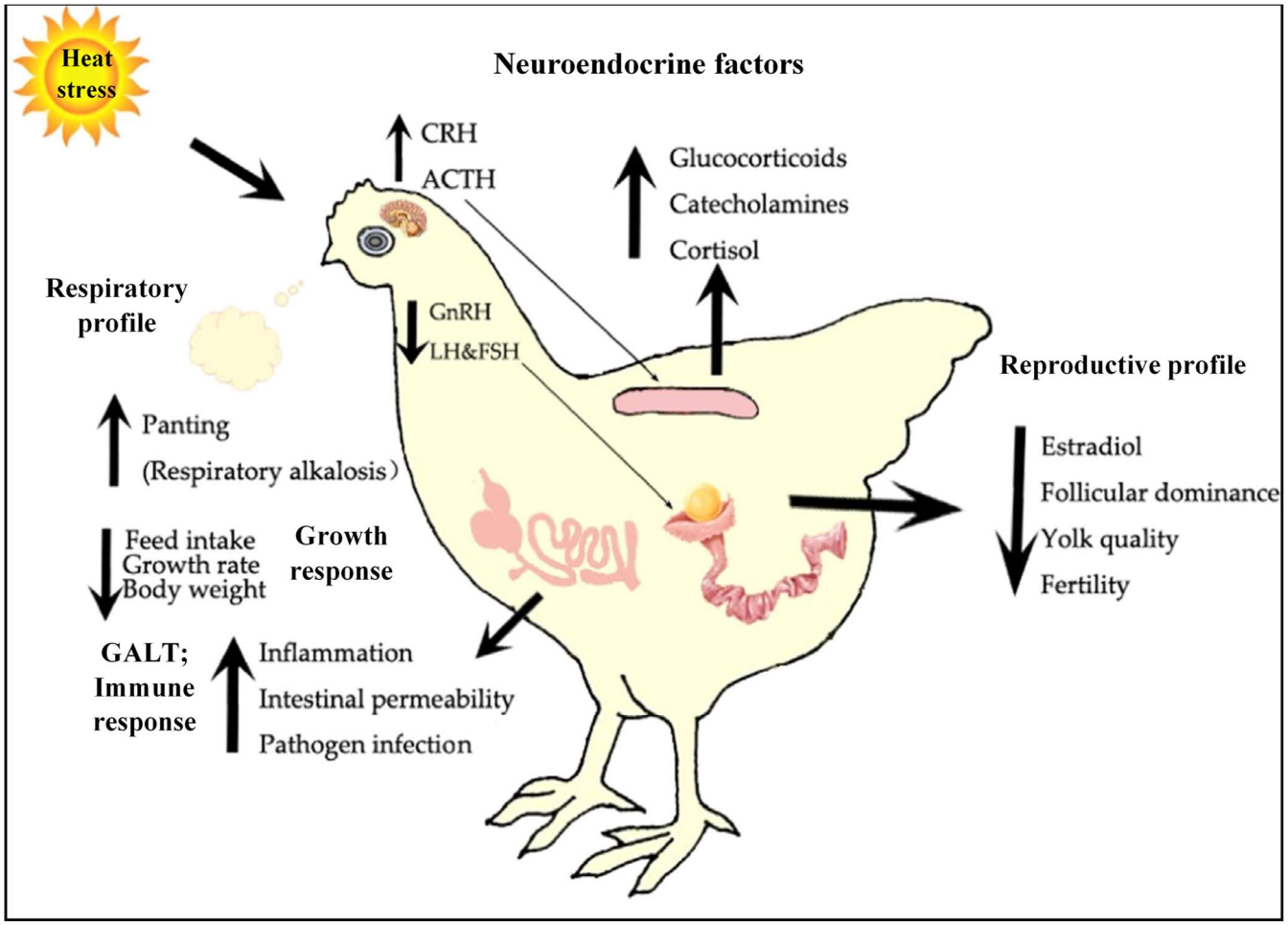

Figure 1.5 Mechanism of heat stress: Its effects on neuroendocrine factors, growth, reproduction parameters, and respiratory profile. Adapted from Nawab et al., 2018.

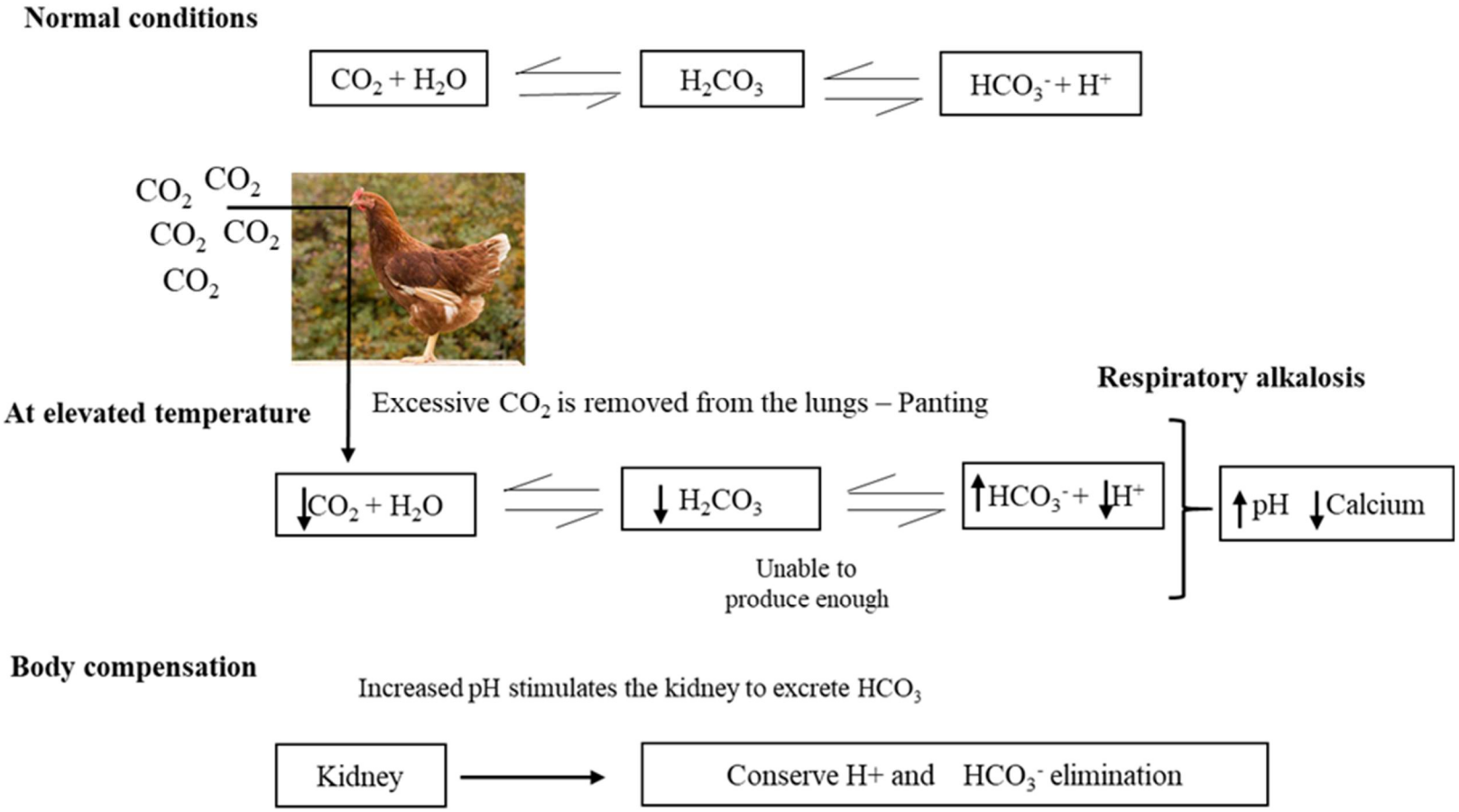

Figure 1.6 The efficient functioning of the acid-base homeostatic mechanisms is an essential feature for optimal growth and production. Blood composition is maintained within narrow limits to allow for a complex interplay of biochemical and physiological events.

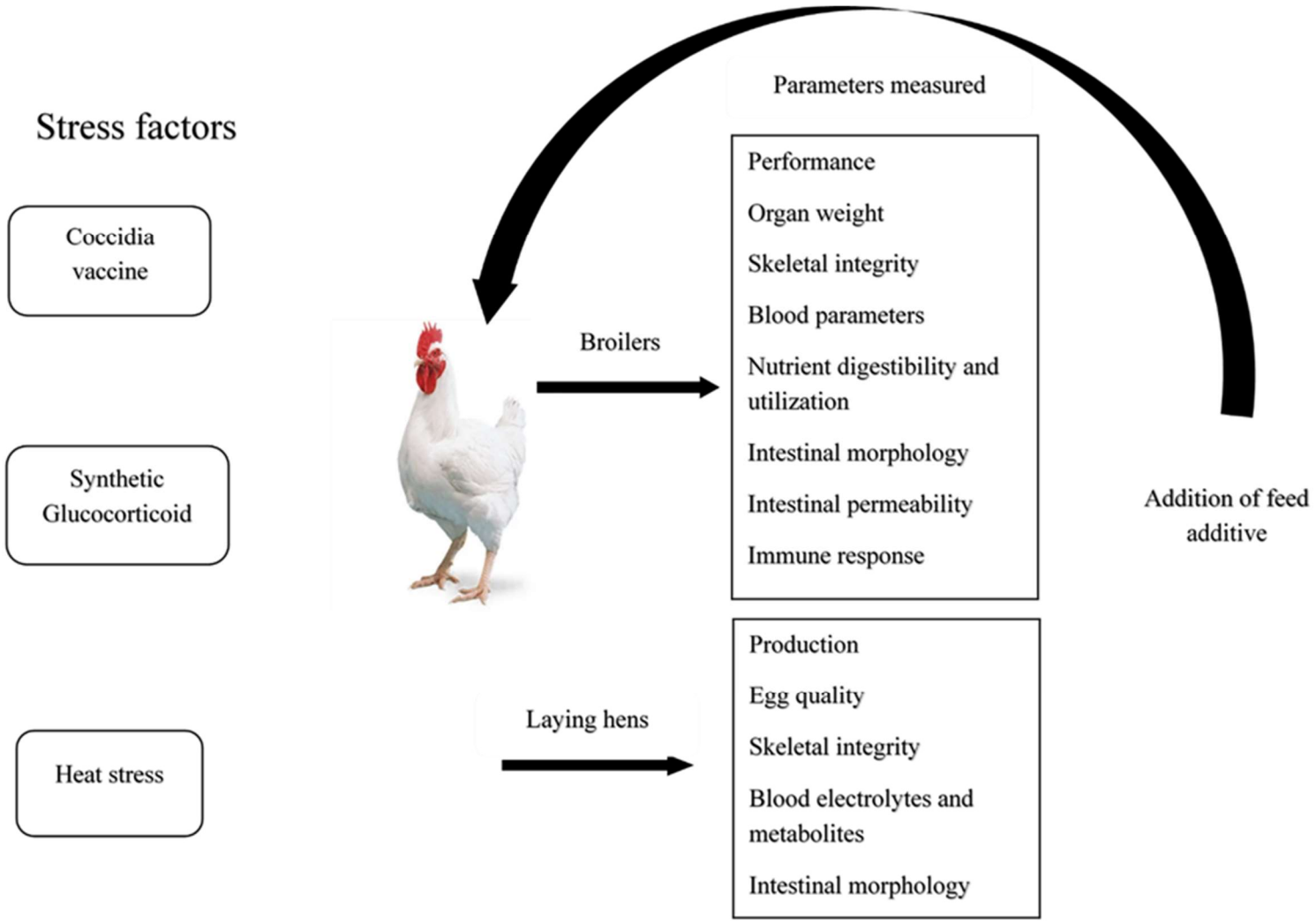

Figure 1.7 Objectives of the entire study.

CHAPTER 2.

EFFECTS OF A DIETARY ANTIOXIDANT AND TWO INORGANIC SODIUM SOURCES ON PERFORMANCE, NUTRIENT DIGESTIBILITY AND UTILIZATION, INTESTINAL PERMEABILITY, AND INFLAMMATORY RESPONSE OF BROILER CHICKENS CHALLENGED WITH DEXAMETHASONE

Abstract

This study was conducted to evaluate the effect of an algae-based antioxidant containing Se yeast, EconomasE™ (EcoE), and two inorganic sodium sources (NaCl and NaCl+$NaHCO_3$) on growth performance, nutrient digestibility and utilization, antioxidant, and immune status of 21-day-old broiler chickens challenged with dexamethasone (DEX). Broilers (336) were randomly assigned to one of four experimental diets: a basal diet with NaCl as the only source of inorganic sodium with no EcoE; a basal diet with a combination of NaCl and $NaHCO_3$ as the sources of inorganic sodium with no EcoE; a basal diet with NaCl as the only source of sodium with EcoE; a basal diet with a combination of NaCl and $NaHCO_3$ as the source of inorganic sodium with EcoE. At 16 d of age, half of the chickens in each dietary treatment were orally gavaged with a 0.6 mL solution of DEX at the rate of 1 mg/kg body weight on 3 alternate days (d 16, 18, and 20). The remaining half (control birds) were gavaged with the same volume of nanopure water. Data were analyzed by a two-way ANOVA. Plasma corticosterone (CORT) levels increased ($P < 0.0001$) with DEX challenge. Bodyweight gain and feed efficiency of the broiler chickens decreased ($P < 0.0001$) with DEX-challenge but not by the dietary treatment. Villus height decreased ($P < 0.05$) with DEX challenge. The addition of NaCl+$NaHCO_3$ as the source of inorganic sodium in the diet increased ($P < 0.05$) the crypt depth of the non-challenge birds. With DEX-challenge, the apparent ileal digestibility (AID) of nitrogen (N) increased ($P =$

0.021). Dietary treatments had no effect on the digestibilities calculated. The non-challenge birds fed diets supplemented with EcoE had a higher ($P < 0.05$) total tract utilization (TTR) of DM compared to the other groups. The TTR of EN and AME and AMEn were higher ($P < 0.05$) in the non-challenged birds fed the diet supplemented with EcoE. Similarly, the TTR of N was higher ($P < 0.05$) in the non-challenge birds fed diets supplemented with EcoE. Finally, jejunal mRNA levels of cytokines and tight junction proteins measured were not affected by DEX-challenge, sodium source, or EcoE supplementation. The results indicate that the inclusion of either of the sodium sources did not attenuate the CORT levels, antioxidant status, growth performance, and immune response of the broilers. The EcoE supplementation did not improve the performance, antioxidant status, or immune response of the broilers however, in non-stress birds reduced CORT plasma levels and increased nutrient and energy utilization in broiler chickens. The DEX challenge was able to mimic the negative effects of an environmental stressor as shown by the depressed growth performance and nutrient utilization.

2.1 INTRODUCTION

The stress response involves the activation of a complex repertoire of the physiologic and behavioral central nervous system and peripheral adaptive responses. If excessive and prolonged, can cause adverse consequences on physiologic functions, such as growth, metabolism, circulation, reproduction, gut health, and the inflammatory/immune response (McEwen, 2008). The corticotropin-releasing hormone **(CRH**) released in the hypothalamus is the first step in the activation of the HPA axis involved in stress response, and this activates the pituitary and the adrenal glands to produce catecholamines and glucocorticoids (Axelrod and Reisine 1984; McEwen, 1998;

Puvadolpirod and Thaxton, 2000a,b; Virden and Kidd, 2009; Kuenzel and Jurkevich, 2010; Konturek et al., 2011). Studies have shown that the CRH plays a key role in stress-induced gut permeability, increases colonic paracellular permeability (Saunders et al., 1994), and visceral hypersensitivity (increased perception of pain).

Given the negative impact of stress on animal welfare and gut health, it has become an important consideration in poultry production. Elevated levels of corticosterone in circulation have been shown to enhance excretion of a water load, inhibit the secretion of several hormones and neuropeptides, impair glucose and mineral metabolism, increase protein catabolism, gastrointestinal lesions, suppress numerous immune reactions, and increase phagocytosis and antibody formation (Selye, 1950; Lupien et al., 2007; Zhao et al., 2012). A composite of interacting stressors exists around broiler chickens and can be mimicked by glucocorticoid (such as corticosterone or dexamethasone) administration (Post et al., 2003; Lin et al., 2004a, b; 2006b). Observation from several studies shows that the administration of glucocorticoid mimics the adverse effects of elevated levels of corticosterone (Binder, 1978; Spitz et al., 1994; Li et al., 2009; Vicuna et al., 2015), and as such a useful tool to understand the negative impact of stress. Dexamethasone (DEX) administration in poultry has been reported to induce oxidative stress (Eid et al., 2003), suppress immune functions (Shini et al., 2010), results in gastrointestinal dysfunction (Saunders et al., 1994), intestinal permeability (Spitz et al., 1994; Vicuna et al., 2015), ultimately impairing the barrier function of the gut (Saunders et al., 1994), and eventually influencing the absorption of nutrients (Puvadolpirod and Thaxton., 2000d).

To improve the resilience of the animal to which its physiological functions can proceed effectively without deviating from their respective set points in the event of stress,

intentional manipulation of the diet even before the occurrence of an infection, will not only confer protection but becomes advantageous in terms of productivity. Among the strategies to improve health through diet, is the use of antioxidants. The most common natural dietary antioxidants capable of neutralizing reactive oxygen species include ascorbic acid (vitamin C), glutathione, lipoic acid, α-tocopherol (vitamin E, VE), carotenoids, coenzyme Q, and selenium (Se) (Masood et al., 2013). Vitamin E, which is part of the chain-breaking antioxidants plays an important role in protecting the cell membranes from oxidative damage (Surai, 2016). Studies have shown that by adding higher levels of VE to poultry diets at doses above the NRC-recommended amount (10 IU/kg of diet; this level is dependent on antioxidants such as vitamin C and selenium, type and amount of lipids in diets, and environmental conditions), can achieve an optimized growth performance, alter the antioxidant capacity (Mahmoud and Hijazi, 2007), and increase the expression of immune parameters (Pitargue et al., 2019). To maximize the benefits of VE when supplemented in the diet, a functional equivalent product was designed to replace VE. EconomasE™ [EcoE, Alltech, Nicholasville, KY] a proprietary blend of ingredients that maximizes the antioxidant status of birds has been shown to significantly reduce the amount of VE required in broiler diets without compromising performance and meat quality (Pierce et al., 2009). The inclusion of EcoE in the diet increased total antioxidant capacity and modulates the expression of certain genes involved in multiple metabolic processes, cell morphology, and immune responses (Xiao et al., 2011). Moreover, these benefits have also been attributed to its high content of antioxidants such as selenium which, alongside other antioxidants play an important role in the antioxidative defense system (Choct et al., 2004).

Similarly, stress responses are integrally involved with the acid-base balance in several species. Through the glucocorticoid receptor and mineralocorticoid receptor, corticosterone can alter mineral and water metabolism (Selye, 1950; Selye, 1976). Brown et al. (1957) reported an increase in the excretion of sodium (Na), potassium (K), total nitrogen, and uric acid, in roosters treated with cortisone acetate. Olanrewaju et al. (2007) reported that birds treated with ACTH had a reduction in plasma concentrations of Na^+ and Cl^- which might be due to an increase in urinary electrolyte excretion. To maintain acid-base homeostasis in poultry, the combined intakes of Na, K, and Cl, are important to determine dietary electrolyte balance (DEB) (Mongin,1981). Salts such as sodium bicarbonate ($NaHCO_3$), potassium chloride (KCl), calcium chloride ($CaCl_2$), and ammonia chloride (NH_4Cl) in water and/or feed proved beneficial for broilers under different heat stress regimens (Borges, 2003; Ahmad et al., 2005). Feeding corticosterone-treated broilers with supplemental water electrolytes resulted in improved body weight gain (Virden et al., 2009). Because the chief goal of the broiler producer is to achieve optimal meat production at the lowest possible cost, research directed to circumventing or alleviating physiological stress during live production is important.

The hypotheses for this study were that the oral administration of DEX will induce stress in the birds and the supplementation of the different inorganic sodium sources and EcoE would ameliorate the negative impact of DEX in 21-d old broiler chickens. Thus, the objective of the study was to understand the effect of stress, induced by the administration of DEX on performance, intestinal morphology, nutrient and energy digestibility and utilization, immune response, intestinal permeability, and the role of different inorganic

sodium sources and EcoE supplementation in mitigating the effect of the stress in broiler chickens.

2.2 MATERIALS AND METHODS

2.2.1 Experimental design

The experiment used day-old male by-product breeder chicks obtained from Cobb Monticello, KY. The chicks were placed in electrically heated battery cages and maintained on a 20 h of light and 4 h of dark lighting schedule. The room temperature was maintained at 32 ℃ for the first week and gradually decreased to 27 ℃ by the third week. Throughout the experiment, broilers had *ad libitum* access to water and diet that met or exceeded NRC recommended nutrient and energy requirement (NRC, 1994). On d 14, 336 broiler chickens were randomly assigned to 8 treatment groups with 7 replicate cages within each treatment, and 6 birds/cage in a completely randomized design. The 8 treatments were factorially arranged to include 2 levels of inorganic sodium sources, 2 levels of EcoE, and 2 levels of DEX challenge. The dietary treatments are listed in Table 2.1. The four diets used in the trial are (A) basal diet with NaCl as the only source of inorganic sodium with no EcoE; (B) basal diet with a combination of NaCl and $NaHCO_3$ as the sources of inorganic sodium with no EcoE; (C) basal diet with NaCl as the only source of sodium with EcoE; (D) basal diet with a combination of NaCl and $NaHCO_3$ as the source of inorganic sodium with EcoE. EconomasE™ was added to the diet at a 0.2 g/kg diet. Within each dietary treatment, chicks were either challenged with DEX (Sigma Chemical Co., St. Loius, MO) or nanopure water on days 16, 18, and 20 via oral gavage at the rate of 1 mg/kg body weight. The DEX dose of 1 mg/kg of BW was chosen based on previous literature (Li et al., 2009; Wideman and Pevzner, 2012). Although both studies administered the DEX intramuscularly, this study

administered the DEX via oral gavage with the expectation that the chosen route will have a high impact on the gut. Titanium dioxide was included in all diets (0.5 %) as an inert marker to determine energy and nutrient digestibility. All diets were made from a single basal diet. All animal handling procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

2.2.2 Sample collection

Birds were weighed individually on 0, 14, 16, and 21 d of age; meanwhile, feed intake (**FI**) was recorded per pen to calculate average body weight gain (**BWG**) and feed efficiency (**FE**). On d 21, all the birds were weighed individually and one bird with the weight closest to the average cage weight was selected for tissue collection. All the birds were euthanized by argon asphyxiation. From the designated tissue bird, blood was drawn from the wing vein into EDTA tubes and centrifuged at 1200 x g for 10 min at 4 ℃. At mid-jejunum, a tissue section was removed, immediately flushed with nanopore water, and divided into 2 segments. One 4-cm was fixed in 10% buffered formalin and processed for villus height, villus width, and crypt depth determination. Another 4 cm segment was taken out for intestinal mucosal samples. The intestinal segment was sliced open longitudinally, and mucosal samples were collected by scraping, using sterile glass microscope slides, into a microtube that contained Trizol (Invitrogen, Grand Island, NY, USA). The samples were rapidly frozen in liquid nitrogen and stored at – 80 ℃ for the gene expression of cytokines, chemokines, and tight junction proteins. The liver, spleen, and bursa of Fabricius were removed and cleaned of adherent tissues. The weight of these organs was determined and expressed as relative to final body weight at sampling [(organs weight/final BW) × 100]. Furthermore, the left, and right tibia were removed and stored at – 20 ℃ for bone-breaking

strength and bone ash. The remaining birds, including the one selected for tissue collection (6 birds per cage), were opened and the contents of the distal 2/3rd of the ileum, were collected by flushing with nanopure water into clean pre-labeled plastic containers. Digesta samples from individual birds within a cage were pooled and immediately frozen at -20 ℃. Ileal digesta samples were subsequently freeze-dried, finely ground using a coffee grinder, and stored in airtight bags until further analysis. Excreta samples were collected on days 20 and 21 for total tract nutrient utilization. Excreta samples collected on d 20 and 21 were weighed to determine the wet weight, dried in a forced-air oven at 55 ℃ for 5 days, and subsequently weighed to determine the dry weight. The dried excreta samples were ground through a 0.5 mm screen using a Wiley Mill laboratory Standard (Model No. 3, Arthur H. Thomas Co., Philadelphia, PA, USA) and stored in airtight plastic bags.

2.2.3 Plasma parameters measurements

Blood samples were centrifuged to separate plasma and then stored at −80 °C until analysis. Plasma samples were analyzed for corticosterone (**CORT**), superoxide dismutase (**SOD**), and catalase (**CAT**) activities using commercially available diagnostic kits. Plasma levels of CORT were determined in duplicates using a Corticosterone ELISA kit (Catalog No ADI-900-097, Enzo Life Science Inc., Farmingdale, NY, USA) according to the manufacturer's instruction. The total SOD activity was determined using a commercial kit (Catalog Number 706002, Cayman, Ann Arbor, MI, USA). Briefly, the activity was quantified using a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. A unit is defined as the amount of enzyme needed to exhibit a 50 % dismutation of the superoxide radical produced. The analysis was carried out according to the manufacturer's instructions in 96-well format, read at 450 nm on a

plate reader Model 680 (Bio-Rad, Hercules, CA, USA). Standard curves for each plate were generated with a final activity of 0.005 to 0.050 U/mL SOD. The activities of CAT (Catalog Number 707002, Cayman Chemical, Ann Arbor, MI, USA) was measured by the rate of hydrogen peroxide (H_2O_2) disappearance. The catalytic activity involves the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water. Samples and standards were measured at an absorbance of 440 nm with a plate reader Model 680 (Bio-Rad, Hercules, CA, USA.

2.2.4 Intestinal morphology

A segment of the jejunum was collected from one bird per cage, flushed with nanopore water to remove the digesta contents. The gut segments were fixed in 10 % neutral buffered formalin (Sigma Chemical Co., St Louis, MO, USA). Subsequently, tissue sections (5 μm) were cut, dehydrated, cleared, and embedded in Polyfin paraffin (Polysciences Inc., Warrington, PA, USA), and stained with hematoxylin and eosin. On each slide, villus height, width, and crypt depth were measured from 10 villi under a magnification of 4X, using a Nikon ECLIPSE Ci-E light microscope equipped with a computer-assisted digital camera (DS-Ri2) using NIS-Elements Br software (Nikon Corporation, Tokyo, Japan). An average value was calculated for each section measured. The villus height: crypt depth ratio was calculated.

2.2.5 Intestinal gene expression

The mRNA expression of the following genes was measured using a real-time quantitative PCR: pro-inflammatory cytokines - interleukin (IL) - IL-1β, IL-6; anti-inflammatory cytokines - IL-10, interferon-gamma (IFN-γ), transforming growth factor-β4 (TGF-β4); chemokines - CCL5 (RANTES), C-C motif, ligand 5; CXCLi1, C-X-C motif

ligand 1 inflammatory; CXCLi2 C-X-C motif ligand 2 inflammatory; and tight junction proteins - ZO-1, claudin 1, occludin. Total RNA was extracted from the jejunum using Trizol® reagent (Invitrogen Canada, Inc.) following the manufacturer's protocol. RNA samples were resuspended in nuclease-free H_2O, and the concentration of the extracted RNA was determined using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) at an optical density of 260 and 280 nm. Subsequently, 1 µg of total RNA from each sample was reverse transcribed into cDNA in a 20 µL RT reaction using Script cDNA super mix for qRT-PCR (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's protocol in a Veriti TM Thermal cycler (Catalog no 4375786, Applied Biosystems). The RNA was incubated for 5 min at 25 ℃, followed by 30 min extension at 42 ℃. The reaction was stopped at 80 ℃ for 5 min and then held at 4 ℃ until removal from the machine. The cDNA was then diluted 1:20 with nuclease-free water before being used for real-time PCR. Briefly, the reaction mix was prepared using 1 µL of cDNA, 0.375 µL of each forward and reverse primer, 6.25 µL SYBR green master mix (Bio-Rad, Hercules, CA), and 4.5 µL of RNase free water to reach a total reaction volume of 12.5 µL. Each sample was tested in duplicate. PCR plate contained target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) - an endogenous housekeeping control and a no-template negative control containing water instead of cDNA. PCR was performed using the Bio-Rad CFX-96 real-time PCR system (Bio-Rad, Hercules, CA), with the following cycle profile: 95 ℃ for 5 min, 95 ℃ for 15 s and then 60 ℃ for 1min ran on a repeat for 40 cycles. The relative levels of mRNA expression were calculated using the $2^{-\Delta\Delta CT}$ method after normalization against the reference gene (Shini and Kaiser, 2009). The average value

of the control group was used as a calibrator. Primers used for real-time PCR are listed in Table 2.2.

2.2.6 Bone breaking strength and bone ash

The frozen tibias were thawed and later stripped of soft tissue. The bones were de-fleshed by hand and the bone cap removed. Subsequently, bone-breaking strength was measured using an Instron Materials tester (model 4301, Instron Corp., Canton, MA) at a loading rate of 40 mm/min. The average breaking strength of the left and right tibia was reported as the breaking strength of the bone. To determine the ash content, the bones were further extracted in anhydrous ether for 24 h (removing nonpolar lipids). After the third extraction, bones were dried at room temperature for 4 h. Bones were individually weighed and placed in a porcelain crucible and ashed in a muffle furnace overnight at 600 ℃ and weighed again after ashing. The percentage of ash was determined relative to the dry weight of the bones.

2.2.7 Chemical analysis

Excreta, ileal, and diet samples were thoroughly mixed, and subset samples were taken for analyses in duplicate. Dry matter (**DM**) content in the excreta, ileal digesta, and diets was determined by oven-drying at 105°C for 24 h (AOAC International 2006; method 934.01). Gross energy of excreta, ileal digesta, and diet samples were determined using a bomb calorimeter (Parr 6200 calorimeter, Parr Instruments Co., Moline, IL, USA) with benzoic acid used as a standard. Titanium (**Ti**) and nitrogen (**N**) contents in the excreta, ileal digesta, and diets were analyzed at the Agricultural Experiment Station Chemical Laboratories, University of Missouri-Columbia (Columbia, MO). The samples were digested using concentrated sulfuric acid and processed as described by Myers et al. (2004)

after which Ti concentration was determined by flame atomic absorption spectroscopy. Nitrogen content was determined by the combustion method using a LECO Trumac Nitrogen Analyzer (LECO, St. Joseph, MI; AOAC International, 2000; method 990.03).

Apparent ileal digestibility (AID) and total tract nutrient utilization (TTR) of dry matter (DM), N, and energy (EN) were calculated using the following equation:

$$AID\ or\ TTR, \% = \left[1 - \left(\frac{Ti_I}{Ti_O}\right) \times \left(\frac{X_O}{X_I}\right)\right] \times 100$$-----Eqn. 1 (Adedokun and Adeola, 2017)

Where Ti_I represents the titanium concentration in the diet and Ti_o represents the titanium concentration in the ileal (AID), or excreta (TTR) samples (%); and X_I represents the concentration of nitrogen or energy in the diet and X_O represents are the concentration of nitrogen or energy in the ileal or excreta samples, respectively, (%).

Ileal digestible energy (DE) and apparent metabolizable energy (AME) were calculated using the following equation.

$$DE\ or\ AME, kcal/kg\ = Calculated\ EN\ (\%) \times\ GE\ of\ diet\ (kcal/kg)$$

Where calculated EN is derived from Eqn. 1 for ileal or excreta samples, and gross energy (GE) of diet is determined by bomb calorimeter. Nitrogen-corrected AME (AMEn) was determined by correcting for N retention by a factor of 8.22 kcal/g of N retained in the body as described by Hill and Anderson (1958).

2.2.8 Statistical analysis

Prior to statistical analysis, outliers (data outside mean ±3 standard deviation) were removed from the data set. For performance and nutrient digestibility data, cage means were considered as experimental units. For the remaining analysis, a bird constituted an experimental unit. Data were subjected to a two-way ANOVA using the GLM procedure

of SAS 9.4 software (SAS Institute Inc., Cary, NC) as a completely randomized design with a 2 x 2 x 2 factorial arrangement of treatments. The significance of the main effects (inorganic sodium source, supplement type (EcoE), and challenge type (DEX) and their interactions were determined. To increase power, when the two-way interactions were not significant, they were pooled into the residual error. Means were separated using Tukey's multiple comparison test and a level of 0.05 was used for statistical significance.

2.3 RESULTS

2.3.1 Plasma antioxidant status (SOD and CAT) and corticosterone

Table 2.3 shows the effect of two inorganic sodium sources, two levels of EcoE, with or without DEX challenge on plasma corticosterone and antioxidant (SOD and CAT) indexes. The interaction effect between the diet and DEX-challenge was not significant for plasma CORT concentration. Plasma CORT level was higher (P = 0.020) in the DEX-challenged broilers compared to the non-challenged broilers. On the other hand, CORT concentration was lower (P = 0.015) in birds fed diets supplemented EcoE. Furthermore, the effect of diet (EcoE or sodium source) and DEX-challenge on antioxidant enzymes SOD and CAT was not significant.

2.3.2 Growth performance

The growth performance of birds in this study is reported in Table 2.4. No two-or three-way interactions were observed for BWG, FI, and FE. Inducing stress in the birds by orally administering DEX starting from day 16 depressed (P <.0001) BWG and FE (16 to 21 d). Dietary supplementation of EcoE and the addition of NaCl or NaCl + $NaHCO_3$ as the sources of inorganic sodium in the diet did not affect BWG, FI, and FE on 16 -21 d.

2.3.3 Relative tissue weights and bone characteristics

The weights of the liver, spleen, and bursa of Fabricius are reported as a percentage of the final body weights (Table 2.5). Relative to body weight, the liver weight increased ($P < .0001$), and the spleen and bursa of Fabricius weight, decreased ($P < 0.05$) with DEX challenge. Dietary treatments did not affect the relative weights of the organs (Table 2.5). Bone breaking strength and bone ash were not affected by the different treatments (Table 2.5).

2.3.4 Intestinal morphology

Data for the jejunal morphology is reported in Table 2.6. Dexamethasone challenge decreased ($P < 0.05$) the VH and CD and did not affect VH: CD. An interaction between NaCl + $NaHCO_3$ as the source of inorganic sodium and DEX-challenge birds increased ($P < 0.05$) the crypt depth. The birds fed the diet supplemented with EcoE had an increase ($P < 0.05$) in CD and a decrease ($P < 0.05$) in VH: CD ratio.

2.3.5 Apparent nutrient digestibility and utilization

The influence of EcoE supplementation, the different inorganic sodium sources, and DEX challenge on AID of DM, N, EN, and DE are presented in Table 2.7. No significant interaction was observed between treatments for the AID of DM, N, EN, and DE. Ileal digestibility of DM, EN, and DE was not influenced by DEX challenge however, N digestibility (84.6 vs. 83.7%) was increased ($P = 0.012$) with the challenge. Supplementation of EcoE and the inclusion of two different inorganic sodium sources to the diet did not affect the AID of DM, EN, N, and DE.

Furthermore, the effect of EcoE, the different inorganic sodium sources, and DEX challenge on apparent TTR of DM, N, and EN and AME and AMEn are presented in Table

2.8. No three-way interaction was observed. A significant two-way interaction of EcoE and DEX for TTR of DM, N, and EN, and AME and AMEn was observed. The non-challenge birds fed diets supplemented with EcoE had a higher ($P < 0.05$) TTR of DM compared to the non-challenged birds fed the diet with no EcoE supplementation. The TTR of EN and AME and AMEn were higher ($P < 0.05$) in the non-challenged birds fed a diet supplemented with EcoE compared to the DEX-challenged birds fed a diet supplemented with EcoE. Similarly, the TTR of N was higher ($P < 0.05$) in the non-challenge birds fed diets supplemented with EcoE compared to the DEX-challenge birds with or without EcoE supplementation in the diet.

2.3.6 Jejunal gene expression

The jejunal mRNA expression levels for cytokines (IL-1β, IL-6, TGF-β4, IL-10), chemokines (CXCLi1, CXCLi2, CCL5), and tight junction proteins (ZO-1, occludin, claudin-1) were unaltered by DEX challenge, inorganic sodium sources, and EcoE supplementation (Table 2.9).

2.4 DISCUSSION

Decades ago, Selye (1936) recognized the conundrum that exists with the activation of stress by physiological systems, and the development of adaptability or resistance to prevent long-term damage to the body. The concept of stress has been constantly revised by several scientists (Selye, 1950; McEwen, 1998.; Moberg, 2000; Kaiser et al., 2009) all of whom concluded that for an animal to experience stress, the body needs to recognize the stressor, develop a biological defense against it, and respond by achieving stability through the change. In cases of an exaggerated activity on the physiological systems, there are several pathophysiological consequences (McEwen, 1998) associated. One of the

mechanisms by which organisms control whole-body homeostasis is the secretion of glucocorticoid steroid hormones, which is corticosterone in birds. High glucocorticoid levels result in a diversity of metabolic effects and impairment of physiological functions. This is an important consideration in poultry production because commercial birds live in an environment with a variety of interacting stressors that can impact their health and productivity. Hence, how we deal with the compromise to their health and productivity through nutrition becomes imperative. This study explored the advantages of an algae-based supplement (EconomasE™) and/or two inorganic sodium sources on performance, nutrient and energy digestibility and utilization, intestinal health, and overall biological response in broilers under immunological stress induced by DEX.

Elevated plasma concentration of CORT in response to stress in poultry has been documented by many researchers (Davison et al., 1985; Puvadolpirod and Thaxton, 2000a; Post et al. 2003; Lin et al. 2006b). By these reports, a significant rise in plasma CORT concentration in DEX-challenged birds was observed compared to the control, which indicates a disruption in homeostasis. This disruption in status-quo can initiate a disproportionate generation of reactive oxygen species (**ROS**), causing oxidative damage to the tissue (Orzechowski et al., 2000; Lin et al., 2004a,b; Costantini et al., 2011). Glucocorticoid mediated stressful conditions, both in vitro and in vivo, have been linked to an increase in ROS, which damages cellular macromolecules, thereby affecting all cellular functions (Lawrence and Sapolsky, 1994; Lin et al., 2004a; Costantini et al., 2008). Acute exposure to elevated temperature (32°C for 6 h) resulted in an increase in the concentration of thiobarbituric acid reactive substances (TBARS) in the plasma and liver of 5-wk-old broiler chickens (Lin et al., 2006b). Similarly, Huang et al. (2015) reported

an increase in TBARS concentration in the breast and thigh muscle, an increase in plasma SOD activity, an increase in SOD and catalase activities in breast muscle, and a decrease in plasma total antioxidant capacity and glutathione peroxidase in heat-stressed (32°C) broiler chickens. In other studies, stress-induced by CORT in broiler chickens, caused an increase in TBARS (Eid et al., 2003; Lin et al., 2004a,b), a decrease in CAT (Zhang et al., 2009), and SOD (Wang et al., 2008) plasma concentration, and an increase in SOD activity in the muscle (Gao et al., 2010).

To protect the body from ROS, it has been suggested that the antioxidant defense system operates through three major lines of defense which, the activity of three enzymes; SOD, glutathione peroxidase (GPX), and CAT is the first line of defense (Surai, 2002). Costantini et al. (2008) observed a significant increase in reactive oxygen metabolites when kestrels were fed CORT for 2 weeks, but serum antioxidant capacity was not affected. Lin et al. (2004a) reported an increase in plasma concentration of TBARS and increased activity of SOD after 3 days of dietary CORT administration. A subsequent study from the same group showed no significant activity of SOD 3 hr after CORT treatment. Likewise, in this study, the oral gavage of DEX did not influence the enzymatic activity of SOD and CAT. Overall, in vitro studies have produced contradictory results regarding steroid effects on host enzymatic antioxidant defense system. However, studies show the possibility of tissue-specific steroid effect. In a rat study by Pereira et al. (1999), the effect of DEX on the antioxidant defense system demonstrated this. According to the group, the TBAR content in the lymphoid organs was decreased and increased in the muscle, Cu/Zn-SOD activity in all tissues was reduced, CAT activity was reduced in the thymus but increased in the muscle and other lymphoid organs, and GPX activity in the lymphoid organs was

elevated and diminished in the muscles. In rats, Mclntosh et al. (1998), observed decreased Cu/Zn SOD activity in the brain but opposite results in the liver and, CAT activity in the brain happens to be unaffected but decreased in the liver. In broilers, Lin et al. (2004b) observed increased SOD activities in the heart, and no effect was observed in the liver. On the other hand, the second line of defense involves non-enzymatic antioxidants like vitamins A, E, C, carotenoids, selenium, uric acid, etc. (Surai, 2016). In this study, EcoE or the inorganic sodium source did not alter the activity of the antioxidant enzymes (SOD and CAT). However, the combined efforts of VE and Se in removing free radicals and detoxifying hydroperoxides cannot be ignored.

In stressful conditions, studies show that there is a redirection of nutrient flow to meet the metabolic requirements where, in a growing animal, the adipose tissue loses its priority (Latshaw, 1991; Elasser et al., 2000; Lupien et al., 2007). In this study, DEX-challenged birds had a decrease in BWG (25%) and FE (26%) (16 - 21 d) 4 d post-challenge compared to the control birds however, FI was comparable between the DEX-challenged and the control birds regardless of the dietary treatment. Although there have been varying reports on the effect of CORT on FI, some report an increase (Gross et al.,1980; Bartov et al., 1980; Nasir et al., 1999; Puvadolpirod and Thaxton, 2000d; Lin et al., 2006b), decrease (Lin et al., 2004a; Shini et al., 2008; Hu et al., 2010) or no effect (Buyse et al., 1987; Malheiros et al., 2003; Wang et al. 2015). This suggests that the observed suppressed growth might be due to an increase in energy waste rather than a decrease in feed consumption (Wang et al., 2015). The unifying explanation is that glucocorticoids exert a diversion of energy to exercising muscle by mobilizing stored energy, inhibiting subsequent energy storage, and stimulating gluconeogenesis (Southwick, 1982; Siegel and

Van Kampen 1984; Davison et al., 1985; Puvadolpirod and Thaxton, 2000a,b; Virden et al., 2007). Through the CORT-induced gluconeogenesis, catabolism of structural protein is enhanced, freeing free amino acids for use as gluconeogenic substrates, which puts meat-producing animals at a disadvantage (Selye, 1950; Southwick, 1982; Puvadolpirod and Thaxton, 2000a; Virden and Kidd, 2009).

There is also a negative feedback response that glucocorticoid exerts on peripheral tissues to insulin, which limits glucose uptake, suppresses muscle development, and alters energy deposition in broilers (Munck, 1971; Zhao et al., 2012). Although we did not measure glucose or insulin levels, observations from Lin et al. (2006b) and Li et al. (2009) demonstrated that CORT-treated broiler chickens expressed a high glucose and insulin plasma concentrations, followed by an increase in abdominal fat accretion (Lin et al., 2006b). Thus, this can in part, explain the BWG decrease observed in the DEX-challenged birds in this study, where energy stores are been redistributed. The two different inorganic sodium sources had no significant effect on the parameters mentioned above. Olanrewaju et al. (2006) reported no alleviation of the negative effects of ACTH on BW that can be attributed to increasing the DEB. Although, they argued that because oxygen demand is increased in chronically stressed birds, maintaining higher levels of dietary electrolyte balance had the potential to ameliorate some of the effects of stress (Olanrewaju et al., 2006). Similarly, EconomasE™ supplementation did not affect the performance parameters. Although VE is said to regulate the production of ROS in the mitochondria, and modulate the expression and activation of signal transduction pathways, which can prevent degenerative tissue changes (Avanzo et al., 2001). Supplementation of EcoE did not prevent the degenerative process – in terms of BW in the birds subjected to DEX. This

could be because DEX-challenge did not affect antioxidant status (SOD and CAT) rather, suppressed growth by limiting glucose uptake.

Other measurements to ascertain a stress response were to demonstrate a difference in lymphoid organ weights. Relative to body weight, the spleen, and bursa weight were significantly decreased, and liver weight increased with DEX compared to the control. One of the common observations of physiological stress after CORT treatment is the involution of immunological organ weights (Gross et al., 1980; Puvadolpirod and Thaxton, 2000b; Post et al., 2003; Shini et al., 2008; Virden et al., 2007; Vicuna et al., 2015) and increased liver weight (Gross et al., 1980; Davison et al., 1985; Puvadolpirod and Thaxton, 2000a,b; Lin et al., 2006b; Virden et al., 2007). The former can be associated with a decrease in B cell differentiation especially since positive selection for B cells seems to occur at the final transition from immature to mature B cells, which occurs in the peripheral lymphoid tissue triggering apoptotic death in immature T and B cells precursor. Several explanations for hypertrophy of the liver based on previous work involves 1) an increase in liver lipid content which invariably increases plasma lipid concentrations providing nutrients to cells involved in host defense and substrates for the regeneration of damaged membranes (Puvadolpirod and Thaxton, 2000b; Lin et al., 2006b; Virden et al., 2007); 2) increase in gluconeogenic activity via a steroid-mediated pathway which increases the activity of key gluconeogenic enzymes (Munck, 1971; Southwick, 1982); and 3) an increase in the production of leukocyte endogenous mediators (LEM) by monocytes which, increase the synthesis of acute-phase proteins, C-reactive proteins, serum amyloid A in the liver associated with the reaction of the host to infection and inflammation (Mireles et al., 2005; Jain et al., 2011).

Often, market age poultry suffers from lameness, bone, and skeletal deformities which can cause bone breakage during catching and transportation, reducing their market value (Weeks et al., 2000). Similarly, bone and skeletal deformities can be exacerbated under adverse conditions such as stress, infections, and inflammation, contributing to further leg problems, (Pattison, 2007; Wideman Jr. and Pevzner, 2012; Zhang et al., 2017). In rats, glucocorticoids have been shown to arrest bone formation and increase bone resorption (Ortoft and Oxlund, 1996). In poultry, synthetic glucocorticoid has been shown to trigger turkey osteomyelitis complex (turkey) and bacterial chondronecrosis with osteomyelitis (broilers). These pathologies occur due to a combined effect of stress-mediated environmental and immunosuppression factors which allows opportunistic pathogens to develop in the joint which affects proliferation and differentiation of chondrocytes retarding bone growth (Zhang et al., 2017), and increases the incidence of lameness (Wideman Jr. and Pevzner, 2012). In this study, bone-breaking strength and bone ash were not affected by DEX challenge, dietary supplementation of EcoE, or either of the two inorganic sodium sources. The effect of DEX on bone breaking strength in this study is the opposite of the observations from Zhang et al. (2017) that reported a lower bone breaking strength in birds treated with methylprednisolone (another glucocorticoid derivative). According to Rath et al. (2000), the severity of DEX on bone strength is age-related. Since Zhang et al. (2017) administered the glucocorticoid at an early age (d 8) compared to d 16 for this study, the severity of the effects of dexamethasone was much reduced when the drug was administered at an age when bone development had nearly reached completion. Hence, the route (oral vs injection) or intensity of dosage (once or continuous) might be a contributing factor to the severity.

Maintenance of normal morphology and structural integrity of the small intestine are imperative for adaptions to the nutrient absorptive function of the epithelium. The running hypothesis is that stressors can invoke significant changes in the intestinal morphology influencing the absorptive capacity of the intestine. According to several authors, exposure to stressors like DEX (Hu and Guo, 2008; Li et al., 2009; Hu et al., 2010; Carvalho et al., 2018), lipopolysaccharide (Liu et al., 2008; Hu et al., 2011; Li et al., 2015), and heat stress (Mitchel and Carlisle, 1992; Burkholder et al., 2008; Deng et al., 2012), decreases the villus height which would correlate with a lowered absorptive capability of the small intestine. However, conflicting results have been observed with crypt depth. Pluske et al. (1996) suggested that an increase in crypt depth is compatible with an increase in crypt-cell production rate and overall stimulation of cell turn-over in the small intestine which has been associated with reduced digestive and absorptive capacity. While observations from elevated temperature conditions have reported an increase in crypt depth (Burkholder et al., 2008; Deng et al., 2012; Liu et al., 2009), results from corticosterone related stress conditions have been conflicting. We observed a decrease in jejunal crypt depth in DEX-challenged birds similar to Hu and Guo, (2008) however, Li et al. (2009) reported an increase in jejunal crypt depth.

A major criterion for a healthy GI system is the ability to break down dietary macromolecules into simple micro molecular moieties for efficient nutrient digestion and absorption. In situations where the animal is stressed, metabolic activity is altered, limiting the digestion or utilization of the nutrients. It could be surmised that the detrimental effects of stress can be mitigated through nutritional regimens that better meet the nutrient requirements of birds during stress. To understand this dynamic, the effects of stress on

nutrient digestion and utilization were elucidated. Ileal digestibility of N in DEX-challenged birds was higher than in the non-challenged birds however, the TTR of N was the reverse. It has been demonstrated that glucocorticoid action increases uric acid excretion and total nitrogen (Brown et al., 1957; Davison et al., 1985; Puvadolpirod and Thaxton., 2000d) so, the digestibility results from this study further support this observation which, suggests that the birds had no problem absorbing the amino acid in the diet, however, the utilization of the nitrogen was subpar. Castellanos and Arroyave (1961) demonstrated that the utilization of proteins from extrahepatic tissues in cortisone treated rats is altered by the level of dietary protein the animal was fed.

Under normal nutritional conditions, rats treated with cortisone experienced an increase in muscle and tissue protein catabolism increasing urea production. The opposite was true in the cortisone-treated rats fed protein-deficient diets because they had low serum urea. By this logic, the relative extent to which gluconeogenesis occurs or not in the presence of increased glucocorticoid activity depends on the level of protein in the diet. Because the DEX-challenge birds in this study already exhibit high levels of CORT, we can speculate that the action will be in favor of gluconeogenesis hence, a reduction in the utilization of nitrogenous compounds is expected (Siegel and Van Kampen, 1984). Thus, the proteins derived from skeletal muscle become the major source of non-carbohydrate compounds, which are preferentially metabolized to provide energy to resist stress. The dietary treatments did not affect the AID of DM, N, EN, and DE calculated. Because GC and catecholamines can cause an increase in urinary electrolyte excretion, it was hypothesized that perhaps, electrolyte deficiencies occur during physiological stress and as such, might influence the absorptive capacity of the birds. Based on our result, the inclusion

of either NaCl or NaCl+$NaHCO_3$ did not influence the absorption and utilization of nutrients. Similarly, EcoE supplementation did not affect the absorption of the nutrients however, EcoE improved the TTR of DM, EN, N, AME, and AMEn in the non-challenged birds.

Much of the impetus for studying the effects of stress on the GIT is to understand the role of stress on gastrointestinal functionality and to offer insights to diagnostic and therapeutic options essential for sustainable animal production. Under physiologic circumstances, among other things, intestinal epithelium represents a barrier that prevents undesirable solutes, microorganisms, and luminal antigens from entering the body. It is therefore easy to envision that the disruption of the epithelial barrier has deleterious effects and decreases the integrity of the intestinal epithelium exposing, tissue compartments to luminal antigens and bacteria. Also, tight junction proteins including claudin-1, occludin, and ZO-1 are complexes that restrict paracellular diffusion of hydrophilic macromolecules and are crucial for the maintenance of barrier integrity. Evidence suggests that claudins are important in establishing the tight junction pore, and ZO-1 and occludin are important in the leaky pathway. Thus, the relevance of gut health is therefore not only restricted to food processing and subsequent nutrient uptake but also its interaction with the epithelial and mucosal immune system that reports to the brain.

In response to a stressor, this interaction signals the onset of both the host innate and acquired immune responses through mediators such as the pro- and anti-inflammatory cytokines and chemokines that regulate each other in an attempt to improve the body's adaptation to stressors. These interactions are complex and often influenced by the time course and level of change of each of the mediators (McEwen, 2008). High concentrations

of CORT have immunosuppressive effects by inhibiting antibody production from B cells, T cells proliferation, and phagocytes (Shini et al., 2010). Acute stress may exacerbate pro-inflammatory responses, whereas chronic stress may induce the suppression of immune responses (Dhabhar, 2009). Shini et al. (2010) showed that 3 to 24 hr (short-term) after CORT treatment led to the up-regulation of pro-inflammatory cytokines and chemokines in the blood whereas the chronic stress induced by CORT down-regulated pro-inflammatory cytokines and chemokines. In this study, mucosal jejunal expressions of proinflammatory cytokines (IL-1β, IL-6, IFN-γ), anti-inflammatory cytokines (IL-10, TGF-β4), and chemokines (CXCLi1, CXCLi2, CCL5) were not influenced by the treatments.

In conclusion, as hypothesized, the DEX administration was able to create some of the negative consequences associated with stress the birds might be exposed to in the industry. However, the inclusion of either of the sodium sources or EcoE supplementation did not mitigate the effect of stress-induced by DEX as evidenced by the insignificant effect on CORT levels, antioxidant status, growth performance, and immune response of the broilers. The magnitude of these results is marked by the decrease in performance, and the and utilization of nutrients observed in the birds. The DEX effect on N digestibility and utilization further demonstrates the complexity of balancing AA and energy levels in the diet during a stressful event. Despite this outcome, EcoE supplementation decreased CORT plasma levels and increased nutrient and energy utilization in non-challenged broiler chickens.

2.5 TABLES

Table 2.1 Ingredients and nutrient composition of the diets fed to broiler chickens for 21 days (on an as-fed basis).

Diet type	A	B	C	D
Sodium source	NaCl		NaCl +$NaHCO_3$	
EconomasE™	No	Yes	No	Yes
Ingredients (g/kg)				
Corn	544.4	534.4	543.7	533.7
Soybean meal, 47%	360.0	360.0	360.0	360.0
Soybean oil	50.0	50.0	50.0	50.0
Limestone (38% Ca)	10.5	10.5	10.5	10.5
Dicalcium phosphate	15.8	15.8	15.8	15.8
NaCl	4.0	4.0	2.4	2.4
$NaHCO_3$	0.0	0.0	2.4	2.4
L-Threonine	1.1	1.1	1.1	1.1
DL-methionine	3.8	3.8	3.8	3.8
L-lysine HCL	2.9	2.9	2.9	2.9
Vitamin mineral premix[1]	2.5	2.5	2.5	2.5
Economase™ premix[2]	0.0	10.0	0.0	10.0
Titanium dioxide premix[3]	5.0	5.0	5.0	5.0
Total	1000	1000	1000	1000
Analyzed nutrient composition[4] `				
GE, kcal/kg		4223		
Crude protein, g/kg		218		
Calcium, g/kg		10.0		
Phosphorus, g/kg		6.4		

[1]Provided the following quantities of vitamins and micro minerals per kilogram of complete diet: iron, 40 mg; copper, 10 mg; manganese, 64 mg; zinc, 75 mg; iodine, 1.85 mg; selenium, 0.3 mg; vitamin A (retinyl acetate), 11,025 IU; vitamin D3 (cholecalciferol), 3,528 IU; vitamin E (dl-α-tocopheryl acetate), 33 IU; vitamin K activity, 0.91 mg; thiamine, 2.21 mg; riboflavin, 7.72 mg; pantothenic acid, 18 mg; niacin, 55 mg; pyridoxine, 5 mg; folic acid, 1.10 mg; biotin, 0.22 mg; vitamin B-12, 0.03 mg; choline, 478 mg.

[2]Economase™ premix was added to diets B and D at the expense of corn to supply 0.2 g of EconomasE™ per kg of diet.

[3]Titanium dioxide was added to the diet at the expense of ground corn.

[4]Because a basal diet was used in the study, the average values of the analyzed nutrients were used to determine digestibility and utilization values.

Table 2.2 Primers used in real-time quantitative PCR

RNA target[1]	Primer/probe sequence (5″-3′)	Accession no.	References
Proinflammatory cytokines			
IL-1β	F GCTCTACATGTCGTGTGTGA′TG	AJ245728	Shini and Kaiser 2009
	R TGTCGATGTCCCGCATGA		
IL-6	F GCTCGCCGGCTTCGA	AJ250838	Shini and Kaiser 2009
	R GGTAGGTCTGAAAGGCGAACAG		
IFN-γ	F GTGAAGAAGGTGAAAGATATCATGGA	Y07922	Shini and Kaiser 2009
	R GCTTTGCGCTGGATTCTCA		
Anti-inflammatory cytokines			
IL-10	F CATGCTGCTGGGCCTGAA	AJ621614	Shini and Kaiser 2009
	R CGTCTCCTTGATCTGCTTGATG		
TGF-β4	F AGGATCTGCAGTGGAAGTGGAT	M31160	Shini and Kaiser 2009
	R CCCCGGGTTGTGTTGGT		
Chemokines			
CXCLi1	F TGGCTCTTCTCCTGATCTCAATG	AF277660	Shini and Kaiser 2009
	R GCACTGGCATCGGAGTTCA		
CXCLi2	F GCCCTCCTCCTGGTTTCAG	AJ009800	Shini and Kaiser 2009
	R TGGCACCGCAGCTCATT		
CCL5	F CCCTCTCCATCCTCCTGGTT	AY037859	Shini and Kaiser 2009

Table 2.2 continued

	R TATCAGCCCCAAACGGAGAT		
Tight junction proteins			
ZO-1	F TGTAGCCACAGCAAGAGGTG	XM 413773.4	Li et al., 2015
	R CTGGAATGGCTCCTTGTGGT		
CLDN-1	F TTGGGGAAATGACTTTACAGG	NM_001013611.2	Cowieson et al., 2017
	R GGAAAGGAAGGTGCTGAAGA		
OCLDN	F ATCAACGACCGCCTCAATCA	NM_205128.1	Cowieson et al., 2017
	R TCCGCTTCAGGTCTTTGAGC		
Housekeeping gene			
GAPDH	F ATGACCACTGTCCATGCCATCCA	NM_204305.1	Cowieson et al., 2017
	R AGGGATGACTTTCCCTACAGCGTT		

*F, forward primer; R, reverse primer; P, probe.
[1]IL-1β, Interleukin 1 beta; IL-6, Interleukin 6; IL-10, interleukin 10; IFN- γ, interferon γ; Tumor necrosis factor- β4, TGF-β4; -CCL5 (RANTES), C-C motif, ligand 5; CXCLi1, C-X-C motif ligand 1 inflammatory; CXCLi2 C-X-C motif ligand 2 inflammatory; OCLN, occludin; CLDN-1, claudin 1; ZO-1, zonula occludens-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2.3 The effects of EconomasE™ (0 or 0.2g/kg diet) supplementation and two inorganic sodium sources (NaCl or $NaCl+NaHCO_3$) on plasma corticosterone, superoxide dismutase, and catalase in dexamethasone-challenged broiler chickens[1].

Treatment Effect			Corticosterone	SOD[2]	CAT[2]
Dexamethasone	Sodium Source	EconomasE™	(ng/mL)	(U/mL)	(nmol/min/ml)
	Main effect				
No			17.58	5.73	12.86
Yes			23.02	5.61	13.14
	NaCl		21.09	5.73	13.34
	$NaCl+ NaHCO_3$		19.52	5.62	12.66
		No	23.14	5.72	12.92
		Yes	17.46	5.65	13.08
	Interaction effect				
No	NaCl	No	21.25[y]	6.04	14.17
No	$NaCl+ NaHCO_3$	No	20.73[y]	5.69	12.68
No	NaCl	Yes	17.50	5.84	12.15[x]
No	$NaCl+ NaHCO_3$	Yes	10.85[y]	5.36	12.43[x]
Yes	NaCl	No	28.69[y]	5.47	10.53[x]
Yes	$NaCl+ NaHCO_3$	No	21.88	5.69[x]	14.32[x]
Yes	NaCl	Yes	16.90[x]	5.57	16.53[x]
Yes	$NaCl+ NaHCO_3$	Yes	24.59	5.73	11.23
	Pooled SD[3]		7.57	1.00	4.14
	P-value				
Dexamethasone			0.020	0.665	0.802
Sodium Source			0.486	0.682	0.560
EconomasE™			0.015	0.720	0.893
Dexamethasone x Sodium x EconomasE™			0.116	0.793	0.083

[1]Values represent means of 7 replicate cages per treatment except for mean values with x and y where the number of replicates was 6 and 5, respectively.
[2]SOD = superoxide dismutase; CAT = catalase.
[3]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 2.4 The effects of EconomasE™ (0 or 0.2g/kg diet) supplementation and two inorganic sodium sources (NaCl or $NaCl+NaHCO_3$) on performance in dexamethasone-challenged broiler chickens[1].

Treatment Effect			16 – 21 d		
Dexamethasone	Sodium Source	EconomasE™	BWG[2] g/bird	Feed Intake g/bird	Feed efficiency g/kg
	Main effect				
No			365	462	784
Yes			274	477	578
	NaCl		315	477	674
	$NaCl+ NaHCO_3$		325	461	688
		No	318	472	678
		Yes	321	466	684
	Interaction effect				
No	NaCl	No	364	467	779
No	$NaCl+ NaHCO_3$	No	360	455	793
No	NaCl	Yes	370	456	776[x]
No	$NaCl+ NaHCO_3$	Yes	367	467	788
Yes	NaCl	No	267	466	575
Yes	$NaCl+ NaHCO_3$	No	281[x]	501	567[x]
Yes	NaCl	Yes	258	456	567
Yes	$NaCl+ NaHCO_3$	Yes	291	483	606
	Pooled SD[3]		24.0	39.2	46.6
	P-value				
Dexamethasone			<.0001	0.160	<.0001
Sodium Source			0.583	0.150	0.264
EconomasE™			0.127	0.563	0.669
Dexamethasone x Sodium x EconomasE™			0.243	0.495	0.682

[1]Values represent means of 7 replicate cages per treatment except for mean values with x where the number of replicates was 6.

[2]BWG, body weight gain.

[3]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$.

Table 2.5 The effects of EconomasE™ (0 or 0.2g/kg diet) supplementation and two inorganic sodium sources (NaCl or $NaCl+NaHCO_3$) on tissue weight and tibia breaking strength and ash in dexamethasone-challenged broiler chickens[1].

Treatment Effect			Relative to body weight (%)			Bone parameters	
Dexamethasone	Sodium Source	EconomasE™	Liver (%)	Spleen (%)	Bursa of Fabricius (%)	Tibia breaking strength (kg/f)	Ash (%)
	Main effect						
No			2.73	0.09	0.16	21.6	52.0
Yes			3.54	0.08	0.08	22.5	51.4
	NaCl		3.20	0.08	0.12	21.6	52.1
	$NaCl+ NaHCO_3$		3.07	0.09	0.12	22.5	51.4
		No	3.13	0.08	0.12	22.1	51.8
		Yes	3.14	0.09	0.12	22.0	51.7
	Interaction effect						
No	NaCl	No	2.78^{x}	0.09	0.16^{x}	20.2	52.0
No	$NaCl+ NaHCO_3$	No	2.76^{x}	0.09	0.17	23.1^{x}	51.7
No	NaCl	Yes	2.75	0.08	0.15^{x}	20.7	52.8
No	$NaCl+ NaHCO_3$	Yes	2.64	0.10^{x}	0.17	22.5	51.6^{x}
Yes	NaCl	No	3.62	0.06^{x}	0.07^{x}	21.9	52.1^{x}
Yes	$NaCl+ NaHCO_3$	No	3.37	0.07^{x}	0.08	23.2	51.4
Yes	NaCl	Yes	3.64	0.09	0.09	23.5	51.4^{x}
Yes	$NaCl+ NaHCO_3$	Yes	3.51	0.09^{x}	0.08^{x}	21.3^{x}	50.8^{y}
	Pooled SD[2]		0.31	0.02	0.03	4.47	1.32
	P-value						
Dexamethasone			<.0001	0.002	<.0001	0.490	0.089
Sodium Source			0.148	0.292	0.536	0.442	0.060
EconomasE™			0.982	0.078	0.787	0.918	0.690
Dexamethasone x Sodium x EconomasE™			0.790	0.125	0.709	0.685	0.586

[1]Values represent means of 7 replicate cages per treatment except for mean values with x and y where the number of replicates was 6 and 5, respectively.

[2]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$.

Table 2.6 The effects of EconomasE™ (0 or 0.2g/kg diet) supplementation and two inorganic sodium sources (NaCl or $NaCl+NaHCO_3$) on intestinal morphology in dexamethasone-challenged broiler chickens[1].

Treatment Effect[1]			Intestinal morphology		
Dexamethasone	Sodium Source	EconomasE™	Villus height (μm)	Crypt depth (μm)	VH: CD
	Main effect				
No			896.0	162.3	5.64
Yes			837.0	145.8	5.74
	NaCl		850.4	151.2	5.79
	$NaCl+ NaHCO_3$		878.3	156.9	5.60
		No	875.1	145.9	6.00
		Yes	853.6	162.2	5.40
	Interaction effect				
No	NaCl	No	869.2	141.2	6.22
No	$NaCl+ NaHCO_3$	No	943.6	161.1^{x}	5.69^{x}
No	NaCl	Yes	873.9	163.8	5.36
No	$NaCl+ NaHCO_3$	Yes	898.6	183.2^{x}	5.12
Yes	NaCl	No	805.4^{x}	138.8^{x}	5.89
Yes	$NaCl+ NaHCO_3$	No	882.0	142.7	5.97^{x}
Yes	NaCl	Yes	852.9	161.2	5.47^{x}
Yes	$NaCl+ NaHCO_3$	Yes	788.9^{y}	140.6^{x}	5.63^{x}
	Pooled SD[2]		101.4	18.4	0.63
	P-value				
Dexamethasone			0.027	0.002	0.622
Sodium Source			0.324	0.277	0.279
EconomasE™			0.447	0.003	0.001
Dexamethasone x Sodium source			0.443	0.009	0.085
Dexamethasone x EconomasE™			0.963	0.240	0.206
Sodium x EconomasE™			0.100	0.227	0.392
Dexamethasone x Sodium x EconomasE™			0.420	0.248	0.531

[1]Values represent means of 7 replicate cages per treatment except for mean values with x and y where the number of replicates was 6 and 5, respectively.

[2]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$.

Table 2.7 The effects of EconomasE™ (0 or 0.2g/kg diet) supplementation and two inorganic sodium sources (NaCl or NaCl+$NaHCO_3$) on apparent ileal nutrient and energy digestibility in dexamethasone-challenged broiler chickens[1].

Treatment Effect			Apparent Ileal Digestibility (%)			
Dexamethasone	Sodium Source	EconomasE™	DM (%)	N (%)	Energy (%)	DE kcal/kg
	Main effect					
No			72.7	83.7	76.0	3642
Yes			72.3	84.7	75.2	3605
	NaCl		72.3	84.2	75.4	3615
	NaCl+ $NaHCO_3$		72.7	84.2	75.7	3632
		No	72.3	84.1	75.4	3621
		Yes	72.8	84.3	75.7	3627
	Interaction effect					
No	NaCl	No	71.8[x]	83.3[x]	75.6	3627
No	NaCl+ $NaHCO_3$	No	72.9	83.9	75.7	3632
No	NaCl	Yes	73.1	83.8[x]	76.6	3661
No	NaCl+ $NaHCO_3$	Yes	73.0	83.9	76.0	3648
Yes	NaCl	No	72.5[x]	85.0	75.0	3605
Yes	NaCl+ $NaHCO_3$	No	72.2[x]	84.3	75.5[x]	3619[x]
Yes	NaCl	Yes	71.8[x]	84.7	74.6	3569
Yes	NaCl+ $NaHCO_3$	Yes	72.7	84.9	75.7[x]	3629[x]
	Pooled SD[3]		1.93	1.53	2.51	116.4
P-value						
Dexamethasone			0.457	0.021	0.26	0.256
Sodium Source			0.511	0.989	0.672	0.605
EconomasE™			0.539	0.640	0.710	0.855
Dexamethasone x Sodium Source			0.878	0.506	0.463	0.527
Dexamethasone x EconomasE™			0.470	0.898	0.575	0.561
Sodium source x EconomasE™			0.983	0.832	0.966	0.825
Dexamethasone x Sodium x EconomasE ™			0.275	0.420	0.635	0.616

[1]Values represent the mean of 7 replicates cages (pooled ileal digesta of 6 birds per replicate) except for mean values with x where the number of replicates was 6.
[2]DM = dry matter; N = nitrogen; DE = ileal digestible energy.
[3]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$.

Table 2.8 The effects of EconomasE™ (0 or 0.2g/kg diet) supplementation and two inorganic sodium sources (NaCl or $NaCl+NaHCO_3$) on apparent total tract utilization of nutrient and energy in dexamethasone-challenged broiler chickens[1].

Treatment Effect			Total tract Digestibility (%)				
Dexamethasone	Sodium Source	EconomasE™	DM %	N (%)	Energy (%)	AME (kcal/kg)	AMEn (kcal/kg)
No			72.4	67.9	76.8	3679	3573
Yes			70.3	54.0	75.7	3630	3476
	NaCl		71.7	61.3	76.5	3668	3540
	$NaCl+ NaHCO_3$		71.0	60.7	76.0	3641	3508
		No	71.1	60.6	76.0	3643	3510
		Yes	71.6	61.4	76.5	3665	3538
Two- way interaction effect							
No	-	No	71.5^{b}	65.7^{a}	75.9^{ab}	3638^{ab}	3523^{ab}
No	-	Yes	73.4^{a}	70.1^{a}	77.7^{a}	3719^{a}	3622^{a}
Yes	-	No	70.8^{b}	55.5^{b}	76.1^{ab}	3649^{ab}	3497^{b}
Yes	-	Yes	69.8^{b}	52.6^{b}	75.4^{b}	3611^{b}	3454^{b}
Pooled SD[3]							
P-value							
Dexamethasone			<.0001	<.0001	0.044	0.046	0.001
Sodium Source			0.166	0.701	0.034	0.258	0.230
EconomasE™			0.348	0.621	0.276	0.356	0.299
Dexamethasone x Sodium source			0.493	0.096	0.823	0.885	0.668
Dexamethasone x EconomasE™			0.003	0.019	0.013	0.015	0.010
Sodium source x EconomasE™			0.166	0.306	0.133	0.194	0.181
Dexamethasone x Sodium x EconomasE™			0.181	0.164	0.286	0.369	0.251

[a–b] Means with different superscripts within the same row differ significantly ($P < 0.05$).

[1]Values represent the mean of 7 replicates cages (pooled ileal digesta of 6 birds per replicate).

[2]DM = dry matter; N = nitrogen; AME = apparent metabolizable energy; AMEn = nitrogen-corrected apparent metabolizable energy.

[3]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 2.9 The effects of EconomasE™ (0 or 0.2g/kg diet) supplementation and two inorganic sodium sources (NaCl or $NaCl+NaHCO_3$) on cytokines, chemokines, and tight junction protein mRNA expressions in the intestinal mucosa of dexamethasone-challenged broiler chickens[1].

Treatment Effect			Pro-inflammatory cytokines[2]			Anti-inflammatory cytokines[2]		Chemokines[2]			Tight junction proteins[2]		
Dexamethasone	Sodium Source	EconomasE	IL-1β	IL-6	IFN-γ	IL-10	TGF-β4	CXCLi1	CXCLi2	CCL5	ZO-1	CLDN1	OCLD
	Main effect												
No			1.15	0.97	1.08	1.44	0.97	1.48	2.20	0.99	1.19	1.04	1.11
Yes			1.47	1.38	1.62	1.92	1.00	1.13	1.65	0.98	1.04	0.98	1.21
	NaCl		1.36	1.15	1.26	1.64	0.97	1.28	1.69	0.96	1.08	1.14	1.11
	$NaCl+ NaHCO_3$		1.26	1.21	1.45	1.71	1.00	1.33	2.15	1.02	1.15	0.88	1.21
		No	1.48	0.81	1.22	1.69	0.91	1.30	2.13	0.88	1.05	1.13	1.19
		Yes	1.15	1.54	1.49	1.66	1.66	1.31	1.72	1.09	1.18	0.89	1.12
	Interaction effect												
No	NaCl	No	0.90x	0.65^{y}	1.00^{x}	1.49	0.88^{x}	0.93^{x}	1.32	0.96^{x}	0.97^{x}	1.31	1.13
No	$NaCl+ NaHCO_3$	No	1.18	1.02^{y}	1.18^{x}	1.61^{x}	1.22	1.45	2.93^{x}	0.91^{x}	1.11	1.06^{x}	1.10^{x}
No	NaCl	Yes	1.88x	1.69^{y}	1.59^{z}	1.94	1.12^{x}	1.93	2.52^{x}	1.08	1.13^{y}	1.32^{z}	1.11
No	$NaCl+ NaHCO_3$	Yes	0.66y	0.53^{y}	0.58^{x}	0.72^{x}	0.67^{x}	1.75	2.01^{x}	1.03^{x}	0.98^{x}	0.49^{y}	1.09
Yes	NaCl	No	1.71y	0.47^{y}	0.89^{z}	1.43^{y}	0.67^{x}	1.35^{x}	1.69^{y}	0.80^{x}	0.92^{y}	1.17^{z}	1.11^{x}
Yes	$NaCl+ NaHCO_3$	No	2.11	1.11^{y}	1.81^{z}	2.25^{x}	0.86	1.61^{x}	2.56^{x}	0.85	1.23^{x}	1.00^{y}	1.43
Yes	NaCl	Yes	0.96x	1.79^{x}	1.56^{y}	1.69	1.22^{x}	0.91	1.25^{x}	0.99^{x}	1.31	0.75^{x}	1.09^{x}
Yes	$NaCl+ NaHCO_3$	Yes	1.08x	2.17^{x}	2.22^{x}	2.29	1.26^{x}	0.63	1.11^{x}	1.27^{x}	1.31	1.00	1.20^{x}
	Pooled SD[3]		0.97	1.34	0.95	1.26	0.62	0.88	1.36	0.42	0.44	0.80	0.55
P-value													
Dexamethasone			0.274	0.327	0.084	0.188	0.857	0.150	0.174	0.905	0.263	0.803	0.533
Sodium Source			0.712	0.886	0.540	0.826	0.865	0.823	0.253	0.630	0.578	0.305	0.517
EconomasE™			0.246	0.087	0.383	0.922	0.373	0.995	0.308	0.079	0.322	0.329	0.653
Dexamethasone x Sodium x EconomasE™			0.067	0.413	0.159	0.298	0.205	0.060	0.169	0.760	0.662	0.638	0.902

[1]Values represent means of 7 replicate cages per treatment except for mean values with x, y, and z where the number of replicates was 6, 5, and 4 respectively.

[2]IL-1β, Interleukin 1 beta; IL-6, Interleukin 6; IL-10, interleukin 10; IFN- γ, interferon γ; Tumor necrosis factor- β4, TGF-β4; - CCL5 (RANTES), C-C motif, ligand 5; CXCLi1, C-X-C motif ligand 1 inflammatory; CXCLi2 C-X-C motif ligand 2 inflammatory; OCLN, occludin; CLDN-1, claudin 1; ZO-1, zonula occludens-1.

[3]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$.

CHAPTER 3.

EFFECT OF DIETARY ANTIOXIDANT AND TWO SODIUM SOURCES ON PRODUCTION PARAMETERS, EGG QUALITY, AND BLOOD ELECTROLYTE RESPONSES OF LAYING HENS EXPOSED TO ELEVATED TEMPERATURES

Abstract

The effect of an antioxidant EconomasE™ (EcoE; 0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+$NaHCO_3$) in laying hens exposed to different environmental temperature on egg quality, performance, blood metabolites, keel bone damage, bone parameters, and mRNA expression of heat shock proteins were investigated. The Hy-line Brown hens at 26 wk of age were randomly assigned to one of four dietary treatments with 9 replicates per diet and 12 hens per replicate. Hens were housed as two birds per cage and photo-stimulated with 16L: 8D. Feed and water were provided ad libitum. The birds were maintained at a thermoneutral (TN1) temperature of 23.8 °C until 31 wk of age. Then, all birds were maintained under cycling elevated temperature conditions (ET1) at 32.2 °C for 8 hours per day (from 9:00 am to 5:00 pm) for 5 wk and allowed to recover at TN for another 5 wk (REC1). This was repeated following the same sequence after a week break. One bird from each 6 replicates (6 bird per treatment) was euthanized during TN1, ET1, REC1, TN2, ET2, and REC2. Serum metabolites and acid-base equilibrium related parameter (HCO^-_3, Na^+, K^+, Cl^-, and Ca) levels were determined. The dietary treatment did not improve performance during the ET1 and ET2 period. A combination of NaCl and $NaHCO_3$ increased ($P < 0.05$) feed intake (FI) during TN1 and REC1, and FCR during the REC1 regimen. The birds fed diets with no EcoE and with NaCl+$NaHCO_3$ had a higher ($P < 0.05$) FI and FCR during the TN2 regimen compared to the birds fed the diet without

EcoE and NaCl. During the ET1 regimen, the albumen height and Haugh unit increased ($P < 0.05$) with EcoE and NaCl+$NaHCO_3$ supplementation compared to birds on a similar diet but without EcoE. Similarly, EcoE improved ($P < 0.05$) albumen height (2.8%), and Haugh unit (1.2%) during the TN2 regimen. The NaCl+$NaHCO_3$ diet decreased ($P < 0.05$) Cl^- (ET1) and increased ($P < 0.05$) HCO_3^- (ET2) levels in the serum. During REC1, the NaCl+$NaHCO_3$ diet decreased ($P < 0.05$) Cl^- and K^+ serum levels while EcoE increased ($P < 0.05$) Cl^- levels. Similarly, Na and Cl levels were decreased ($P < 0.05$) with NaCl+$NaHCO_3$ inclusion in the diet (TN2). EconomasE™ and NaCl supplementation decreased ($P < 0.05$) serum HCO_3^- (REC2) compared to the other three dietary combinations. During the REC2 regimen, serum Na^+ level increased ($P < 0.05$) in the birds fed the diet supplemented with EcoE and NaCl+$NaHCO_3$ compared to those fed diets with No EcoE + NaCl+$NaHCO_3$ and EcoE + NaCl. The EcoE and NaCl+$NaHCO_3$ diet increased ($P < 0.05$) villus height compared to birds in the other dietary treatments and increased ($P < 0.05$) VH: CD ratio compared to birds fed no EcoE and NaCl+ $NaHCO_3$ and EcoE + NaCl (TN1). During the REC2 regimen, EcoE and NaCl+$NaHCO_3$ in the diet reduced ($P < 0.05$) VH and CD compared to the diet without EcoE and NaCl+$NaHCO_3$ supplementation. The NaCl+$NaHCO_3$ diet reduced ($P < 0.05$) tibia and femur breaking strength while EcoE improved ($P < 0.05$) the breaking strength in the femur (REC2). In summary, dietary treatment did not improve performance, egg quality, intestinal morphology, keel bone, bone-breaking strength, and HSP 70 and 90 during ET1 and ET2. Albumen height and Haugh unit with EcoE and NaCl+$NaHCO_3$ (TN2) and EcoE alone (ET2), which suggests that the supplementation can improve the fresh appearance of the egg during ET conditions.

3.1 INTRODUCTION

High environmental temperatures coupled with high humidity have been shown to influence thermoregulation and impact the production and physiological responses of commercial poultry. The deleterious effects of heat stress in laying hens have been discussed extensively (Sahin et al., 2002; Babinszky et al. 2011; Quinteiro-Filho et al., 2012; Sohail et al., 2012). Impaired performance due to a decrease in feed intake with a concomitant reduction in body weight gain and increased feed conversion ratio has been observed (Scott and Balnave, 1988; Mashaly et al., 2004; Babinszky et al. 2011; Quinteiro-Filho et al., 2012; Sohail et al., 2012). To put in perspective, Mitchell and Carlisle (1992) reported a decrease in feed intake (29%) and growth rate (37%) when birds were maintained at elevated environmental temperatures (35°C) for two weeks. Consequently, live weight, egg production, and mean egg weight decreases (Hsu et al., 1998; Mashaly et al., 2004), and some egg quality traits, such as eggshell weight, thickness, breaking strength, Haugh unit, and egg specific gravity were also influenced (Hsu et al., 1998; Sahin et al., 2002). Another adverse effect of elevated temperature is the imbalance of acid-base that stems from the excessive loss of carbon dioxide (**CO_2**) via increased panting. The partial pressure of CO_2 in the blood is reduced with panting, causing a decrease in the concentration of carbonic acid (**H_2CO_3**) and hydrogen ion (H^+) (Allahverdi et al., 2013). In response, the kidney increases bicarbonate ions (**HCO_3^-**) excretion and conserves H^+ which, inadvertently increases pH in an attempt to keep the bird's acid-base balance. This condition results in respiratory alkalosis (Borges et al., 2007). On the other hand, metabolic compensation will automatically result in the retention of chloride – because of the anion gap which, results in a little bit of acidosis on top of the compensation. In laying hens, the

loss of CO_2 is accentuated by the need for blood bicarbonate to increase the hydrogen ions and free calcium into circulation for the mineralization of the eggshell. This reduces the blood ionized calcium (**Ca**) pool, increasing the concentration of Ca that is bound to protein, which in turn limits the availability of Ca for eggshell formation (Odom et al., 1986). A 19% reduction in blood ionized calcium level was observed in laying hens exposed to 35℃ temperature (Odom et al., 1986). Similarly, Allahverdi et al. (2013) reported a decrease in Ca concentration in birds exposed to elevated temperature and a subsequent decrease in egg production and egg quality.

Also, it is important to note that acid-base and electrolytes status inter-relate through the body's homeostatic mechanisms, specifically sodium (**Na^+**), potassium (**K^+**), and chloride (**Cl^-**) which are essential ions for the maintenance of the acid-base balance of the body fluids Mongin (1981). Hence, in response to respiratory alkalosis, decreased levels of plasma Na^+, K^+, Ca^+, magnesium (**Mg**), and increased levels of Cl^- (Kohne and Jones, 1975; Borges et al., 2004) has been observed. Moreover, heat stress causes a variety of alterations in cellular physiology, altering the normal biochemical processes in the body. As a result, an increase in reactive oxygen species (ROS) production ensue (Lin et al., 2006b). To protect the body from the deleterious effect of ROS, a conserved mechanism via the induction of heat shock proteins (HSP) have been reported (Wang and Edens, 1994; Mahmoud and Edens, 2005). Heat shock proteins have been implicated in the development of thermotolerance in protein folding and translocation, in steroid receptor protein binding, and the onset of human autoimmune diseases. In vivo, Wang and Edens (1994, 1998) demonstrated that broiler chickens were able to synthesize HSPs as a way to resist acute heat stress. Broiler chickens exposed to 41°C for 60 min induced the expression of HSP70

protein in blood leukocytes, testes, and bursa of Fabricius (Wang and Edens, 1998). Mahmoud and Edens (2005) reported an increase in HSP70 protein levels in the liver of broiler chickens subjected to HS (40-C for 1 h).

Most of the common ways to mitigate the effect of heat stress involved environmental control, either by increasing airflow in the house, the use of evaporative cooling systems, reducing stocking densities, or implementing some nutritional modifications. Some statistics on heat stress management show that without the use of heat management strategies, U.S. livestock industry producers incur an average loss of $2.4 billion annually and when heat management strategies were accounted for, incur an estimated loss of $1.7 billion. From the overall total, a $128 million loss is attributed to the poultry industry (St-Pierre et al., 2003). Hence, since environmental control strategies are said to be an expensive option, nutritional modification strategies are becoming more favorable. Supplemental dietary vitamins E (**VE**) has been shown to improve egg production, feed intake, and egg qualities in hens reared under elevated temperature conditions (Sahin and Kucuk, 2001; Sahin et al., 2002). EconomasE, a cheaper alternative to VE can be advantageous in improving feed intake, feed efficiency, and egg quality.

Similarly, the addition of electrolyte salts such as sodium bicarbonate ($NaHCO_3$), potassium bicarbonate ($KHCO_3$), potassium chloride (KCl), calcium chloride ($CaC1_2$), and ammonium chloride (NH_4C1) (Ahmad and Sawar., 2005; Borges, et al., 2007) are important in maintaining acid-base balance during exposure to high environmental temperatures (Hayat et al., 1999; Borges et al., 2004; Gezen et al., 2005). Some researchers have reported the beneficial effect of substituting a part of the dietary inorganic Na provided in the diet as NaCl with $NaHCO_3$ (Frank and Burger, 1965; Makled and

El- Gammal, 1977; Makled and Charles, 1987; Balnave and Muheereza, 1997; Yörük et al., 2004), others have reported no benefits (Cox and Balloun, 1968; Ernst et al., 1975; Grizzle et al., 1992). The Na and Cl dietary requirements are interdependent and are supplied at precise and adequate levels for optimum growth, bone development, good litter quality, and egg quality (Murakami et al., 2001). Sodium is closely associated with Cl^- and HCO_3 in managing the basal metabolism while Cl^- competes with HCO_3 for cations in the extracellular fluid to maintain electrical conductivity (Sandercock et al., 2001). Hence supplementing $NaHCO_3$ may help to maintain proper pH balance, eliminate acidosis, and facilitate the metabolic process, ensuring maximum growth and productivity (Ahmad et al., 2005; Naseem et al., 2005). Similarly, to correct for the reduced HCO_3 concentration in the lumen of the shell gland that negatively affects eggshell quality in high temperatures, (Hall and Helbacka, 1959; Wideman Jr. and Buss, 1985), a bicarbonate source is supplemented in laying hens diet as a source of alkaline (Ghorbani and Fayazi, 2009).

There is contradicting information regarding the potential benefits of $NaHCO_3$ in addition to NaCl in the diet, during elevated temperature on laying hen performance, egg quality, and blood profile. This is also true for the supplementation of VE in laying hens diet. Thus, the study hypothesized that if the level of Na in the diet is met by substituting half of the requirement with $NaHCO_3$ and supplementing EcoE, this can mitigate the negative effect of elevated temperature in laying hens. The objective of the present study was to determine the effects of EconomasE™ (a blend of ingredients that maximizes and maintains the antioxidant status of the animal) and two inorganic sodium sources (NaCl or $NaCl+NaHCO_3$), supplemented in the diet of laying hens on their production parameters,

egg quality, blood metabolites, intestinal morphology, keel bone, and bone-breaking strength and ash at different environmental temperatures.

3.2 MATERIALS AND METHODS

The birds in this experiment were maintained and used following the protocol approved by the University of Kentucky Animal care and Use Committee.

3.2.1 Birds management and housing

A total of 432 commercial brown laying hens (Hy-line Brown) at 26 weeks of age were used in this study. Hens were housed in a two-tier cage battery and randomly assigned to one of four dietary treatments replicated nine times, with 12 hens per replicate. The experimental house was a 2-tier cage facility and hens were housed 2 birds per cage. The hens in 3 adjacent cages on the top and bottom tier were considered an experimental replicate. Each cage (dimensions were approximately 65 cm wide, 103 cm deep, and 116 cm tall) was equipped with a wire floor, automatic nipple drinkers, and a trough feeder located in front of the cage. The feed was manually distributed. Throughout the experimental period, feed and water were available *ad libitum*. Photo stimulation was provided as 16 hours of light and 8 hours of darkness throughout the entire laying period. A ventilation fan ran between 0800 h to 2000 h to maintain uniform distribution of air and temperature within the room. The experiment lasted between February 26 and June 22, 2018, a 15-wk period for the first phase, and after a week break, the second phase lasted between July 2 and October 13, 2018, another 15-wk period. Within each phase, the experimental period consisted of 3 temperature regimen that lasted for 5 weeks.

The first temperature regimen lasted between 26 and 30 wk of age, and the birds were maintained at thermoneutral temperature (TN1, 23.8 ± 1 °C). The second temperature

regimen lasted between 33 and 37 wk of age, representing the high environmental temperature (ET1, 32.2 ± 1 °C). The third temperature regimen lasted between 38 and 42 wk of age, which was designated as the recovery period also at thermoneutral room temperature (REC1, 23.8 ± 1 °C). This same trend was followed for the second phase with each temperature and hen age represented as follows; TN2 (44 and 48 wk of age), ET2 (49 and 53 wk of age), and REC2 (54 and 58 wk of age). During the TN and REC regimen, hens were maintained at the thermoneutral temperature for 24 h/d. While during the elevated temperature regimen (ET1 and ET2), hens were kept at the elevated temperature for 8 h/day (from 9:00 am to 5:00 pm) Monday to Friday, and reduced to 23.2 ℃ by the end of the day (5:30 pm to 7:30 am) and throughout the weekends; the same temperature as the TN and the REC groups. Ambient temperature and humidity values were recorded using an electronic data recorder (HOBO ZW series wireless, Onset Computer Corporation, Bourne, MA). It is important to point out that the initial elevated temperature for this experiment was set at 35 ℃ and set to start at hen age 31 weeks. However, due to issues with airflow that resulted in a lack of uniform heat distribution around the room, the study was terminated after about three hours. The elevated temperature regimen was eventually initiated two weeks (hen-age 33 weeks) after the heat circulation problem within the room was resolved.

3.2.2 Experimental diet

Table 3.1 shows the ingredients and nutrient composition of the experimental diets fed in the study. The basal diet was a typical commercial layer diet formulated to meet or exceed nutrients and energy requirements (NRC, 1994). The hens were randomly assigned to four dietary treatments that include two different inorganic sodium sources (NaCl or

NaCl+$NaHCO_3$) and two levels of EconomasE™ supplementation (0 or 0.2 g/kg of diet) (Table 3.1). The diets given were A) a basal diet supplemented with NaCl as the only source of inorganic sodium with no EconomasE™ supplementation (0 g/kg diet); B) a basal diet with NaCl + $NaHCO_3$ as the source of inorganic sodium (by substituting an equal amount of sodium (Na), as provided by NaCl) with no EconomasE™ supplementation (0 g/kg diet); C) a basal diet supplemented with NaCl as the only source of inorganic sodium with EconomasE™ supplementation (0.2 g/kg diet); D) a basal diet with NaCl + $NaHCO_3$ as sources of inorganic sodium with EconomasE™ supplementation (0.2 g/kg diet). Hens were fed these diets throughout the experimental period with necessary nutrient adjustments made to accommodate for different production phases.

3.2.3 Egg production and measurement of egg quality

Egg production and mortality were recorded daily on a per-replicate basis and percent hen-day egg production was calculated for each time regimen (Eqn. 1). Feed consumption was determined at the end of each regimen, and feed intake was calculated as g/bird/d. The feed conversion ratio was expressed as kilograms of feed consumed per kilogram of egg produced (Eqn. 2). Throughout the experiment, production variables such as feed intake and egg production were adjusted for hen mortalities. All the eggs laid on two consecutive days on the first, third, and fifth week of each 5-wk regimen were collected to obtain the egg weight. Egg mass was calculated by multiplying percent hen-d production by egg weight. Subsequently, six eggs from each replicate (9) per dietary treatment were randomly chosen to determine egg quality (approximately 1,296 eggs were used for every temperature-period). Eggshell breaking strength was measured using an electronic eggshell tester equipment (Egg Force Reader, ORKA Technology LLC, USA) and expressed as a

unit of compression force exposed to unit eggshell surface area (kg/f). Then, eggs were cracked, carefully separating the eggshell and egg content. The eggshells were cleaned, dried, weighed, and expressed as a proportion (%) of the whole egg. Using a tripod micrometer (Sanovo), the albumen height was measured midway between the yolk and the edge of the albumen. Haugh unit was calculated from the HU formula (Eqn. 3). The summary of all the data collected as well as collection frequencies are reported in Table 3.2.

3.2.4 Sample collection

Hens were weighed at the end of each temperature-regimen; TN1, ET1, REC1, TN2, ET2, and REC2. Furthermore, one bird was randomly chosen from 6 of the 9 replicates (consistently from the first 6 replicates) per dietary treatment to collect samples, and the remaining birds were carried through the experiment. A blood sample was taken via the wing vein into heparinized tubes. Blood was allowed to clot and sent out for immediate analysis of blood chemistry panel. The birds were then euthanized by argon asphyxiation. At necropsy, the hen's liver, jejunal segments, mucosa, and bones (tibia and femur) were harvested rapidly. The liver weights were recorded and used to calculate relative weight determinations. For mucosa collection, segments of the mid-jejunum were cut off and the mucosal side was exposed. The mucosal side was nipped by forceps and gently rinsed in nanopore water until the content was cleaned. Jejunal mucosa was scraped using a clean microscope slide, the mucosal scrapings were collected in microtubes containing TRIzol, snap-frozen in liquid nitrogen, and stored at −80°C until analysis of the mRNA expression of HSPs. Subsequently, segments of mid-jejunum harvested immediately after euthanasia were fixed in 10% formalin for morphology measurements

3.2.5 Blood electrolytes and metabolites analysis

Blood samples collected were analyzed at the Rood and Riddle equine hospital (Lexington, KY, 40511). The blood chemistry panel [Alkaline Phosphatase (Alk. Phos.), creatinine kinase (CK), lactose dehydrogenase (LDH), Albumin, Calcium, Phosphorus, Glucose] and electrolytes (Na^+, K^+, HCO_3^-, and Cl^-) analysis were performed using the AU480 Chemistry Analyzer (Beckman Coulter, Inc. CA. USA).

3.2.6 Intestinal morphology

The intestinal segment of the jejunum was collected from one bird per cage (using 6 replicates per treatment) during each phase, flushed with nanopore water to remove the digesta contents. The cut sections were fixed in 10% neutral buffered formalin (Sigma Chemical Co., St Louis, MO, USA). In brief, tissue sections were cut, dehydrated, cleared, and embedded in Polyfin paraffin (Polysciences Inc., Warrington, PA, USA). Then, the paraffin-embedded jejunal samples were sliced to approximately 5 μm with a microtome and mounted on slides. These sections were deparaffinized in xylene, rehydrated in a graded alcohol series, and stained with hematoxylin and eosin. From each slide, villus height, width, and crypt depth were measured from 10 villi under a magnification of 4X using a Nikon ECLIPSE Ci-E light microscope equipped with a computer-assisted digital camera (DS-Ri2) using NIS-Elements Br software (Nikon Corporation, Tokyo, Japan). An average value was calculated for each section measured. The villus height: crypt depth ratio was calculated.

3.2.7 Intestinal gene expression analysis

The mRNA expression levels of HSP 70 and 90 were measured using real-time quantitative PCR. Jejunal mucosal samples in TRIzol reagent were allowed to thaw on ice

before the isolation process begins. Samples were homogenized using an Omni tissue homogenizer (TH) with 5 mm plastic disposable probes (Omni International, GA, USA) and placed on ice afterward. Total RNA extraction was performed on the homogenates according to the manufacturer's instructions (Invitrogen Inc., Carlsbad, CA). RNA samples were resuspended in nuclease-free water, and the concentration and purity of the extracted RNA were determined using a NanoDrop one (Thermo Fisher Scientific, Wilmington, DE USA) spectrophotometer at an optical density of 260 and 280 nm. A 260/280 ratio above 2.0 and 260/230 ratio in the range of 2.0–2.2 was accepted as "pure" for RNA. Lower ratios may indicate the presence of protein, phenol, EDTA, carbohydrates, or other contaminants that absorb at or near 260, 230, or 280 nm and were subjected to further purification. Subsequently, 1 μg of total RNA from each sample was reverse transcribed into cDNA in a 20 μL RT reaction using Script cDNA super mix for qRT-PCR (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's protocol in a Veriti TM Thermal cycler (Catalog no 4375786, Applied Biosystems). The RNA was incubated for 5 min at 25 ℃, followed by 30 min extension at 42 ℃. The reaction was stopped at 80 ℃ for 5 min and then held at 4 ℃ until removal from the machine. The cDNA was then diluted 1:20 with nuclease-free water before being used for real-time PCR. Briefly, the reaction mix was prepared using 1μL of cDNA, 0.375μL of each forward and reverse primer, 6.25 μL SYBR green master mix (Bio-Rad, Hercules, CA), and 4.5 μL of RNase free water to reach a total reaction volume of 12.5 μL. Each sample was tested in duplicate. PCR plate contained target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) - an endogenous housekeeping control, and negative controls, which consisted of all the components of the qRTPCR mix except cDNA, were used for all primers. Thermal cycling was carried out

using a Bio-Rad CFX-96 real-time PCR system (Bio-Rad, Hercules, CA) with the following cycle profile: 95 ℃ for 5 min, 95 ℃ for 10 s, followed by 30s at and then 60 ℃ and 72 ℃ for 10s with final melting at 95 ℃ for 20 s on repeat for 40 cycles. For each gene examined (GAPDH: F -5′ GTG TTA TCA TCT CAG CTC CCT CAG 3′, R- 5′ GGT CAT AAG ACC CTC CAC AAT G 3′ (GenBank No.FJ_217667); cHsp70: F - 5′ GAC AAG AGT ACA GGG AAG GAG AAC 3′, R-5′ CTG GTC ACT GAT CTT TCC CTT CAG 3′ (GenBank No. FJ_217667.1; Al-Zhgoul et al., 2013), HSP 90: F – TCA TCA ACA CGT TCT ACT CCA ACA AG, R – CGG AGG CGT TGG AGA TGA G (Rimoldi et al., 2015) duplicate from each cDNA was analyzed and the formation of single PCR products was confirmed using melting curves. The relative levels of mRNA expression were calculated using the $2^{-\Delta\Delta CT}$ method after normalization against the reference gene (Shini and Kaiser, 2009). In the $2^{-\Delta\Delta CT}$ analysis, the threshold cycle (CT; cycle number at which the expression exceeds threshold level) from control birds was used as a calibrator sample.

3.2.8 Keel bone damage, bone-breaking strength, and bone ash

At the end of each environmental period, eighteen hens per treatment (two random hens were selected in each replicate cage) were assessed for levels of keel bone damage (KBD). Keel bone scores were not determined during the REC1 regimen because the investigator was not available during that time. After all, consistency is important to limit variations from different investigators. The hens were palpated by the same investigator throughout the experiment, an observer who has extensive experience in poultry handling and carcass dissection techniques. Palpations were performed by running the thumb and index finger down the edge and length of the keel bone, feeling for alterations such as S-derivations, bumps or depressions, all indicators of keel bone damage. The keel was

assessed for fractures and deviations associated with KBD using a scoring scheme adapted from Scholz et al. (2008). For each damage type assessed, the scores were condensed into three categories showing increasing severity (Table 3.3). The severity of damage was assigned a numerical value between zero and two.

The frozen tibias and femurs were thawed and later stripped of soft tissue. Extraneous muscles were removed by hand and the bone cap removed. Subsequently, bone-breaking strength was measured using an Instron Materials tester (model 4301, Instron Corp., Canton, MA) at a loading rate of 40 mm/min. The average breaking strength of the left and right tibia and femur was reported as the breaking strength of the bone. To determine the ash content, the bones were further extracted in anhydrous ether for 72 h during which the ether was replaced every 24 hours for three consecutive days or until the ether becomes completely clear. After the extraction process, bones were dried at room temperature, under the hood, for 4 h after which they were placed in the oven at 105 °C for a minimum of 16 hours (overnight). The weight of an empty porcelain crucible and bones were determined before ashing in a muffle furnace overnight at 600 °C. The weight of each porcelain crucible and its content (ash) was thereafter determined. The percentage of ash was determined relative to the dry weight of the bones.

3.2.9 Calculations

$$\text{Hen-d egg production \%} = \frac{\text{Total number of eggs laid during the period}}{\text{Total number of hen-days in the same period}} \times 100 \quad \text{---------Eqn. 1}$$

where period represents the 5-wk period for each temperature regimen.

$$\text{Feed conversion ratio (per kg egg mass)} = \frac{\text{kg of feed consumed}}{\text{kg of egg produced}} \quad \text{----------Eqn. 2}$$

$$\text{Haugh Unit} = 100log\,[\text{H} + 7.57 - 1.7\text{W}^{0.37}] \quad \text{----------Eqn. 3}$$

where, H = albumen height in mm, W = weight of whole egg in gram (Eisen et al., 1962).

3.2.10 Statistical analysis

In this study, the dietary treatment was not analyzed across the temperature regimens because the same birds were used throughout the experiment and, there is a potential inherent carryover of temperature effect that would not be accounted for. As such, analysis of the dietary treatment was conducted within each temperature regimen. The experimental unit for the performance parameters (body weight, feed intake, feed conversion, egg production), egg quality parameters, was a replicate consisting of two adjacently caged laying hens (3 adjacent cages; top and bottom tier) fed as a group, with a total of 9 replicates. The experimental unit for the blood parameters, histology, gene expression was a single bird from 6 replicates rather than 9 replicates. Data were subjected to a two-way ANOVA using the GLM procedure of SAS 9.4 software (SAS Institute Inc., Cary, NC) for a completely randomized design. The model included a 2 x 2 factorial arrangement of treatments, were the main effects of inorganic sodium source, EconomasE™, and their interaction within each temperature-period was tested. The mean values were compared using Tukey and significance is based on a probability of < 0.05. To assess the occurrence of keel bone damage (curvature and fracture) and the relationship to the dietary modifications (EconomasE™ and two different sodium sources) within each temperature regimen, PROC FREQ of SAS 9.4 software (SAS Institute Inc., Cary, NC) was used. In total, a count of 72 birds (2 birds per cage) were used to determine the frequencies of keel bone curvature or fracture occurring. Fisher's exact test was used to determine the differences of the frequencies ($P < 0.05$).

3.3 RESULTS

The effect of dietary supplementation of EconomasE™ and two inorganic sodium sources at different environmental temperature regimens on different parameters are presented. The experimental temperature regimen are as follows; Phase 1 [TN1 (23.2℃; 26-30 weeks), ET1 (32.2℃; 33 – 37 weeks), REC1 (23.2℃; 38 – 42 weeks), and Phase 2 TN2 (23.2℃; 44 - 48 weeks), ET2 (32.2℃; 49 - 53 weeks), REC2 (23.2℃; 54 -58 weeks)].

The performance data are presented in Table 3.4. During the REC2 regimen, birds fed the diet without EcoE and NaCl+$NaHCO_3$, and with EcoE and NaCl as the source of inorganic sodium had a higher ($P < .0001$) body weight compared to the other combinations. Feed intake increased ($P < 0.05$) with NaCl+$NaHCO_3$ supplementation during TN1 and REC1. Feed intake and FCR decreased ($P < 0.05$) in birds fed the diet with NaCl as the only source of inorganic Na without EcoE supplementation compared to birds on a similar diet but with inorganic Na coming from a combination of NaCl and $NaHCO_3$ (Phase 2 TN2; Table 3.4). Feed conversion ratio increased ($P < 0.05$) in birds fed diets with NaCl+$NaHCO_3$ as the source of inorganic Na during the REC1 regimen. EconomasE™ supplementation improved ($P < 0.05$) HDEP during the REC2 period.

Egg quality parameters observed in this study include average egg weight, eggshell weight, eggshell breaking strength, albumen height, and Haugh unit (Table 3.5). An interaction effect was observed during the ET1 regimen where albumen height and Haugh unit improved ($P < 0.05$) in birds on diets supplemented with EcoE and NaCl+$NaHCO_3$ as the source of inorganic Na compared to birds on a similar diet without EcoE supplementation. Average egg weight decreased ($P < 0.05$) in birds fed the diet with EcoE supplementation and NaCl as the only source of inorganic Na during the REC1 temperature

regimen compared to those fed diets without EcoE supplementation and NaCl as the source of inorganic Na (Table 3.5). During the TN2 regimen, EcoE supplementation improved ($P < 0.05$) albumen height and Haugh unit (Table 3.5). The other egg quality parameters were not affected by the diet supplementation of EcoE and the two inorganic sodium sources.

The effects of the experimental treatments on laying hen blood chemistry are reported in Table 3.6. An interaction ($P = 0.015$) between the dietary supplementation of EcoE and the two different inorganic Na sources was observed for HCO_3^- during REC1. A combination of inorganic Na from NaCl and EcoE supplementation resulted in a reduced ($P < 0.05$) serum HCO_3^- compared to the other three treatments (REC1). Serum K^+ decreased during the REC2 with NaCl+$NaHCO_3$ in the diet. Serum Na and Cl levels were higher ($P < 0.050$) in birds on diets with inorganic Na from NaCl only (TN2) while serum HCO_3^- was increased ($P <0.001$) in birds with inorganic Na from a combination of NaCl and $NaHCO_3$ (ET2; Table 3.6). EconomasE™ supplementation increased ($P = 0.031$) serum Na level (ET2). Serum Na level was increased ($P < 0.05$) with a combination of NaCl and $NaHCO_3$ compared to birds on diets with NaCl as the only source of inorganic Na but was not different from that of birds on diet with NaCl as the only source of inorganic Na without EcoE supplementation. However, serum Cl level was higher ($P = 0.011$) in birds on the diet with only NaCl as the only source of inorganic Na without EcoE supplementation (REC2; Table 3.6).

Blood metabolites are reported in Table 3.7. An interaction effect observed was on the levels of LDH, glucose, Ca, and P in the blood. During the TN environmental temperature, LDH levels in the blood decreased ($P < 0.01$) in birds fed the diet supplemented with NaCl +$NaHCO_3$ and no EcoE compared to those that had EcoE in the

diet (Table 3.7). Serum Ca, P, and Albumin levels decreased ($P < 0.05$) and glucose levels increased ($P < 0.05$) in birds fed diets supplemented with EcoE (TN1; Table 3.7). Glucose levels, on the other hand, increased in birds fed the diet containing NaCl+$NaHCO_3$ as the only source of Na in the diet (TN1). In the REC1 regimen, the serum CK levels decreased ($P < 0.05$) in birds fed diets supplemented with EcoE. The EcoE supplementation in the diet increased ($P < 0.05$) P and alkaline phosphatase levels in the blood during the ET2 regimen. Furthermore, EcoE supplementation and inclusion of NaCl +$NaHCO_3$ in the diet as the only source of inorganic Na decreased ($P < 0.05$) Ca and P levels in the serum compared to the treatment during the REC2 regimen. While glucose levels increased ($P = 0.003$) and Ck serum levels decreased ($P < 0.05$) in the blood with the inclusion of NaCl +$NaHCO_3$ in the diet as the only source of inorganic Na during REC2.

Relative to the body weight, liver weight was not affected by environmental temperature and the diet supplementation of the different inorganic Na sources and EcoE (Table 3.8).

The jejunal morphology of birds subjected to different environmental temperatures and fed diets supplemented with or without EcoE and two different inorganic Na sources are presented in Table 3.9. The data showed that during the TN1 regimen, supplementation of EcoE and NaCl+$NaHCO_3$ as the only source of inorganic Na improved ($P < 0.05$) villus height and VH: CD ratio compared to the other 3 diet combinations. By phase 2, no significant effect was observed until the REC2 period where birds fed a diet supplemented with EcoE and NaCl+$NaHCO_3$ had a lower ($P < 0.05$) VH and CD compared to birds fed the diet without EcoE and NaCl+$NaHCO_3$ in the diet.

The occurrence of the keel bone curvature was low (P = 0.013) in the birds fed NaCl as the source of inorganic sodium during the TN1 temperature regimen. Eighty-three percent of the birds fed NaCl had no curvature, 14% had a slight curvature, and 3% had a severe curvature compared to the 53%, 44%, and 3% respectively, fed a diet containing NaCl+$NaHCO_3$ (Table 3.10).

We observed no interaction effect of EcoE supplementation and the two inorganic sodium sources on bone breaking strength except during the REC1 regimen where birds fed diets with EcoE and NaCl as the only source of Na had a decrease in femur breaking strength compared to birds fed diets without EcoE supplementation and NaCl. During the REC2 regimen, EcoE supplementation, improved (P < 0.05) femur breaking strength (5.4%; Table 3.11). On the other hand, birds fed a diet with NaCl+$NaHCO_3$ as the only source of inorganic Na had a decrease (P < 0.05) in femur and tibia breaking strength (Table 3.11). Percent tibia ash was improved (P = 0.02) by EcoE supplementation during the ET1 period. Similarly, NaCl+$NaHCO_3$ as the only source of inorganic Na resulted in a higher (P = 0.017) percent tibia ash (ET1; Table 12). During the REC2, regimen, EcoE supplementation improved (3.9%; P = 0.002) percent ash of the tibia (Table 3.12).

The mRNA expression of HSP 70 and 90 in the jejunum was not influenced by EcoE supplementation and the two inorganic Na source during each temperature period (Table 3.13).

3.4 DISCUSSION

It has been inferred that the decline in egg production and egg weight is more influenced by the reduction in feed consumption, while eggshell and overall egg quality is influenced primarily by high temperature (Balnave and Muheereza, 1997; Mashaly et al.,

2004). Dietary inclusion of a combination of NaCl and $NaHCO_3$ as the source of inorganic Na significantly increased FI during the TN1 and REC1 regimen and FCR during the REC1 regimen. Performance results have shown that the addition of 1.0% of $NaHCO_3$ to diets fed to broilers subjected to elevated temperatures from 34 to 36 ºC led to a trend of improved feed consumption, weight gain, and feed conversion (March, 1984). Similar to Ahmad et al. (2005) report, we did not observe any significant difference with NaCl+ $NaHCO_3$ in the diet for FI and FCR during the ET1 and ET2 regimen, however, Borges et al. (2003) reported an increase in FI and FCR with $NaHCO_3$ and NaCl as the Na sources under heat stress conditions. Consequently, body weight was not affected during the different environmental temperature regimens except during the REC2 period where body weight increased in birds fed a diet supplemented with EcoE and NaCl as the source of inorganic Na in the diet. Although, during the same temperature regimen (REC2), the bodyweight of hens on diets supplemented with EcoE with a combination of NaCl and $NaHCO_3$ as sources of inorganic Na was lower compared to the hens fed a diet without EcoE supplementation with a combination of NaCl and $NaHCO_3$.

In thermoneutral temperature, Borges et al. (2003) reported an increase in body weight gain of broilers with a DEB of 240. Similarly, Ahmad et al. (2005) reported that $NaHCO_3$, Na_2CO_3, and Na_2SO_4 increased body weight gain after 42 days of age. Under elevated temperature, Hassan et al. (2011) suggested that the higher body weight gain observed in birds fed diet containing electrolytes such as $NaHCO_3$ and KCl can be attributed to the partial correction in acid-base balance which acts as a heat sink and resulted in a better metabolism in the hens. Hen-d egg production was not impacted by the two different inorganic Na sources however, during REC1, HDEP decreased with EcoE

supplementation but increased during REC2. Numerous studies have documented the beneficial effects of VE (Whitehead et al., 1998; Puthpongsiriporn et al., 2001) supplementation on egg production and egg quality in heat-stressed poultry. Panda et al. (2008) reported that supplementing VE in the diet influenced egg production and feed efficiency, and by increasing the level of VE from 25 to 125 IU/kg diet, egg production and feed conversion efficiency were significantly improved.

Egg quality parameters, excluding albumen height, Haugh unit, and average egg weight were affected by the dietary treatments during the different environmental temperature regimens. El-Gammal and Makled (1977) reported that by replacing NaCl (0.67%) with $NaHCO_3$ (1%), egg production increased by 6%. However, no change in egg production was reported in hens supplemented with 1% $NaHCO_3$ during peak period but eggshell weight increased (Balnave and Muheereza, 1997; Grizzle et al., 1992). Hassan et al. (2011) reported the beneficial effects of $NaHCO_3$ in the diet on different egg production traits (egg number, egg weight). Jiang et al. (2015) reported the benefit of supplementing $NaHCO_3$ in laying hens' diet on eggshell breaking strength. In this study, a decrease in the albumen height and Haugh unit during the ET1 regimen in birds fed the diet supplemented without EcoE and with the inclusion of NaCl and $NaHCO_3$ as the inorganic Na sources, was observed. Ghorbani and Fayazi (2009) indicated that the Haugh unit, albumen index, yolk index shell strength, shell weight, were not significantly affected by the addition of dietary $NaHCO_3$. Sahin et al. (2002) reported that VE supplementation positively influenced, egg weight, egg specific gravity, eggshell thickness, and Haugh unit which demonstrates the beneficial effect of EcoE supplementation with NaCl+ $NaHCO_3$ on albumen height and Haugh unit that was observed in this study. This is beneficial to the

industry because albumen quality is a very important parameter in the raw egg consumption market. Both albumen height and Haugh unit are used as a global indicator of egg freshness. Eggs with higher albumen and greater Haugh unit values can be stored for a long time while still maintaining their fresh appearance when used by the consumer.

Although we did not analyze across the different environmental temperatures to determine their effect on blood metabolites and electrolytes, studies have demonstrated that birds subjected to elevated temperatures can express respiratory alkalosis and a decrease in plasma levels of Ca, Na, P, and Mg (Kohne and Jones, 1975; Bogin et al., 1981). On the other hand, Koelkebeck and Odom (1995) reported that exposing laying hens to elevated temperature 38 °C did not affect blood plasma glucose, alkaline phosphatase, total protein, uric acid, and creatinine. The increase in plasma Cl^- and reduction of Na^+ and K^+ levels as a function of heat stress has been well documented (Belay and Teeter, 1993). In the current study, Cl^- levels were significantly reduced during ET1, REC1, and TN2 in birds fed diets with inorganic Na from both NaCl and $NaHCO_3$. Cohen and Hurwitz (1974) suggested that one of the effects of dietary supplementation with one of the ionic components is the increase in its concentration in plasma. Ahmad et al. (2005) also reported that blood Na^+, K^+, and Cl^- levels were a direct response to the respective supplemented minerals. This result indicates that the elevated levels of Cl^- during HS because of respiratory alkalosis can be counteracted by supplementing with salts containing less Cl^-. Though the decrease in Na^+ levels with NaCl+ $NaHCO_3$ during the TN2 failed to follow this logic, the increase in Na levels during the REC2 period in birds fed diets supplemented with NaCl+ $NaHCO_3$ and EcoE further supports this. Moreover, HCO_3^- levels were increased with NaCl+ $NaHCO_3$ supplementation during ET2 which according to Junqueira et al. (1984)

and Gorman and Balnave (1994), heat stress may induce a metabolic requirement for the HCO_3^-, and dietary supplements of both bicarbonate and carbonate salts may be considered as a substrate for HCO_3^-. This means the supplementation of $NaHCO_3$ in the diet might counteract the need for HCO_3^- associated with elevated temperature.

Maintenance of normal microarchitecture in the small intestine is very important for the proper growth and development of the bird. Several reports have documented the effect of HS on intestinal morphology including a decrease in VH (Mitchell and Carlisle, 1992; Sohail et al., 2012), CD (Burkholder et al., 2008; Sohail et al., 2012), and VH: CD while, others have reported no effect (Quinteiro-Filho et al., 2010, 2012). In this study, before exposing the birds to the ET, we observed an increase in VH and VH: CD ratio in birds fed diets supplemented with EcoE and NaCl+ $NaHCO_3$. However, at the end of the experiment during the REC2 regimen, this interaction effect negatively affected VH and CD. Since there is a potential for an imbalance between pro- and antioxidant systems in animals leading to oxidative stress, EcoE an antioxidant is intended to confer protection limiting oxidative damage in the intestinal tract. This study did not support that hypothesis during the REC2 regimen. So probably, EcoE with a combination of NaCl and $NaHCO_3$ can improve the intestinal tract environment when conditions are favorable, as evidenced by the increased villus height observed in birds fed diets inclusion during TN1. However, in unfavorable conditions (elevated temperature) or after unfavorable conditions (recovery period), the beneficial effects are limited and an increase in the level supplemented might be advantageous.

It has been shown that high temperatures (above the thermoneutral zone) have a negative effect on feed intake and body mass growth in poultry (Deaton et al., 1978; Deaton

et al., 1984) which, can result in the deficits of nutrients in the body that participate in bone building. Bone-breaking strength, bone ash, and bone mineral retention are criteria for assessing bone quality. Similarly, the relationship between bone strength and the overall prevalence of keel bone deformities is complicated by competing influences. The current opinion appears to be that deviations/deformities to the keel bone, to a large extent, are due to perching behavior in combination with hard, thin perches whereas fractures are the result of impact collisions with housing structures (Thofner et al., 2020). Layer fatigue or poor bone health have also been suggested to be contributing factors to keel bone fractures as well as the genetics of the bird, lack of specific feedstuff components, and high egg production (Fleming et al., 2004; Casey-Trott et al., 2017). Siegel et al. (1973) reported that thermal stress could reduce bone mass and the bones' mechanical strength. Moreover, the bone strength of caged layers is said to significantly correlate with the percentage of bone ash (Rowland et al., 1968). No significant difference was observed with NaCl+ $NaHCO_3$ during the ET1 and ET2 regimen in this study. However, during the REC2 regimen, birds fed diets supplemented with NaCl+ $NaHCO_3$ had a decrease in both the tibia and femur breaking strength. This is unlike the results Ferguson et al. (1974) reported where no difference was observed in layers fed diets supplemented with $NaHCO_3$. EconomasE™ supplementation on the other hand improved the femur breaking strength during the REC2 regimen. The progressive loss of bone strength in hens starts early but continues throughout the production period with no time to recuperate (Wilson et al., 1992) causing osteoporosis to be most severe at the end of the laying period (Whitehead and Fleming, 2000). Hence, even though NaCl+ $NaHCO_3$ did not improve the bone-breaking strength and the decrease in bone breaking strength might be due to the age of the birds.

Murakami et al. (1997a, b) demonstrated experimentally that disorders in mineralization processes, manifested as reduced bone ash content, resulted from an increased amount of Na in chickens' diet, while a higher dietary content of chlorine counteracted this effect. Percent ash improved in birds fed diets supplemented with either EcoE or with NaCl+ $NaHCO_3$ during the ET1 regimen and a further increase during REC2 (EcoE). In Moghaddam et al. (2005) study, the tibia ash of pullets fed different dietary electrolyte levels (187, 230, 251, and 284 mEq Kg-1) was not significantly different. Keel bone damage was not observed during any of the environmental temperature periods except for the TN1 regimen where supplementation of NaCl+ $NaHCO_3$ in the diet increased the chances of keel bone deviation. In concert, the severity of deviation of the keel bone was reduced with NaCl+ $NaHCO_3$ and no EcoE in the diet which does not explain the tibia and femur breaking strength results where NaCl+ $NaHCO_3$ in the diet reduced the breaking strength.

Furthermore, cellular exposure to thermal stress induces several anomalies in the functioning of cells which ultimately alters the biological molecules. Heat shock proteins (HSPs) mediate important endogenous protective mechanisms to assist acclimatization to change environments and protect against various stressors such as heat, cold, bacteria, viruses, and UV (Garrido et al., 2001; Panda et al., 2008) Also, HSP is one of the cellular proteins found most abundantly under non-stress conditions. The expression of HSPs provides protection against hyperthermia, circulatory shock, and cerebral ischemia during heatstroke which signifies the ability of cells to resist damage and adapt to environmental stress (Garrido et al., 2001; Xu et al., 2017). Of the HSP proteins known, HSP70 is the most abundant and temperature-sensitive of the HSPs and can be activated by numerous

physical and physiological stressors (Al-Aqil and Zulkifli, 2009; Zulkifli et al., 2009). Also, HS has been shown to induce HSP90, which can help the proteins mostly associated with cellular pro-survival/anti-apoptotic signal transduction pathways to maintain their correct molecular structure (Chrisostomos et al., 2000; Wang and Edens, 1998). Gu et al. (2012) reported an increase in HSP70 at 2 h and 3 h after birds were subjected to an elevated temperature at 35 ℃. Similarly, Pearce et al. (2013) reported that HS in pigs resulted in an elevated ileal mucosa HSP70. During each of the environmental temperature conditions, the supplementation of EcoE or the two sodium sources did not affect the activities of HSP 70 and 90. Although, dietary supplementation with VE is beneficial against oxidative stress and the production of ROS. The production of HSP induced by HS has a close relationship with the generation of ROS. Panda et al. (2008) reported an increase in the activities of glutathione reductase, an enzymatic antioxidant that play a vital role in scavenging oxidative radicals. Yin et al. (2018) reported that in vitro, pre-treatment with vitamin C-Na for 16 h induced basal expression of HSP70 upon heat stress.

In conclusion, the supplementation of NaCl+ $NaHCO_3$ yielded comparable results in terms of feed intake and feed efficiency with the inclusion of NaCl+EcoE in the diet. While we see a decrease in egg quality parameters during the ET1 and ET2 regimen, the diet did not improve these parameters except in the albumen height and Haugh unit. Overall, NaCl+ $NaHCO_3$ helped circumvent the effects of respiratory alkalosis by reducing Cl^- levels during ET and increase $Na+$ and HCO_3^- levels during the REC1 regimen. Also, the beneficial effect of EcoE with NaCl+ $NaHCO_3$ was observed during the TN1 regimen on VH and VH: CD. Bone breaking strength, on the other hand, was not improved with

NaCl+ $NaHCO_3$ supplementation however, percent tibia ash was improved by both EcoE and $NaHCO_3$.

3.5 TABLES

Table 3.1 Ingredient and nutrient composition of the experimental diets (on an as-fed basis).

Ingredients, %	A NaCl	B NaCl + $NaHCO_3$	C NaCl + EconomasE™	D NaCl + $NaHCO_3$ + EconomasE™
Corn	56.0	55.9	56.0	55.9
Soybean meal (48% CP)	28.0	28.0	28.0	28.0
Soybean oil	3.75	3.75	3.75	3.75
Limestone (38% Ca)	7.20	7.20	7.20	7.20
Dicalcium phosphate	1.00	1.00	1.00	1.00
NaCl	0.38	0.18	0.38	0.18
$NaHCO_3$	0.0	0.30	0.0	0.30
DL- methionine	0.17	0.17	0.17	0.17
Oyster shell	3.00	3.00	3.00	3.00
Vitamin premix (no mineral)[1]	0.25	0.25	0.25	0.25
Mineral premix[2]	0.25	0.25	-	-
Mineral premix[3]	-	-	0.25	0.25
EconomasE™ premix[4]	-	-	0.02	0.02
Total	100.0	100.0	100.0	100.0
Analyzed nutrients and energy (%)				
Crude protein	17.36	17.73		
Crude fat	6.27	5.87		
Fiber	6.01	4.56		
Ash	13.57	15.19		
Calcium	4.26	4.92		
Total phosphorus	0.53	0.58		
Metabolizable energy[5], kcal/kg	2,874	2,871		
Non-phytate P[4]	0.31	0.31		
Ca: tP[5]	0.79	0.79		
Sodium[5]	0.20	0.20		
Chlorine[5]	0.40	0.28		
Dietary electrolyte balance (DEB)[6], mEq/kg	183.59	217.65		

[1]Supplied per kg of diet: vitamin A, 9921 IU; vitamin D3, 2756 ICU; vitamin E, 33 IU, vitamin B12, 22.0 µg; vitamin K (as menadione), 1.98 mg; riboflavin, 6.6 mg; d-pantothenic acid, 11 mg; thiamine, 1.98 mg; niacin, 44 mg; vitamin B6, 3.97 mg; folic acid, 1.32 mg; choline, 496 mg; biotin, 0.11 mg.

[2]Minerals supplied per kg of diet: Selenium, 0.2 mg; Copper, 10.0 mg; Iodine, 3.0 mg; Iron, 79.8 mg; Manganese, 79.9 mg; Zn, 80.0 mg.

[3]Minerals supplied per kg of diet: ; Copper, 10.0 mg; Iodine, 3.0 mg; Iron, 79.8 mg; Manganese, 79.9 mg; Zn, 80.0 mg.

[4]Economase™ premix was added to diets C and D at the expense of corn to supply 0.2 g of EconomasE™/kg of diet.

[5]Calculated values.

[6]Electrolyte balance was calculated as Na+K-Cl in mEq/kg.

Table 3.2 Summary of measurements taken and sampling intervals

Collection interval	Parameter
[1]Biweekly	Egg weight (g)
	Eggshell weight (g)
	Yolk weight (g)
	Albumen height; calculated Haugh units
	Yolk color
[2]End of each period	Bodyweight (g)
	Feed intake (g); calculated Feed conversion ratio (g)
	Egg production (%)
	Blood metabolites (alkaline phosphatase, creatine kinase, lactose dehydrogenase, albumin, calcium, phosphorus, glucose, and electrolytes (Na^+, K^+, HCO_3^-, and Cl^-).

[1]Biweekly: Two consecutive days in the 1st, 3rd, and 5th week of each period
[2]TN1 (23.2℃; 26-30 weeks), ET1 (32.2℃; 33 – 37 weeks), REC1 (23.2℃; 38 – 42 weeks), TN2 (23.2℃; 44 - 48 weeks), ET2 (32.2℃; 49 - 53 weeks), REC2 (23.2℃; 54 -58 weeks).

Table 3.3 Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimens on performance variables in laying hens[1].

Environmental Temperature[2]	Performance[3]	Main effect				Interaction effect				SD[4]	P-value		
		EconomasE™		Sodium source		No EconomasE™		EconomasE™			EconomasE™	Sodium source	EconomasE™ x Sodium source
		No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$				
TN1 (23.8 ℃)	Bodyweight (kg)	1.98	1.97	1.97	1.97	1.99	1.97^{x}	1.96	1.98	0.05	0.722	0.925	0.283
26 – 30 wk	Feed intake(g/hen/d)	104.2	107.4	103.8	107.8	103.0	105.3	104.5	110.2^{x}	6.08	0.130	0.059	0.412
	FCR	1.78	1.81	1.77	1.82	1.76	1.79	1.77^{x}	1.85	0.10	0.358	0.127	0.501
	HDEP (%)	97.2	97.7	97.5	97.3	97.9^{x}	96.5^{x}	97.1	98.2^{x}	2.09	0.521	0.794	0.094
ET1 (32.2 ℃)	Bodyweight (kg)	1.93	1.91	1.92	1.93	1.92	1.95^{x}	1.91^{x}	1.91	0.04	0.157	0.437	0.245
33 – 37 wk	Feed intake (g/hen/d)	102.3	101.8	101.2	103.0	101.8^{x}	102.9	100.6	103.1	2.88	0.602	0.070	0.512
	FCR	1.74	1.74	1.73	1.75	1.73^{y}	1.76	1.74	1.74^{x}	0.04	0.744	0.131	0.310
	HDEP (%)	96.1	95.8	96.4	95.5	97.2^{x}	95.0	95.7	96.0	2.49	0.766	0.306	0.150
REC1 (23.8 ℃)	Bodyweight (kg)	1.96	1.94	1.94	1.96	1.95	1.97	1.93	1.95	0.06	0.473	0.331	0.877
38 – 42 wk	Feed intake (g/hen/d)	100.4	99.8	98.8	101.4	98.7	102.1	98.90	100.6	3.88	0.652	0.057	0.508
	FCR	1.70	1.70	1.68	1.72	1.66	1.74	1.69	1.70	0.07	1.000	0.056	0.103
	HDEP (%)	92.9	90.1	91.1	92.0	92.4^{x}	93.4^{x}	89.7	90.6	2.79	0.007	0.350	0.961
TN2 (23.8 ℃)	Bodyweight (kg)	2.03	2.03	2.01	2.05	2.00^{x}	2.06	2.03	2.00^{x}	0.07	0.884	0.194	0.331
44 - 48 wk	Feed intake (g/hen/d)	102.9	103.2	102.0	104.1	100.3$^{b, x}$	105.4^{a}	103.6^{ab}	102.8$^{ab, x}$	3.67	0.778	0.096	0.027
	FCR	1.72	1.71	1.71	1.72	1.69^{b}	1.75^{a}	1.72^{ab}	1.70$^{b, x}$	0.05	0.483	0.298	0.014
	HDEP (%)	89.7	89.6	89.7	89.5	90.8^{x}	88.6^{x}	88.7	90.4^{x}	3.31	0.937	0.830	0.105
ET2 (32.2 ℃)	Bodyweight (kg)	1.93	1.92	1.92	1.93	1.90	1.95	1.93	1.90	0.08	0.720	0.816	0.130
49 - 53 wk	Feed intake(g/hen/d)	95.5	96.0	94.7	96.8	94.0	97.1	95.5	96.5	5.48	0.793	0.273	0.565
	FCR	1.61	1.62	1.61	1.62	1.60	1.62^{x}	1.62	1.62	0.09	0.613	0.703	0.758
	HDEP (%)	83.9	84.3	84.0	84.2	84.7	83.0	83.3	85.3	4.49	0.791	0.916	0.222
REC2 (23.8 ℃)	Bodyweight (kg)	2.01	2.03	2.01	2.03	1.95^{b}	2.07$^{a, x}$	2.06$^{a, x}$	1.99$^{b, x}$	0.05	0.437	0.271	<.0001
54 - 58 wk	Feed intake(g/hen/d)	110.1	110.5	108	112.6	107.3	112.9	108.7	112.2	9.05	0.911	0.142	0.722
	FCR	1.93	1.81	1.79	1.86	1.79	1.88	1.78	1.84	0.15	0.677	0.154	0.826
	HDEP (%)	84.9	88.0	86.8	86.1	85.7	84.1^{x}	88.0^{x}	88.0^{x}	4.19	0.041	0.599	0.577

$^{a-b}$ Means within environmental temperature lacking a common superscript are different ($P < 0.05$).

[1]Mean values represent means of 9 replicate cages per treatment except for mean values with x where the number of replicates was 8. Hens were subjected to environmental temperature for 5 consecutive weeks.

[2]Environmental temperature: (Phase 1 and 2) TN= Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature

[3]FCR- Feed conversion ratio; HDEP- hen-d egg production

[4]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$.

Table 3.4 Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimens on egg quality in laying hens[1].

Environmental Temperature[2]	Egg quality	Main effect				Interaction effect				SEM	P-value		
		EconomasE™		Sodium source		No EconomasE™		EconomasE™			EconomasE™	Sodium source	EconomasE™ x Sodium source
		No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$				
TN1 (23.8 ℃) 26 – 30 wk	Average egg weight (g)	58.6	58.5	58.2	58.8	58.5	58.8	58.0	58.9	0.31	0.697	0.185	0.507
	Eggshell weight (%)	9.78	9.79	9.78	9.79	9.80	9.77	9.77	9.82	0.04	0.858	0.858	0.476
	Eggshell breaking strength (kg/cm^2)	4.24	4.27	4.25	4.26	4.23	4.25	4.27	4.27	0.04	0.662	0.793	0.861
	Albumen height (mm)	8.70	8.93	8.81	8.82	8.66	8.74	8.95	8.90	0.09	0.080	0.886	0.612
	Haugh unit	93.3	94.2	93.7	93.8	93.1	93.5	94.3	94.2	0.37	0.082	0.818	0.660
ET1 (32.2 ℃) 33 – 37 wk	Average egg weight (g)	58.5	58.3	58.2	58.5	58.5	58.4	57.9	58.7	0.38	0.687	0.391	0.331
	Eggshell weight (%)	9.74	9.77	9.76	9.8	9.71	9.77	9.81	9.73	0.07	0.631	0.873	0.339
	Eggshell breaking strength (kg/cm^2)	3.64	3.68	3.66	3.66	3.60	3.67	3.72	3.64	0.06	0.483	1.000	0.299
	Albumen height (mm)	8.08	8.1	8.17	8.04	8.24^{a}	7.91^{b}	8.09^{ab}	8.17^{a}	0.07	0.476	0.084	0.005
	Haugh unit	90.2	90.4	90.6	90.0	91.0^{a}	89.3^{b}	90.3^{ab}	90.6^{a}	0.38	0.487	0.099	0.012
REC1 (23.8 ℃) 38 – 42 wk	Average egg weight (g)	59.2	58.8	59.0	59.0	59.6^{a}	58.8^{ab}	58.4^{b}	59.2^{ab}	0.36	0.262	0.938	0.035
	Eggshell weight (%)	9.72	9.82	9.75	9.79	9.74	9.70	9.76	9.88	0.06	0.150	0.548	0.202
	Eggshell breaking strength (kg/cm^2)	3.56	3.67	3.61	3.62	3.59	3.53	3.65	3.68	0.06	0.072	0.892	0.450
	Albumen height (mm)	7.89	7.92	7.96	7.86	7.98	7.82	7.94	7.90	0.07	0.771	0.152	0.412
	Haugh unit	89.1	89.3	89.5	88.9	89.56	88.67	89.51	89.08	0.40	0.649	0.107	0.572

[a-b] Means within environmental temperature lacking a common superscript are different ($P < 0.05$).

[1]Values are expressed as mean ± SEM. Hens were subjected to each environmental temperature for 5 consecutive weeks.

[2]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature

Table 3.4 contd. Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimens on egg quality in laying hens[1].

Environmental Temperature[2]	Egg quality	Main effect				Interaction effect				SEM	P-value		
		EconomasE™		Sodium source		No EconomasE™		EconomasE™			EconomasE™	Sodium source	EconomasE™ x Sodium source
		No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$				
TN2 (23.8 ℃)	Average egg weight (g)	60.0	60.4	60.0	60.4	59.8	60.2	60.1	60.6	0.41	0.375	0.348	0.905
44 - 48 wk	Eggshell weight (%)	10.07	10.06	10.08	10.04	10.08	10.06	10.08	10.03	0.07	0.867	0.642	0.876
	Eggshell breaking strength (kg/cm^2)	3.69	3.76	3.74	3.70	3.69	3.70	3.79	3.70	0.05	0.291	0.449	0.321
	Albumen height (mm)	7.92	8.14	8.07	7.99	7.99	7.84	8.15	8.13	0.09	0.016	0.350	0.460
	Haugh unit	89.0	90.1	89.8	89.3	89.4	88.5	90.2	90.1	0.50	0.026	0.314	0.468
ET2 (32.2 ℃)	Average egg weight (g)	58.9	59.3	59.0	59.3	58.9	59.0	59.0	59.6	0.41	0.425	0.440	0.507
49 - 53 wk	Eggshell weight (%)	9.55	9.57	9.59	9.52	9.62	9.48	9.48	9.57	0.07	0.820	0.326	0.326
	Eggshell breaking strength (kg/cm^2)	3.21	3.29	3.23	3.26	3.16	3.26	3.31	3.27	0.07	0.257	0.655	0.284
	Albumen height (mm)	7.52	7.63	7.62	7.53	7.65	7.38	7.58	7.68	0.10	0.242	0.372	0.066
	Haugh unit	86.6	87.3	87.3	86.6	87.5	85.7	87.2	87.5	0.59	0.213	0.219	0.073
REC2 (23.8 ℃)	Average egg weight (g)	60.1	60.9	60.5	60.5	59.9	59.9	61.0	60.9	0.46	0.063	0.876	0.640
54 - 58 wk	Eggshell weight (%)	10.07	10.01	10.06	10.02	10.16	9.98	9.97	10.06	0.08	0.492	0.582	0.105
	Eggshell breaking strength (kg/cm^2)	3.64	3.63	3.69	3.57	3.72	3.56	3.67	3.58	0.70	0.844	0.099	0.633
	Albumen height (mm)	7.37	7.46	7.46	7.37	7.47	7.27	7.44	7.48	0.12	0.480	0.497	0.325
	Haugh unit	85.6	86.0	86.1	85.5	86.4	84.9	85.8	86.2	0.76	0.649	0.466	0.254

[1]Hens were subjected to each environmental temperature for 5 consecutive weeks.
[2]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature.

Table 3.5 Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimen on blood electrolytes in laying hens[1].

Environmental Temperature[2]	Blood electrolytes[3]	Main effect				Interaction effect				SD[4]	P-value		
		EconomasE™		Sodium source		No EconomasE™		EconomasE™			EconomasE™	Sodium source	EconomasE™ x Sodium source
		No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$				
TN1 (23.8 °C)	HCO_3^- (mmol/L)	21.8	21.7	21.2	22.2	21.6^{x}	21.7	20.9	22.6	1.41	0.889	0.131	0.192
26 – 30 wk	Na (mmol/L)	157.5	157.73	157.8	157.4	156.8	158.2^{x}	158.8^{x}	156.7	2.13	0.815	0.679	0.071
	K (mmol/L)	4.83	4.90	5.01	4.73	4.83	4.83	5.18^{x}	4.62^{x}	0.51	0.763	0.215	0.215
	Cl (mmol/L)	113.6	114	114.5	113.1	113.6^{x}	113.5	115.4^{x}	112.7	1.84	0.548	0.090	0.113
ET1 (32.2 °C)	HCO_3^- (mmol/L)	19.5	19.8	19.1	20.2	18.8^{x}	20.2^{x}	19.4^{x}	20.2^{x}	1.36	0.629	0.089	0.629
33 – 37 wk	Na (mmol/L)	151.3	152.8	152.9	151.2	153.3	149.2^{x}	152.5	153.2	2.89	0.210	0.168	0.062
	K (mmol/L)	4.26	4.46	4.30	4.42	4.16^{x}	4.36^{x}	4.43	4.48^{x}	0.38	0.253	0.468	0.650
	Cl (mmol/L)	115.8	117.5	118.8	114.5	119.0	112.6^{x}	118.7	116.3	2.90	0.177	0.002	0.110
REC1 (23.8 °C)	HCO_3^- (mmol/L)	23.3	21.5	21.6	23.3	23.7$^{a, x}$	23.0^{a}	19.5$^{b, x}$	23.5^{a}	1.99	0.048	0.070	0.015
38 – 42 wk	Na (mmol/L)	152.7	154.2	153.2	153.8	151.8^{x}	153.7^{x}	154.6^{x}	153.8^{x}	3.33	0.313	0.705	0.369
	K (mmol/L)	4.29	4.37	4.66	4.00	4.67^{x}	3.92	4.66^{x}	4.08^{x}	0.33	0.607	<.0001	0.577
	Cl (mmol/L)	114.2	116.3	117.0	113.5	116.0	112.4^{x}	118.0	114.6^{x}	2.21	0.050	0.003	0.921

[a-b] Means within environmental temperature lacking a common superscript are different ($P < 0.05$).

[1]Mean values represent means of 6 replicate cages per treatment except for mean values with superscript x where the number of replicates was 5. Hens were subjected to each environmental temperature for 5 consecutive weeks.

[2]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature.

[3]Blood electrolytes: HCO_3^- = bicarbonate; Na = Sodium; K = Potassium; Cl = Chloride

[4]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 3.5 contd. Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimen on blood electrolytes in laying hens[1].

Environmental Temperature[2]	[3]Blood electrolytes	Main effect				Interaction effect				SD[4]	P-value		
		EconomasE™		Sodium source		No EconomasE™		EconomasE™			EconomasE™	Sodium source	EconomasE™ x Sodium source
		No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$				
TN2 (23.8 ℃)	HCO_3^- (mmol/L)	22.1	20.5	21.6	21.0	22.6^{x}	21.7	20.6^{x}	20.3	4.23	0.370	0.745	0.865
44 - 48 wk	Na (mmol/L)	154.7	155.1	156.2	153.6	155.2^{x}	154.2^{x}	157.2^{x}	153.0^{x}	2.04	0.667	0.012	0.098
	K (mmol/L)	4.68	4.30	4.67	4.31	4.94^{x}	4.42	4.40^{x}	4.20^{x}	0.53	0.119	0.135	0.493
	Cl (mmol/L)	116.8	116.4	118.2	115.0	118.3	115.2^{x}	118.0	114.8^{x}	2.07	0.684	0.002	0.970
ET2 (32.2 ℃)	HCO_3^- (mmol/L)	22.3	21.9	20.4	23.8	20.3	24.2^{x}	20.4^{x}	23.4^{x}	1.47	0.575	<.0001	0.509
49 - 53 wk	Na (mmol/L)	152.4	154.3	153.7	153	152.2^{x}	152.7	155.2^{x}	153.4^{x}	1.82	0.031	0.414	0.172
	K (mmol/L)	4.73	4.94	4.92	4.75	4.72^{x}	4.74^{x}	5.12^{x}	4.76^{x}	0.37	0.224	0.321	0.269
	Cl (mmol/L)	117	117.7	117.9	116.8	117.4^{x}	116.7	118.3	117.0^{x}	2.64	0.563	0.374	0.794
REC2 (23.8 ℃)	HCO_3^- (mmol/L)	27.1	25.2	25.4	26.9	24.9	29.2^{x}	25.8^{x}	24.5	4.49	0.332	0.450	0.165
54 - 58 wk	Na (mmol/L)	153.3	153.4	153.0	153.8	154.7^{ab}	152.0^{bc}	151.3^{c}	155.5^{a}	2.33	0.931	0.439	0.002
	K (mmol/L)	4.90	4.81	4.96	4.75	4.98	4.82^{x}	4.94^{x}	4.68	0.39	0.599	0.228	0.785
	Cl (mmol/L)	116.0	115.4	116.2	115.2	117.8^{a}	114.2^{b}	114.6$^{b, x}$	116.2^{ab}	2.22	0.515	0.272	0.011

[a-c] Means within environmental temperature lacking a common superscript are different ($P < 0.05$).

[1]Mean values represent means of 6 replicate cages per treatment except for mean values with superscript x where the number of replicates was 5. Hens were subjected to each environmental temperature for 5 consecutive weeks for each temperature.

[2]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature.

[3]Blood electrolytes: HCO_3^- = bicarbonate; Na = Sodium; K = Potassium; Cl = Chloride

[4]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 3.6 Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimen on blood metabolites in laying hens[1].

Environmental Temperature[2]	Blood metabolites[3]	Main effect				Interaction effect				SD[4]	P-value		
		EconomasE™		Sodium source		No EconomasE™		EconomasE™					
		No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$		EconomasE™	Sodium source	EconomasE™ x Sodium source
TN1 (23.8 ℃)	Ca (mg/dL)	28.2	25.9	27.4	26.7	27.7^{x}	28.6	27.1^{x}	24.8	2.42	0.043	0.498	0.146
26 – 30 wk	Phos (mg/dL)	6.15	4.85	5.57	5.43	6.10	6.20	5.04^{x}	4.66^{x}	0.94	0.005	0.733	0.560
	Glucose (mg/dL)	247.7	260.8	244.6	263.9	240.6^{x}	254.8^{x}	248.7	273.0^{x}	9.17	0.005	0.0002	0.224
	Albumin (g/dL)	1.78	1.65	1.73	1.70	1.83	1.72^{x}	1.62^{x}	1.68	0.13	0.041	0.666	0.138
	AlkPhos (U/L)	333.3	322.5	344.5	311.3	343.0^{x}	323.6^{x}	346.0^{y}	299.0^{x}	114.2	0.840	0.538	0.797
	CK (U/L)	1280.1	1217.8	1237.6	1260.3	1223.6^{x}	1336.5	1251.7	1184^{x}	235.9	0.546	0.825	0.383
	LDH (U/L)	485.1	363.8	392.9	460	342.8^{ab}	627.4$^{a, y}$	443.0$^{ab, x}$	284.6$^{b, x}$	171.6	0.137	0.428	0.011
ET1 (32.2 ℃)	Ca (mg/dL)	26.5	27.8	25.9	28.2	24.0	28.9^{x}	28.0^{x}	27.6	5.01	0.556	0.309	0.232
33 – 37 wk	Phos. (mg/dL)	5.91	5.50	5.19	6.22	5.22^{x}	6.60^{x}	5.16^{x}	5.83	2.00	0.643	0.258	0.692
	Glucose (mg/dL)	227.5	221.9	225.3	224.2	233.7	221.3	216.8	227.0^{x}	20.1	0.514	0.899	0.196
	Albumin (g/dL)	1.62	1.68	1.64	1.60	1.60	1.64^{x}	1.68	1.68^{x}	0.17	0.412	0.806	0.771
	AlkPhos (U/L)	546.4	596.5	631.1	511.8	584.5^{y}	508.2^{x}	677.6^{x}	515.4^{x}	232.1	0.646	0.283	0.694
	CK (U/L)	1206.8	1293.0	1266.0	1233.8	1311.0^{x}	1102.6^{x}	1221.0^{x}	1365.0^{x}	225.3	0.405	0.753	0.099
	LDH (U/L)	723.6	491.6	568.7	646.6	655.5^{x}	791.7	481.8	501.4^{x}	358.3	0.148	0.618	0.708
REC1 (23.8 ℃)	Ca (mg/dL)	27.1	26.2	27.1	26.2	27.5^{x}	26.7^{x}	26.6^{x}	25.7^{x}	2.79	0.467	0.486	0.969
38 – 42 wk	Phos. (mg/dL)	5.16	5.12	5.15	5.12	5.18	5.13^{x}	5.12	5.12	1.03	0.932	0.955	0.955
	Glucose (mg/dL)	213	217.9	217.7	213.2	213.7	212.3^{x}	221.8^{x}	214.0	10.7	0.297	0.33	0.488
	Albumin (g/dL)	1.8	1.69	1.76	1.73	1.82	1.78^{x}	1.70^{x}	1.68	0.13	0.072	0.647	0.909
	AlkPhos (U/L)	371.9	378.6	426.7	323.8	356.2^{x}	387.7^{x}	497.2	260.0^{x}	171.7	0.931	0.194	0.095
	CK (U/L)	1628.8	1149.3	1530.3	1247.8	1945.7^{x}	1312.0^{x}	1115.0^{x}	1183.7^{x}	510.3	0.049	0.228	0.138
	LDH (U/L)	840.6	646.3	817.6	669.3	979.2^{x}	701.9^{x}	660.0^{x}	636.7^{x}	361.4	0.256	0.382	0.446

[a-b] Means within environmental temperature lacking a common superscript are different ($P < 0.05$).

[1]Mean values represent means of 6 replicate cages per treatment except for mean values with superscript x and y where the number of replicates was 5 and 4, respectively. Hens were subjected to each environmental temperature for 5 consecutive weeks.

[2]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature.

[3]Blood metabolites: Ca = Calcium; Phos = Phosphorus; AlkPhos = Alkaline phosphatase; CK = Creatine kinase; LDH = Lactate dehydrogenase

[4]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 3.6 contd. Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimens on blood metabolites in laying hens[1].

Environmental Temperature[2]	Blood metabolites[3]	Main effect				Interaction effect				SD[4]	P-value		
		EconomasE™		Sodium source		No EconomasE™		EconomasE™					
		No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$		EconomasE™	Sodium source	EconomasE™ x Sodium source
TN2 (23.8 ℃)	Ca (mg/dL)	25.5	26.5	25.6	26.4	25.7^{x}	25.3^{x}	25.5^{x}	27.5^{x}	1.65	0.203	0.295	0.118
44 - 48 wk	Phos. (mg/dL)	5.40	5.40	5.46	5.34	5.25	5.54^{x}	5.67	5.13	0.75	0.988	0.704	0.208
	Glucose (mg/dL)	215.2	216.3	220.4	211.1	219.5	210.8^{x}	221.2^{x}	211.3	15.5	0.868	0.179	0.931
	Albumin (g/dL)	1.78	1.74	1.76	1.76	1.75	1.80^{x}	1.76^{x}	1.72	0.10	0.422	0.941	0.309
	AlkPhos (U/L)	260.0	337.5	341.4	256.1	284.4^{x}	235.6^{x}	398.3	276.6^{x}	134.5	0.206	0.166	0.544
	CK (U/L)	2296.9	1900.8	2230.8	1966.9	2663.2^{x}	1930.6^{x}	1798.4^{x}	2003.2^{x}	685.7	0.215	0.402	0.146
	LDH (U/L)	1093.7	931.6	1105.6	919.8	1320.5^{x}	867.0^{x}	890.7^{x}	972.5^{x}	354.3	0.337	0.273	0.122
ET2 (32.2 ℃)	Ca (mg/dL)	24.0	24.0	23.4	24.6	23.7^{x}	24.2^{x}	23.0^{y}	25.0^{x}	3.25	0.973	0.441	0.616
49 - 53 wk	Phos. (mg/dL)	4.05	4.98	4.51	4.51	4.30^{x}	3.80^{x}	4.73^{y}	5.23^{y}	0.84	0.036	1.000	0.230
	Glucose (mg/dL)	232.2	225.6	227.5	230.3	224.8^{x}	239.5^{y}	230.3^{y}	221.0^{x}	11.7	0.260	0.631	0.049
	Albumin (g/dL)	1.75	1.74	1.81	1.68	1.78	1.72	1.84^{x}	1.64^{x}	0.23	0.921	0.194	0.509
	AlkPhos (U/L)	320.5	464	324.4	460.1	272.2^{x}	368.8^{x}	376.7	551.4^{x}	158.2	0.054	0.067	0.580
	CK (U/L)	865.5	1028.1	996.9	896.7	848.4^{x}	882.6^{x}	1145.4^{x}	910.8^{x}	373.9	0.345	0.558	0.433
	LDH (U/L)	474.4	455.0	496.9	432.5	526.7^{x}	422.2^{x}	467.2^{x}	442.7^{x}	217.7	0.844	0.517	0.687
REC2 (23.8 ℃)	Ca (mg/dL)	28.6	28.2	29.2	27.6	28.3$^{a, x}$	28.9$^{a, x}$	30.2$^{a, x}$	26.3$^{b, x}$	20.0	0.643	0.041	0.008
54 - 58 wk	Phos. (mg/dL)	6.14	6.38	6.52	6.00	5.88^{ab}	6.40^{ab}	7.16$^{a, x}$	5.60^{b}	1.15	0.625	0.290	0.043
	Glucose (mg/dL)	237.8	235.5	232.1	241.2	231.8^{x}	243.8^{x}	232.3	238.6x	6.07	0.392	0.003	0.296
	Albumin (g/dL)	1.76	1.76	1.79	1.73	1.77^{ab}	1.75^{ab}	1.82^{a}	1.70$^{b, x}$	0.09	1.000	0.095	0.204
	AlkPhos (U/L)	294.2	259.6	250.9	302.8	290.7	297.7	211.2^{x}	308.0	98.5	0.412	0.223	0.289
	CK (U/L)	656.9	846.7	886.3	617.3	741.3^{ab}	572.4$^{b, x}$	1031.2^{a}	662.2$^{ab, x}$	300.6	0.158	0.051	0.447
	LDH (U/L)	215.2	263.3	258.0	220.5	229.7^{x}	200.7^{x}	286.4^{x}	240.3^{x}	92.9	0.264	0.380	0.839

[a-b] Means within environmental temperature lacking a common superscript are different ($P < 0.05$).

[1]Mean values represent means of 6 replicate cages per treatment except for mean values with superscript x and y where the number of replicates was 5 and 4, respectively. Hens were subjected to each environmental temperature for 5 consecutive weeks for each temperature.

[2]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature.

[3]Blood metabolites: Ca = Calcium; Phos = Phosphorus; AlkPhos = Alkaline phosphatase; CK = Creatine kinase; LDH = Lactate dehydrogenase

[4]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 3.7 Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimens on relative liver weight (%) in laying hens[1].

Environmental Temperature[2]	Main effect				SEM	P-value		
	EconomasE™		Sodium source			EconomasE™	Sodium source	EconomasE™ x Sodium source
	No	Yes	NaCl	NaCl + $NaHCO_3$				
TN1 (23.8 °C) 26 – 30 wk	1.78	1.93	1.78	1.93	0.06	0.079	0.113	0.220
ET1 (32.2 °C) 33 – 37 wk	1.84	1.82	1.84	1.82	0.05	0.744	0.744	0.448
REC1 (23.8 °C) 38 – 42 wk	1.85	1.89	1.93	1.82	0.07	0.698	0.318	0.816
TN2 (23.8 °C) 44 - 48 wk	2.23	2.24	2.12	2.35	0.10	0.909	0.122	0.570
ET2 (32.2 °C) 49 - 53 wk	1.84	1.74	1.86	1.73	0.07	0.346	0.213	0.874
REC2 (23.8 °C) 54 - 58 wk	2.13	2.09	2.12	2.11	0.07	0.684	0.935	0.466

[2]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature.

Table 3.8 Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimens on intestinal morphology in laying hens[1].

Environmental Temperature[2]	Intestinal morphology[3]	Main effect				Interaction effect				SD[4]	P-value		
		EconomasE™		Sodium source		No EconomasE™		EconomasE™					
		No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$		EconomasE™	Sodium source	EconomasE™ x Sodium source
TN1 (23.8 ℃)	VH	1127.8	1157.0	1132.0	1152.9	1179.2^{b}	1076.5$^{b, x}$	1084.7$^{b, x}$	1229.3$^{a, x}$	104.20	0.531	0.652	0.015
26 – 30 wk	CD	177.3	175.4	175.5	177.1	178.0	176.5^{x}	173.2	177.7	26.15	0.867	0.887	0.787
	VH:CD	6.49	6.72	6.47	6.73	6.88$^{ab, x}$	6.10$^{b, x}$	6.07^{b}	7.37^{a}	0.72	0.470	0.408	0.003
ET1 (32.2 ℃)	VH	1126.7	1168.6	1149.3	1146.0	1115.4	1138.1^{x}	1183.3	1153.9	129.6	0.450	0.951	0.636
33 – 37 wk	CD	152.6	156.5	154.1	155.1	154.7^{x}	150.5	153.4	160.0^{x}	25.1	0.716	0.926	0.631
	VH:CD	7.56	7.42	7.29	7.69	7.24^{x}	7.88	7.34^{x}	7.50^{x}	0.8	0.699	0.280	0.511
REC1 (23.8 ℃)	VH	1091.8	1156.6	1099.2	1149.2	1092.8^{x}	1090.7	1105.5	1207.6	106	0.161	0.274	0.255
38 – 42 wk	CD	139.6	136.7	136.9	139.4	136.6	142.7	137.2	136.1^{x}	16.30	0.667	0.714	0.604
	VH:CD	8.04	8.36	8.01	8.39	8.28	7.80	7.74^{x}	8.98	0.99	0.461	0.382	0.056
TN2 (23.8 ℃)	VH	1204.4	1340.4	1259.6	1285.2	1217.4	1191.4	1301.8^{x}	1379.0	215.4	0.149	0.780	0.574
44 - 48 wk	CD	148.8	148.2	148.8	148.3	152.5	145.1^{x}	145.1^{x}	151.4^{x}	14.9	0.931	0.938	0.313
	VH:CD	7.90	8.03	7.97	7.96	8.05	7.75	7.90	8.17	1.81	0.858	0.982	0.705
ET2 (32.2 ℃)	VH	1286.7	1313.4	1302.0	1298.0	1282.1^{x}	1291.2	1321.9	1304.9^{x}	146.2	0.675	0.950	0.837
49 - 53 wk	CD	159.7	170.3	160.9	169.0	151.4^{x}	167.9	170.4	170.1^{x}	19.3	0.216	0.340	0.322
	VH:CD	8.14	7.55	7.96	7.73	8.50^{x}	7.78	7.42^{x}	7.68^{x}	0.78	0.101	0.512	0.170
REC2 (23.8 ℃)	VH	1376.9	1363.5	1386.6	1353.7	1309.5$^{ab, x}$	1444.2$^{a, x}$	1463.8^{a}	1263.2^{b}	121.5	0.800	0.535	0.005
54 - 58 wk	CD	181.4	170.6	178.5	173.6	174.5^{ab}	188.4$^{a, x}$	182.4$^{ab, x}$	158.8$^{b, x}$	20.6	0.247	0.596	0.053
	VH:CD	7.83	7.58	7.83	7.57	7.95	7.70	7.72	7.44^{x}	0.87	0.505	0.477	0.971

$^{a-b}$ Means within environmental temperature lacking a common superscript are different ($P < 0.05$).

[1]Mean values represent means of 6 replicate cages per treatment except for mean values with superscript x where the number of replicates was 5. Hens were subjected to each environmental temperature for 5 consecutive weeks.

[2]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature.

[3]Intestinal morphology: VH = villus height; CD = crypt depth; VH:CD = villus height crypt depth ratio.

[4]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 3.9 Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimens on keel bone curvature occurrence in laying hens[1].

[2]Curvature score (%)	Main effect				Interaction effect				P-value		
	EconomasE™		Sodium source		No EconomasE™		EconomasE™				
	No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	EconomasE™	Sodium source	EconomasE™ x Sodium source
TN1 (23.8 ℃) 26 - 30 wk											
0	67	69	83	53	78	56	89	50			
1	28	31	14	44	17	39	11	50	0.552	0.013	0.480
2	6	0	3	3	6	6	0	0			
ET1 (32.2 ℃) 33 - 37 wk											
0	50	53	53	50	44	56	61	44			
1	3	8	6	6	6	0	6	11	0.559	1.000	0.607
2	47	39	42	44	50	44	33	44			
TN2 (23.8 ℃) 44 - 48 wk											
0	42	36	39	39	39	47	39	33			
1	48	44	47	45	56	40	39	50	0.488	1.000	0.830
2	9	19	14	15	6	13	22	17			
ET2 (32.2 ℃) 49 - 53 wk											
0	61	61	53	69	50	72	56	67			
1	0	0	0	0	0	0	0	0	1.000	0.227	0.733
2	39	39	47	31	50	28	44	33			
REC2 (23.8 ℃) 54 - 58 wk											
0	61	44	64	42	72	50	56	33			
1	39	56	36	58	28	50	44	67	0.238	0.098	0.315
2	0	0	0	0	0	0	0	0			

[1]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature.
[2]The severity of the keel bone curvature was assigned a numerical value between zero and two.

Table 3.9 contd. Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimens on keel bone fracture occurrence in laying hens[1].

	Main effect				Interaction effect						
	EconomasE™		Sodium source		No EconomasE™		EconomasE™				
[2]Fracture score (%)	No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	EconomasE™	Sodium source	EconomasE™ x Sodium source
TN1 (23.8 °C) 26 - 30 wk											
0	78	92	89	81	83	72	94	89			
1	22	8	11	19	17	28	6	11	0.189	0.514	1.000
2	0	0	0	0	0	0	0	0			
ET1 (32.2 °C) 33 - 37 wk											
0	81	78	78	81	78	83	78	78			
1	3	6	3	6	6	0	0	11	1.000	0.812	0.411
2	17	17	19	14	17	17	22	11			
TN2 (23.8 °C) 44 - 48 wk											
0	76	83	86	73	83	67	89	78			
1	9	6	0	15	0	20	0	11	0.740	0.063	0.539
2	15	11	14	12	17	13	11	11			
ET2 (32.2 °C) 49 - 53 wk											
0	68	67	69	65	72	63	67	67			
1	23	31	25	29	17	31	33	28	0.564	0.918	1.000
2	9	3	6	6	11	6	0	6			
REC2 (23.8 °C) 54 - 58 wk											
0	72	64	69	67	78	67	61	67			
1	14	31	25	19	11	17	39	22	0.182	0.506	0.304
2	14	6	6	14	11	17	0	11			

[1]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature.
[2]The severity of the keel bone fracture was assigned a numerical value between zero and two.

Table 3.10 Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimens on bone breaking strength in laying hens[1].

Environmental Temperature[2]	Bone breaking strength (kg/f)	Main effect				Interaction effect				SD[3]	P-value		
		EconomasE™		Sodium source		No EconomasE™		EconomasE™			EconomasE™	Sodium source	EconomasE™ x Sodium source
		No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$				
TN1 (23.8 ℃)	Tibia	20.34	20.25	20.20	20.39	20.50^{x}	20.18^{x}	19.90	20.60^{x}	2.57	0.937	0.868	0.656
26 – 30 wk	Femur	20.30	22.06	20.47	21.85	18.86^{x}	21.66^{x}	22.08	22.04^{x}	3.68	0.279	0.404	0.390
ET1 (32.2 ℃)	Tibia	17.72	18.75	17.33	19.14	16.75	19.62^{x}	19.84^{x}	20.63	4.15	0.559	0.308	0.590
33 – 37 wk	Femur	18.19	20.24	18.30	20.13	16.75	19.62^{x}	19.84	20.63	5.21	0.370	0.423	0.647
REC1 (23.8 ℃)	Tibia	19.40	17.96	18.16	19.20	16.33	19.10	18.32^{x}	19.18^{x}	3.63	0.366	0.514	0.116
38 – 42 wk	Femur	19.29	18.43	18.39	19.34	20.95^{a}	17.63^{ab}	15.82$^{b, x}$	21.04$^{a.x}$	3.65	0.589	0.551	0.014
TN2 (23.8 ℃)	Tibia	21.03	21.01	21.61	20.43	21.20^{x}	20.87^{x}	22.02	20.00	3.55	0.987	0.439	0.578
44 - 48 wk	Femur	22.20	21.69	20.94	20.95	23.86^{x}	20.54	22.07	21.37	3.68	0.751	0.225	0.409
ET2 (32.2 ℃)	Tibia	18.61	18.54	18.78	18.37	17.76	19.47^{x}	18.10^{x}	18.98^{x}	3.62	0.964	0.794	0.414
49 - 53 wk	Femur	18.71	20.03	19.01	19.73	18.72	18.70^{x}	19.30^{x}	20.76	3.13	0.349	0.606	0.597
REC2 (23.8 ℃)	Tibia	20.64	22.25	23.29	19.60	23.20	18.08	23.38^{x}	21.12	3.26	0.240	0.012	0.296
54 - 58 wk	Femur	19.85	23.53	23.48	19.89	22.37	17.33	24.60	22.45	3.69	0.028	0.031	0.363

[a-b] Means within environmental temperature lacking a common superscript are different ($P < 0.05$).

[1]Mean values represent means of 6 replicate cages per treatment except for mean values with superscript x where the number of replicates was 5. Hens were subjected to each environmental temperature for 5 consecutive weeks.

[2]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature.

[3]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 3.11 Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimens on bone ash in laying hens[1].

Environmental Temperature[2]	Ash (%)	Main effect				Interaction effect				SD[3]	P-value		
		EconomasE™		Sodium source		No EconomasE™		EconomasE™			EconomasE™	Sodium source	EconomasE™ x Sodium source
		No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$				
TN1 (23.8 ℃)	Femur	46.56	46.49	46.06	46.99	46.30^{x}	46.82	45.82	47.16^{x}	2.33	0.945	0.363	0.683
26 – 30 wk	Tibia	48.02	48.80	47.97	48.85	48.74^{x}	47.30^{x}	47.20	50.40	4.27	0.674	0.636	0.220
ET1 (32.2 ℃)	Femur	47.14	46.78	46.16	47.76	45.57	48.72^{x}	46.76^{x}	46.80	2.28	0.714	0.119	0.128
33 – 37 wk	Tibia	48.99	51.63	48.93	51.70	47.58	50.40	50.27^{x}	53.00^{x}	2.46	0.022	0.017	0.969
REC1 (23.8 ℃)	Femur	51.53	52.60	52.03	52.12	50.95	52.12	51.93	53.26^{x}	2.29	0.282	0.934	0.210
38 – 42 wk	Tibia	58.64	57.54	58.38	57.80	59.45	57.82^{x}	57.30	57.78^{x}	2.72	0.359	0.627	0.376
TN2 (23.8 ℃)	Femur	55.09	54.38	54.68	54.80	54.40	55.78^{x}	54.96^{x}	53.82	1.56	0.308	0.862	0.076
44 - 48 wk	Tibia	59.38	60.04	60.36	59.06	59.40	59.37	61.32^{x}	58.76^{x}	1.90	0.429	0.128	0.137
ET2 (32.2 ℃)	Femur	54.05	53.70	53.20	54.56	53.22	54.88	53.18^{x}	54.22	1.82	0.649	0.092	0.684
49 - 53 wk	Tibia	56.29	57.43	56.57	57.15	55.90	56.69	57.25	57.61	1.99	0.176	0.484	0.792
REC2 (23.8 ℃)	Femur	57.14	57.17	57.96	56.35	58.26	56.03	57.66	56.56	2.28	0.979	0.099	0.511
54 - 58 wk	Tibia	58.03	60.30	59.38	58.94	58.47	57.58	60.29	60.31	1.61	0.002	0.519	0.495

[1]Mean values represent means of 6 replicate cages per treatment except for mean values with superscript x where the number of replicates was 5. Hens were subjected to each environmental temperature for 5 consecutive weeks for each temperature.

[2]Environmental temperature: (Phase 1 and 2) TN = Thermoneutral temperature; ET = Elevated temperature; REC = Recovery temperature.

[3]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 3.12 Effect of dietary supplementation of EconomasE™ (0 or 0.2 g/kg) and two inorganic sodium sources (NaCl or NaCl+ $NaHCO_3$) during different environmental temperature regimen on the relative mRNA expression of heat shock protein 70 and 90 in laying hens[1].

Environmental Temperature[2]	Heat shock proteins[3]	Main effect				Interaction effect				SD[4]	P-value		
		EconomasE™		Sodium source		No EconomasE™		EconomasE™			EconomasE™	Sodium source	EconomasE™ x Sodium source
		No	Yes	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$	NaCl	NaCl + $NaHCO_3$				
TN1 (23.8 ℃)	HSP 70	0.85	0.93	0.90	0.88	0.94^{x}	0.76^{x}	0.85	1.02	0.46	0.680	0.974	0.394
26 – 30 wk	HSP 90	1.54	0.80	0.92	1.42	1.06^{x}	2.02	0.78^{x}	0.82^{x}	1.27	0.203	0.384	0.422
ET1 (32.2 ℃)	HSP 70	1.48	1.03	1.28	1.22	1.32	1.64^{x}	1.25	0.80^{x}	0.69	0.140	0.832	0.205
33 – 37 wk	HSP 90	1.34	1.06	1.04	1.36	1.08^{x}	1.60^{x}	1.00^{x}	1.12^{x}	0.58	0.284	0.223	0.440
REC1 (23.8 ℃)	HSP 70	1.13	1.19	1.05	1.27	1.07	1.18	1.03	1.35	0.39	0.683	0.193	0.542
38 – 42 wk	HSP 90	1.04	0.85	0.82	1.08	1.03	1.05	0.60	1.10	0.40	0.265	0.138	0.164

[1]Mean values represent means of 6 replicate cages per treatment except for mean values with superscript x where the number of replicates was 5. Hens were subjected to each environmental temperature for 5 consecutive weeks.

[2]Environmental temperature: (Phase 1) TN1 = Thermoneutral temperature; ET1 = Elevated temperature; REC1 = Recovery temperature

[3]Phase 2 samples were not analyzed for HSP 70 and 90 to minimize the cost associated with the RT-PCR analysis of those samples.

[4]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

CHAPTER 4.

THE EFFECT OF DEXAMETHASONE AND COCCIDIA VACCINE CHALLENGE WITH THE SUPPLEMENTATION OF A FEED ADDITIVE ON GROWTH PERFORMANCE, NUTRIENT DIGESTIBILITIES AND UTILIZATION, INTESTINAL BARRIER INTEGRITY, AND IMMUNE RESPONSE IN BROILER CHICKENS

Abstract

This study was conducted to evaluate the effect of stress and intestinal parasitic conditions typical of poultry production, on growth performance, nutrient digestibilities and utilization, intestinal barrier integrity, and immune response with Natustat™ supplementation. At day-of-hatch, birds were placed on two standard broiler diets supplemented with or without Natustat™ until d 28. On d 14, within each diet group, 448 chicks were randomly assigned to four challenge type: no-challenge (Control), dexamethasone (DEX), coccidia vaccine (Cocci), and a combination of DEX and Cocci (CocciDex) challenge. The DEX and CocciDex group received DEX in the diet at 1.5mg/kg of diet for 7 days, while the Cocci and CocciDex groups were orally gavaged with coccidia vaccine 20 times the recommended dosage. This experiment lasted until d 28, and performance data, blood, ileal digesta, excreta samples, jejunal segment, and mucosal samples were collected to determine growth performance, nutrient digestibilities and utilization, immune response, and intestinal permeability markers on d 21 and 28. Coccidia vaccine challenge did not affect BWG, FI, and feed efficiency (FE) on d 21 and 28. In contrast, DEX and CocciDex challenge reduced (P <.0001) BWG, FI, and FE compared to the Control and Cocci groups on d 21 and 28 except for FE that increased (P < .0001) with DEX and CocciDex-challenge (d 28). CocciDex decreased (P < 0.05) the

apparent ileal digestibility (AID) of dry matter (DM) and energy (EN), and digestible energy (DE), and total tract utilization (TTR) of DM, N, and EN, and the AME and AMEn, compared to the other challenge types on d 21. Both the DEX and CocciDex-challenge reduced ($P < 0.05$) AID of calcium (Ca) and phosphorus (P) on d 21 however, on d 28 the DEX-challenge increased ($P < 0.05$) AID of Ca. Cocci challenge reduced ($P < 0.05$) the TTR of DM, EN, AME, and AMEn (d 21), and N, AME, and AMEn (d 28) compared to the Control group. No significant differences were observed with Natustat™ supplementation on the digestibilities (d 21 and 28) and utilization (d 21) coefficients calculated. On d 28, TTR of EN, and the AME and AMEn increased ($P < 0.05$) in birds of the Control group fed diets without Natustat™. The FITC-d concentration in the serum increased ($P <.0001$) with CocciDex and DEX challenge on d 21 compared to the other groups. Cocci challenge increased ($P < 0.05$) the mRNA expression of TLR4, IL-1β, IL- 6, IFN-γ (d 21), and decreased ($P < 0.05$) SGLT1, NaPi-IIb, and IgA (d21), and reduced ($P < 0.05$) TLR4, NF-κB, IL-10 and NaPi-IIb (d 28). The DEX challenge reduced ($P < 0.05$) the expression of TLR4, IL-1β, IL-6, IFN-γ, and increased ($P < 0.05$) SGLT1 and IgA in the jejunum. In conclusion, DEX and CocciDex were able to induce stress and reduce performance, digestibility, intestinal permeability, and immune response. The coccidia vaccine challenge did not affect performance, and intestinal permeability however, TTR was impaired, and immune response was initiated. Finally, Natustat™ supplementation did not influence production parameters, intestinal permeability, or morphology however, increased the jejunal mRNA expression of IL-6 and MUC 2 and decreased IL-10.

4.1 INTRODUCTION

Gut health challenges are becoming increasingly important in the animal food industry. The current understanding of the gastrointestinal tract (GIT), suggests it contributes to health in many ways. Gut health encompasses the healthy GIT which allows effective digestion and absorption, a stable intestinal microbiota, and a functioning immune status (Bischoff, 2011). Hence, the relevance of gut health in animal production highlights the need to maximize food processing and nutrient utilization, improve production performance, and enhance vitality. At the forefront of potential causes of impaired intestinal health is the prevalence of coccidiosis in poultry. Essentially, coccidiosis is a major recurring intestinal parasitic problem in poultry production caused by intracellular protozoan parasites of the *Eimeria* species which, invade and reside in the lining of the intestine or ceca (Lillehoj and Lillehoj, 2000; Laurent et al., 2001). These parasites form environmentally resistant oocysts which undergo a fecal-oral transmission between hosts. Depending on the species, *Eimeria* undergoes an endogenous development at different locations in the intestine and ceca, with varying degrees of pathogenicity (Rose, 1987; Lillehoj and Trout, 1993; Williams, 2005). Ultimately, this results in hemorrhagic enteritis with severe erosion of the mucosal membranes, loss of gut absorptive capacity, inflammation, and death (Lillehoj and Trout, 1993; Williams, 2005).

In response to coccidia infection, the host initiates a complex protective mechanism to maintain the integrity of the gut barrier. Both the innate and adaptive arms of the immune system are involved. Protection via the activation of innate immune receptors in response to protozoan parasite infection has been observed (Swaggerty et al., 2011; Tan et al., 2014). To activate the innate immune system, certain sensor cells detect pathogen-associated

molecular patterns (PAMPs) associated with conserved microbial patterns and endogenous danger signals via the pattern recognition receptors (PRRs) (Keestra and van Putten, 2008; Temperley et al., 2008). Toll-like receptors (TLRs) are types of PRRs that serve to amplify the immune response via the MyD88-dependent and TRIF-dependent pathways to initiate a signaling pathway that leads to the activation of the transcription factor NF-κB (Juul- Madsen et al., 2008; Zhou et al., 2013). The NF-κB acts primarily to induce the expression of proinflammatory mediators including tumor necrosis factor (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and type I interferons (IFNs) (Barton and Medzhitov, 2002; Temperley et al., 2008; Zhou et al., 2013). In an in-vitro study, expression of ChTLR4 and MyD88 were increased following a live *Eimeria tenella* sporozoites stimulation (Zhou et al., 2013). Similarly, Tan et al. (2014) reported an increase in the expression of ChTLR4 with coccidia challenge in broilers. Since *Eimeria sp* actively targets the gut, the GALT act as the front line of defense.

Both the non-specific (antimicrobials, gastric secretions, mucus production by goblet cells, and activating phagocytosis by macrophages and dendritic cells), and specific (T- lymphocytes and, to a lesser extent, antibodies from B- lymphocytes) protective mechanisms are activated, to defend against the intestinal pathogen (Giambrone et al., 1980; Lillehoj and Trout, 1993; Lillehoj and Lillehoj, 2000; Laurent et al., 2001; Dalloul and Lillehoj 2006; Tan et al., 2014). This often results in the activation of T lymphocytes and the expression of their corresponding cytokines, and the activation of IgA precursor B cells in GALT. The PAMPs of the parasite are recognized by TLR-mediated lamina propria macrophages, which activate the pathways that lead to the signaling of the Th1 cells and the subsequent release of the cytokines (Laurent et al., 2001; Dalloul and Lillehoj, 2005).

Key support for this is the proven involvement of IFN-γ during coccidia infection (Lillehoj and Trout, 1993; Lillehoj and Lillehoj, 2000; Yun et al., 2000; Laurent et al., 2001). Moreover, the activation of TLR4 can directly increase paracellular permeability (Camillerri et al., 2012). As such, a disrupted barrier function comprises of alterations in epithelial tight junction protein (TJ) and mucin gene expression. Following coccidia infection, there is evidence of intestinal epithelial sloughing and villus tip damage as seen by the reduced villus height, crypt dilation, and goblet cell depletion (Tan et al., 2014; Dersjant-Li et al., 2016).

Indeed, the control and eradication of intestinal infections are some of the challenges faced by livestock producers worldwide. Successful commercial poultry production relies heavily on effective pathogen control, among other things. Vaccinating d-old birds with a low dose of live oocyst of important Eimeria sp is a common practice in the poultry industry (Chapman et al., 2005). This often results in a low-level infection with prospects that it confers protection on the birds against future coccidiosis occurrence. Accompanying this procedure is a decrease in some performance parameters – BWG and feed efficiency, impaired nutrient digestion and absorption, which are undesirable for efficient broiler production (Lee et al., 2011; Chapman, 2014). Similarly, reports also show that the birds have an increased susceptibility to secondary infections resulting in diseases such as necrotic enteritis (Kogut et al., 1998; Chapman et al., 2002; Williams, 2005; Chapman, 2014). This is because it requires at least 7 to 10 d, following vaccination, for the stimulation of the acquired immune response.

Consequently, researchers capitalize on this and other pathophysiological effects that arise from coccidia challenge to understand and propose actions to counteract the effect

of coccidiosis in birds. Inducing mild coccidia challenge via oral gavage using a vaccine with live oocyst at doses more than the recommended dosage by the manufacturer (Adedokun et al., 2012; Adedokun et al., 2016; Adedokun and Adeola, 2017), live oocyst of specific *Eimeria sp* (Isobe and Lillehoj, 1993; Laurent et al. 2001; Bortoluzzi et al., 2019), or sporulated oocyst of a combination of several *Eimeria sp* (Lee et al., 2011; Amerah and Ravindran, 2015) has been used to induce enteric diseases. To further understand the dynamics of an additional stressor during a coccidia challenge, the known immunosuppressant dexamethasone (DEX) was added to the diet. Corticosteroids are useful in the study of various parasitic diseases since they suppress the host's immune responses and thus modify the course of a disease (Nilo, 1970; Rose, 1970; Isobe and Lillehoj, 1993). By acting on glucocorticoid receptors, DEX mimics the effects of corticosterone as part of the feedback mechanism that turns the immune response (inflammation) down, impairing the ability to cope with stress. In chickens, an increase in oocyst production, susceptibility to *Eimeria* infections, prolonged patent period (Nilo, 1970; Rose, 1970; Isobe and Lillehoj, 1993), and enhanced disease susceptibility as a result of reduced T- cell proliferation, IL-2, and IFN-γ (Isobe and Lillehoj, 1993) have been observed.

In addition to vaccination, prophylactic control of coccidiosis using anticoccidial drugs has been used to maintain or restore gut-related infections. However, the increase in anticoccidial resistance has raised concerns about the need for new alternatives for the control of coccidia infections. New strategies have been embraced in recent years to induce potent protective immune responses in poultry (Allen et al., 1998; Yang et al., 2009). These strategies consider that any disturbance on the balance of the microbiome and the mucosal

immune system will lead to impairment of the GIT barrier, and subsequently an increased risk to gut health and development of intestinal infection. There are various chemicals and biologic substances considered immunomodulators in poultry; of interest are those with known influence on the intestinal integrity and immune system. Prebiotics, probiotics, synbiotics, essential oils, and plant extracts are considered alternative products to antibiotics used to improve chicken intestine health and growth performance in the poultry industry. Specifically, mannan oligosaccharide (MOS), a type of commercial prebiotic, has been reported to prevent gram-negative pathogen infection by competitive exclusion in chicken GIT (Baurhoo et al., 2007; Yang et al., 2009). The benefits of the yeast-derived MOS as natural feed additives in livestock and poultry on performance (Sims et al., 2004; Reisinger et al., 2012; Fowler et al., 2015) and gastrointestinal health (Gomez-Verduzco et al., 2009; Munyaka et al., 2012; Alizadeh et al., 2016), have been well documented. Others have suggested that probiotics may enhance host defenses and improve vaccine response against enteric parasites (Dalloul et al., 2003; Farnell et al., 2003; Koenen et al., 2004). The beneficial effects of Natustat™ (Alltech, Inc., Nicholasville, KY), a natural plant-derived proprietary product composed of at least 1 yeast-derived MOS plus organic mineral nutrients and plant extracts have been reported. Improved body weight gain, feed conversion ratio, reduced oocyst shedding in *Eimeria* challenged broilers (Duffy et al, 2005a), and improved performance, a reduction in intestinal lesion severity in Cochlosoma anatis and *Eimeria* challenged turkeys (Duffy et al., 2005b) fed diets supplemented with Natustat™ has been observed.

Thus, this study hypothesized that Natustat™ supplementation will ameliorate the effect of DEX, coccidia vaccine challenge, and their combination in broiler chickens 7-

and 14- days post-challenge. The objective of the present study was to outline the metabolic failings from a well-established enteric broiler coccidia vaccine and DEX challenge model with or without Natustat™ supplementation. The analysis adopted a multifaceted approach that considered the effect of the challenge on performance, ileal nutrient and energy digestibility, intestinal morphology, gut permeability, and immune response. Furthermore, to understand how these stress factors alter intestinal inflammation-associated permeability, fluorescein isothiocyanate (FITC-d) a 3 – 5 kDa, a marker used to measure tight junction permeability in enteric inflammation models was evaluated.

4.2 MATERIALS AND METHODS

Experimental procedures followed the approved protocols of the Animal Care and Use Committee of the University of Kentucky.

4.2.1 Bird husbandry and experimental diets

The experiment used 500 day-old male by-product breeder chicks obtained from Cobb Monticello, KY. For the first 7 days, the chicks were housed in electrically heated battery cages with wire floors in an environmentally controlled room. Room temperature was maintained at 37 ℃ for the first week and gradually decreased to 27 ℃ by the end of the experiment. Birds were reared up to 28 d post-hatch and fed a corn-soybean meal-based starter (d 0-14) and grower (14-28) diets, that met the National Research Council (NRC, 1994) energy and nutrient requirements. The ingredient composition and analyzed nutrient and energy contents of the diets are shown in Table 4.1. Included in the diets was titanium dioxide (0.5%) as an inert marker. Chicks consumed feed and water ad libitum during the entire time of the experiment. At the start of the experiment, all the chicks were tagged at the wing, weighed individually, and randomly assigned to two dietary treatments - (I) a

basal diet not supplemented with Natustat™ or (II) a basal diet supplemented with 1g/kg of Natustat™ (Alltech, Inc., Nicholasville, KY). The birds were on the two-dietary treatments from d 0 – 28.

On d 14, birds were weighed individually and randomized to treatments with similar weights between cages and across treatments. Four hundred and forty-eight chicks were used in this experiment and were randomly assigned to eight treatments with seven replicate cages per treatment and eight birds per cage in a 2 x 4 factorial arrangement. Using the Experimental Animal Allotment Program by Kim and Lindemann (2007), in a completely randomized design, the birds within each dietary treatments mentioned above were assigned to four challenge types; no-challenge (Control), dexamethasone challenge (DEX), coccidia vaccine challenge (Cocci), and a combination of dexamethasone and coccidia vaccine challenge (CocciDex). To minimize cross-contamination, treatments were randomized to battery cages in different sections of the room, with the Control and DEX group in the front section of the room and Cocci and CocciDex group in the rear section of the room. Moreover, daily monitoring and care of the birds started with non-challenged birds followed by the challenge birds. Dexamethasone was supplemented to the basal diet at 1.5 mg/kg of diet and fed to treatment groups DEX and CocciDex. Diet containing DEX was fed for 7 consecutive days. Furthermore, Coccivac-B-52 a live oocyst vaccine containing strains of *E. acervulina, E. mivati, E. maxima, and E. tenella* (Coccivac®-B- 52; Merck Animal Health), was used as an immune stimulus. According to the manufacturer's recommendation, one dose of the coccidia vaccine is administered to day-old broiler chicks. Day-old chicks are approximately 45 g in weight. To induce an immune response at d 14, accounting for the weight of the birds (at d 14; an average of 450 g), 20 times the

recommended dosage for day-old chicks were used to achieve the equivalent of ~2X of what is recommended for 45 g day-old chick. Birds in the Cocci and CocciDex groups were orally gavaged with 0.6 mL solution containing Coccivac-B-52 diluted in nanopure water. Birds in the unchallenged group received the same volume of nanopure water via oral gavage. On d 21, following sampling, birds on the DEX diet were switched to a standard grower diet with or without Natustat™ supplementation for an additional 7 days (until d 28) (Figure 4.1).

4.2.2 Sampling

Bodyweight (BW) and feed intake (FI) were monitored pre- (d 0 to 14), during (d 14 -21), and post (d 21 – 28) challenge periods for calculation of performance. Average FI and BW gain were adjusted for mortality. Similarly, tissue samples were collected on d 21 and d 28 to determine the effect of the treatments during and post-challenge periods. On d 21, all the birds were weighed and 4 (2 heaviest and 2 lightest) were randomly selected from each cage and euthanized for sample collection. The remaining birds were raised for an additional seven days. Out of the 4 birds selected for sampling, one bird was returned to the cage for FITC-d administration and blood collection. Subsequently, the birds were euthanized by CO_2 asphyxiation. A blood sample was drawn from the jugular vein into EDTA tubes, spun down at 1200 x g for 10 mins at 4 ℃ for plasma. Plasma samples were stored at -80 ℃ for further analysis. Liver and spleen samples were excised, cleaned and the weights were recorded. The determined organ weight was expressed relative to the bird's final body weight.

Five centimeters of the mid-jejunum was taken for mucosa gene expression determination. The jejunal sections were cleaned by flushing with nanopore water, sliced

open longitudinally, and the mucosa layer was scraped into a microtube containing 1 mL of Trizol (Invitrogen, Grand Island, NY, USA), snap-frozen in liquid nitrogen, and stored at – 80 ℃ for gene expression of immunity and inflammation-related genes, cytokines, and tight junction proteins. Additional jejunal tissue was collected to determine intestinal morphology. Ileal digesta was collected from the distal two-thirds of the ileum The contents of the posterior ileum were collected by gently flushing with nanopore water into labeled plastic containers and stored at – 20 ℃ until freeze-dried. Ileal digesta within a cage was pooled, lyophilized, ground using a coffee grinder, and stored in corresponding labeled bags. Excreta samples were collected from each cage on days 20 and 21, and 27 and 28 stored at –20 °C, and subsequently oven-dried at 55 °C for six days. Diets and the dried excreta samples were ground through a 0.5 mm screen using a Wiley Mill laboratory Standard (Model No. 3, Arthur H. Thomas Co., Philadelphia, PA, USA) and stored in airtight plastic bags. Dried ileal digesta, excreta, and diet samples were stored until they were analyzed for dry matter (DM), gross energy (GE), nitrogen (N), calcium (Ca), and Phosphorus (P).

4.2.3 Chemical analyses

Ground excreta, ileal digesta, and diet samples were thoroughly mixed, and subset samples were analyzed in duplicate. Dry matter content in the excreta, ileal digesta, and diets was determined by oven-drying at 105 °C for 24 h (AOAC International 2006; method 934.01). Gross energy of excreta, ileal digesta, and diet samples were determined using a bomb calorimeter (Parr 6200 calorimeter, Parr Instruments Co., Moline, IL, USA) which was standardized with benzoic acid. Titanium (**Ti**) and N, Ca, and P content in the excreta, ileal digesta, and diets were determined at the Agricultural Experiment Station Chemical

Laboratories, University of Missouri-Columbia (Columbia, MO). The samples were digested using concentrated sulfuric acid and processed as described by Myers et al. (2004) after which Ti concentration was determined by flame atomic absorption spectroscopy**.** Nitrogen content was determined by the combustion method using a LECO Trumac Nitrogen Analyzer (LECO, St. Joseph, MI; AOAC International, 2000; method 990.03). The samples were wet acid digested with nitric and perchloric acid mixture (AOAC International, 2005; method 990.08), and concentrations of Ca and P were determined at specific wavelengths for each element (Ca, 393.3 nm; P, 185.9 nm) by inductively coupled plasma-optical emission spectroscopy using a Thermo Jarrell Ash IRIS instrument (Thermo Jarrell Ash Corporation, Franklin, MA). The instrument was calibrated against standards (Junsei Chemical Co., Ltd., Tokyo, Japan) of known concentration. Apparent ileal digestibility (**AID**) and total tract nutrient utilization (**TTR**) of DM, N, energy (**EN**), Ca, and P were calculated using the following equation:

$$AID\ or\ TTR, \% = \left[1 - \left(\frac{Ti_1}{Ti_0}\right) \times \left(\frac{X_0}{X_1}\right)\right] \times 100$$ ----------Equation 1 (Kiarie et al., 2014)

Where Ti_I represents the titanium concentration in the diet and Ti_o represents the titanium concentration in the ileal (AID), or excreta (TTR) samples (%); and X_I represents the concentration of nitrogen or energy in the diet and X_O represents are the concentration of nitrogen or energy in the ileal or excreta samples, respectively, (%).

Ileal digestible energy (DE) and apparent metabolizable energy (AME) were calculated using the following equation.

$$\text{DE or AME, kcal/kg} = Calculated\ EN\ (\%) \times GE\ of\ diet\ (kcal/kg)$$

Where calculated EN is derived from Eqn. 1 for ileal or excreta samples, and gross energy (GE) of diet is determined by bomb calorimeter.

Nitrogen-corrected AME (AMEn) was determined by correcting for N retention by a factor of 8.22 kcal/g of N retained in the body as described by Hill and Anderson (1958).

$$\text{Relative organ weight, \%} = \frac{Organ\ weight\ (g)}{Final\ body\ weight\ (g)} \times 100$$ ----------Equation 3

4.2.4 Jejunal morphology analysis

On d 21 and 28, tissue samples of the mid-jejunum were collected from one bird per cage and flushed with nanopure water to remove the contents. In brief, the gut segments were fixed in 10% neutral buffered formalin (Sigma Chemical Co., St Louis, MO, USA). Subsequently, tissue sections (5 μm) were cut, dehydrated, cleared and embedded in paraffin (Polyfin paraffin, Sigma Polysciences, St. Louis, MO), and stained with hematoxylin and eosin. On each slide, villus height, width, and crypt depth were measured using a Nikon ECLIPSE 80i light microscope (Eclipse E600, Nikon Corp., Tokyo, Japan) equipped with computer-assisted imaging software (Nikon's NIS Elements Basic Research Microscope Imaging morphometric system), and a camera (XC77E, Sony Corp., Tokyo, Japan). Approximately ten intact villi were randomly selected per slide and measured under a 4X magnification. Villus height was measured from the tip of the villus to the villus-crypt junction, whereas crypt depth was defined as the length between the crypt opening and base. Villus width was measured at the basal (crypt-villus junction) and apical ends (Iji et al., 2001). An average value was calculated for each section measured and the villus height: crypt depth ratio was calculated.

4.2.5 Administration and determination of FITC-d

Mucosal barrier dysfunction was determined by measuring the appearance of a marker - FITC-d MW 3–5 KDa (Sigma Aldrich Co., St. Louis, MO, USA) in the blood.

All administration and detection procedures of FITC-d was done as described by Kuttappan et al. (2015); Vicuna et al. (2015); Baxter et al. (2017); and Duff et al. (2019). Briefly, on d 21 and 28, FITC-d was prepared at 4.17mg/mL of the mean weight of birds in the pen and orally gavaged 0.6 ml of the solution. Two hours post-gavage, birds were euthanized via CO_2 asphyxiation, and blood samples were collected from the jugular vein to quantify levels of FITC-d. To determine FITC-d, the blood was kept at room temperature for 2 h to allow clotting and centrifuged at 1000×g for 15 min to separate the serum. The serum samples were then diluted in phosphate buffer saline at 1:5 and readings were performed at a gain 70. A non-FITC-d serum was used as blank and FITC-d concentrations were calculated using the standard curve adapted for each plate. Fluorescence was measured spectrophotometrically at 485 nm excitation and 528 nm emission wavelength (Synergy HT, multimode microplate reader, BioTek Instruments, Inc., VT, USA).

4.2.6 Jejunal gene expression

Two-step quantitative real-time PCR (qRT-PCR) was used to determine the expression levels of selected genes in the jejunum. Jejunal mucosa samples collected on d 21 and 28 were analyzed for mucosal immune-related genes – mucin-2 and IgA; inflammation-related genes – TLR4, NF-κB, IL-1β, IL-6, IFN-γ, and IL-10; tight junction protein – occludin; and nutrient transporters – SGLT-1 and NaPi-IIb. Jejunal mucosa samples suspended in 1 mL of Trizol® were homogenized using a power homogenizer (Omni tissue homogenizer TH) with 5 mm plastic disposable probes (Omni International, GA, USA) at room temperature before RNA isolation. Subsequently, total RNA was extracted using a TRIzol/chloroform/isopropanol method followed by the removal of supernatants. The RNA pellet was then resuspended in nuclease-free H_2O, and the

concentration and purity of the extracted RNA were determined using a NanoDrop One (Thermo Fisher scientific™, Wilmington, DE, USA) spectrophotometer at an optical density of 260 and 280 nm. Total RNA was quantified at 260/280nm using the Acclaro™ Sample Intelligence technology built into the NanoDrop One instruments ND-1000 spectrophotometer (Thermo Fisher scientific™, Wilmington, DE, USA) and stored at −80 ℃. Total RNA (1µg) from each sample was reverse transcribed into cDNA in a 20 µL RT reaction using Script cDNA supermix (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's protocol in a Veriti Dx Thermal cycler (Applied Biosystems, Foster City, CA). The RNA was incubated for 5 min at 25 ℃, followed by 30 min extension at 42 ℃. The reaction was stopped at 80 ℃ for 5 min and then held at 4 ℃ until removal from the machine. Real-time PCR detection of the cDNA was conducted using the SYBR Green assay. The primers used for real-time PCR are listed in Table 4.2. The cDNA was diluted to 1:20 with nuclease-free water before being used for real-time PCR. Amplification was carried out in a total volume of 12.5 µL containing, 1µL of cDNA, 0.375 µL of each forward and reverse primer, and 6.25 µL SYBR green master mix (Bio-Rad Laboratories, Hercules, CA), and 4.5 µL of RNase free water. Each sample was tested in duplicate. PCR plate contained target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) an endogenous housekeeping control and a no-template negative control containing water instead of cDNA. PCR was performed using the Bio-Rad CFX-96 real-time PCR system (Bio-Rad, Hercules, CA), with the following cycle profile: an initial denaturation step at 95 ℃ for 5 min, followed by 95 ℃ for 15 s and then 60 ℃ for 1min ran on a repeat for 40 cycles. The relative levels of mRNA expression were calculated

using the $2^{-\Delta\Delta CT}$ method after normalization against the reference gene (Shini and Kaiser, 2009). The average value of the control group was used as the calibrator.

4.2.7 Statistical analysis

Data were analyzed using a two-way ANOVA to determine differences between the main effects of the 2 dietary levels, 4 challenge measures, and their interaction using the general linear model procedure of SAS 9.4 software (SAS Institute Inc., Cary, NC). For performance and nutrient digestibility data, cage means were considered as the experimental unit. For the remaining analysis, a bird constituted the experimental unit. Differences were separated using Tukey multiple comparison test and a probability value of less than 0.05 was defined as statistically significant.

4.3 RESULTS

4.3.1 Growth performance

To understand the dynamics between birds fed the diet supplemented with or without Natustat™ and the challenge type (Control, Cocci, DEX, and CocciDex), we evaluated the growth performance of broilers from d 0 -14 (pre-challenge), d 14 -21 (during-challenge), and d 21 -28 (post-challenge). These data are presented in Table 4.3. During the pre-challenge period, the supplementation of Natustat™ in the diet did not affect the BWG, FI, and FE. During the challenge period (d 14 -21), the coccidia vaccine challenge had no significant effect on BWG, FI, and FE rather, they were comparable to the Control birds. However, these parameters were reduced (P <.0001) by DEX-challenge, with further reduction (P <.0001) by CocciDex-challenge. Post-challenge (d 21 – 28), the BWG and FI were similar in the Control and Cocci groups but lower (P <.0001) with DEX and CocciDex-challenge. Furthermore, FE was higher ((P <.0001) in the DEX- challenged

birds compared to the Control and Cocci birds. Natustat™ supplementation did not impact any of the observed performance parameters pre, during, and post-challenge.

4.3.2 Organ weights

Liver weight relative to the BW on d 21, was higher (P <.0001) in the DEX- challenged birds compared to the remaining groups with the Cocci and Control birds having the lowest (P < 0.05) liver weight (Table 4.4). In contrast, spleen weight was lower (P <.0001) in DEX and CocciDex-challenge birds compared to the Control and Cocci group where the Cocci group had the highest (P < 0.05) spleen weight (Table 4.4). Post- challenge (d 28), liver weight was similar between the Cocci, DEX, and CocciDex- challenge birds but higher (P < 0.05) than the Control birds while spleen weight was lower (P <.0001) in the Control and Cocci-challenge birds compared to the CocciDex- challenge birds. Natustat™ supplementation did not affect organ weights measured during and post-challenge periods (Table 4.4).

4.3.3 Jejunal morphology

Jejunal morphological effects in response to the challenge-type and dietary treatment for d 21 and 28 are presented in Table 4.5. There was no significant interaction between the challenge-type and dietary treatment on VH, CD, VH: CD ratio on d 21. The birds in the Control group had a higher (P <.0001) VH compared to the birds in the other challenge-types (Cocci, DEX, CocciDex) on d 21. The CD of birds in the Cocci group was higher (P <.0001) compared to the other challenge-types, while the VH: CD ratio was lower (P <0.0001) in the Cocci and CocciDex compared to the Control and DEX-challenge birds (d 21). On d 28, an interaction effect was only observed for the VH. The birds in the Cocci group fed the diet supplemented with Natustat™ had a higher (P < 0.05) VH compared to

birds in the Control group fed diets with no Natustat™ supplemented, and birds in the DEX and CocciDex group with Natustat™ supplemented in the diet. An inverse outcome was observed for the measured CD and VH: CD within the challenge-types on d 28. The CD in the Cocci and CocciDex group was higher (P <0.0001) compared to the DEX and Control group while the VH: CD ratio was lower (P <0.0001) in the Cocci and CocciDex group compared to the DEX and Control group. Natustat™ supplementation did not affect the CD and VH: CD ratio (d 28).

4.3.4 Ileal digestibility and total tract utilization

The effects of DEX, coccidia vaccine challenge with or without Natustat™ supplementation on AID of DM, N, EN, Ca, P, and DE as well TTR of DM, N, EN, Ca, and P, and AME, and AMEn in 21 and 28 day-old broilers, are presented in Table 4.6 and 4.7, respectively. Results for the ileal digestibility showed no significant interaction between the treatments on d 21 and 28 (Table 4.6). CocciDex-challenge resulted in a lower (P < 0.05) ileal DM, EN, Ca, and DE digestibility compared to the Control. Apparent ileal P digestibility values in the DEX challenged birds, although higher (P < 0.05) than the CocciDex-challenge birds, was lower (P < 0.05) than the Control and Cocci birds. (d 21; Table 4.6). Similarly, AID of Ca was lower (P <.0001) in the DEX-challenged birds compared to the Control and Cocci birds but comparable to the CocciDex birds. On d 28, the challenge type did not affect the apparent ileal digestibility of DM, N, and EN, and DE. However, ileal Ca digestibility was higher (P <.0001) in the DEX-challenge group compared to the remaining challenge groups. The Cocci and CocciDex-challenge reduced (P <.0001) AID of P on d 28 compared to the Control and DEX-challenge birds. On the

other hand, Natustat™ supplementation did not influence the AID of DM, N, EN, Ca, P, and DE, on days 21 and 28 (Table 4.6).

The TTR of DM and AMEn for both the Control and DEX groups were similar and higher (P <.0001) than Cocci and CocciDex groups (Table 4.7). The TTR of EN and AME in the DEX-challenged birds was higher (P <.0001) compared to the other challenge type with the CocciDex-challenged birds having the lowest utilization. Nitrogen TTR was lower (P <.0001) in the DEX group compared to the Cocci and Control groups and considerably lower (P < 0.05) in the CocciDex-challenge group compared to the other treatments. Apparent TTR of P was highest (P < .0001) in the Control group compared with the other challenge groups. Coccidia challenge decreased (P <.0001) the TTR of DM, EN, Ca, P, and the AME and AMEn compared to the Control. Total tract utilization of N was higher (P <.0001) in the DEX group compared to the Control and Cocci group (d 28). Calcium utilization was higher (P <.0001) in the Control, Cocci, and DEX group compared to the CocciDex birds, while P utilization was higher (P <.0001) in the Cocci, DEX, and CocciDex-challenged groups compared to the Control group. Natustat™ supplementation did not affect the TTR of DM, N, EN, Ca, P, and the AME and AMEn on d 21 (Table 4.7). On d 28, an interaction effect (P < 0.05) between Natustat™ and the challenge type was observed for EN, and the AME and AMEn, with an increase (P <.0001) in these parameters in birds of the Control, DEX, and CocciDex birds fed diets without Natustat™ (d 28; Table 4.7).

4.3.5 Intestinal permeability determined by FITC-d

Intestinal permeability was assessed through the translocation of FITC-d into the bloodstream. The FITC-d concentration in blood serum of birds in the CocciDex-

challenged type increased (P <.0001) compared to the other challenge types on d 21 and 28 of age (Table 4.8). Similarly, DEX-challenge in the birds increased (P <.0001) FITC-d concentration in blood serum compared to the Cocci and Control birds. FITC-d concentration in the blood in both the Cocci and Control birds were not different. Natustat™ supplementation did not influence the translocation of FITC-d (Table 4.8).

4.3.6 Jejunal gene expression

The jejunal expression profiles of genes involved in inflammation (TLR4, NF-κB, IL-Iβ, IL-6, IFN-γ, and IL-10), intestinal nutrient transporters (SGLT-1 and NaPi-IIb), and gut integrity (MUC-2, IgA, and Occludin) for all treatment groups on d 21 and 28 are presented in Table 4.9 and 4.10 respectively. On d 21, no significant interaction was observed between Natustat™ supplementation and the challenge type for the mRNA expressions of the genes evaluated in this study except for SGLT-1. This interaction effect reveals that supplementation of Natustat™ increased (P <.0003) the expression of SGLT- 1 in the CocciDex-challenged group compared to the birds in the DEX and Cocci-challenged groups (Table 4.9). Individual main effects for the mRNA expression of jejunal samples analyzed on d 21 are summarized as follows: TLR4 expression increased (P <.0001) with Cocci-challenge compared to the other groups; NaPi-IIb expression increased (P <.0001) with DEX and CocciDex-challenge compared to the Cocci and Control birds; MUC-2 gene expression levels was higher (P <. 0001) in the Control birds compared to the challenge birds; Coccidia vaccine challenge increased (P <.0001) the expression levels of IFN-γ compared to the other groups and was considerably lower (P < 0.05) in the DEX challenge birds. Similarly, IL-1β and IL-6 gene expression levels were lower (P < 0.05) in the DEX group compared to other challenge groups; expression levels of NF-κB occludin was not

influenced by the challenge type. Overall, Natustat™ supplementation did not influence the mRNA expression of the genes analyzed except for SGLT-1 on d 21 (Table 4.9).

On d 28, an interaction effect was observed in 1L- 6 and IL-10 mRNA expression (Table 4.10). With Natustat™ supplementation, IL-6 mRNA expression was lower ($P < 0.011$) in the Cocci group and IL -10 was higher ($P < 0.011$) in the CocciDex- challenge group compared to birds in the same group fed diets without Natustat™. Other main effects include - decreased ($P < 0.05$) TLR4, NFκB, SGLT1, and NaPi-IIb and increased ($P < 0.05$) occludin mRNA mucosal expression in the Cocci- challenge group. The DEX-challenge increased ($P < 0.05$) TLR4, NaPi-IIb and decreased ($P < 0.05$) NFκB, IL-1β, IFN-γ, and SGLT1 expression. Finally, CocciDex and DEX challenge increased ($P < 0.05$) TLR-4 gene expression but unlike DEX, it also resulted in higher mRNA expression of NFκB, IL-1β, IFN-γ, IL-10, SGLT-1 (Table 4.10).

4.4 DISCUSSION

In the present study, the coccidia vaccine (at 20x the manufacturer's recommended dose for d-old chicks) and/or the addition of DEX to the feed, were used to induce the experimental intestinal challenge. Challenging the birds with the 20x coccidia vaccine on d 14 did not induce a significant decrease in performance. This observation is contrary to several reports that showed the coccidia vaccine challenge significantly reduced BWG, FI, and FE in broiler chickens (Isobe and Lillehoj, 1993; Tan et al., 2014; Amerah and Ravindran, 2015; Adedokun et al., 2016; Dersjant-Li et al., 2016; Osho et al., 2019). The observed BWG, FI, FE the during-challenge (d 14 -21) and post-challenge (d 21-28) periods were comparable to the birds not challenged with the vaccine. Only very few published articles reported similar results (Kettunen et al., 2001; Bortoluzzi et al., 2019).

The cause for this is unknown but the rationale is that the vaccine was not virulent enough to induce a mild infection to allow the birds to express the characteristic outcomes of coccidiosis, such as reduced performance. Conversely, DEX-induced stress resulted in depressed BWG, FI, and FE, similar to other published articles (Isobe and Lillehoj, 1993; Li et al., 2009; Barekatain et al., 2019) except for FE that was improved significantly post-challenge (d 28), and further depressed the BWG, FI, FE in combination with Cocci during- the challenge period. However, post-challenge, BWG, and FI were comparable between DEX and CocciDex groups.

As an exogenous glucocorticoid, DEX has a profound effect on energy homeostasis and glucose metabolism. Well established actions of glucocorticoids exert that it acts on hepatic production of glucose from amino acids, enhancing protein catabolism; and simultaneously decreasing glucose uptake (Munck, 1971; Post et al., 2003; Lin et al., 2004a). Although plasma glucose of broilers has been reported to increase significantly with DEX administration (Li et al., 2009), the decrease glucose uptake can explain the negative impacts DEX has on the performance of the animal as reflected by the depressed BWG and FE. Despite the large variety of stressors, birds are exposed to in the industry, the effects are often similar, and their combined effect can exacerbate an infectious disease (Isobe and Lillehoj, 1993; Huff et al., 1999; Huff et al., 2001). Similarly, in addition to direct measurements of corticosteroids, there is other (indirect) evidence of pituitary-adrenal involvement in the stress response of birds which is, for the most part, based on the target tissue responses to the corticosteroids. One important target is the lymphatic tissue the corresponding heavy liver weight and small spleen weight associated with DEX stress models were observed in this study. However, with the CocciDex challenge, the liver was

smaller than the DEX but considerably bigger than the Control and Cocci groups (Siegel, 1980).

In this study, the supplementation of Natustat™ did not yield a better growth performance in the challenged birds. Duffy et al. (2005c) also reported no significant improvement with Natustat™ supplementation in the performance of turkey tom challenged with *Histomonas meleagridis* on d 28. However, in another study, they reported a higher live weight gain and lower FCR in the *Eimeria*-challenged birds fed a diet supplemented with Natustat™ (Duffy et al., 2005a). The improved performance in birds fed MOS supplemented diets is suggested to be due to the prebiotic functionality, which tends to promote the colonization of beneficial bacteria, improve intestinal integrity, and enhance immune functions. Some researchers have found that the inclusion of yeast-derived MOS in the diet improved the growth performance of broiler chickens (Muthusamy et al., 2011; Ghosh et al., 2012; Gomez-Verduzco et al., 2009). Other studies have demonstrated no significant difference in growth performance parameters (Midilli et al., 2008; Cox et al., 2010; Munyaka et al., 2012). The rationale for this inconsistency with regards to the benefits of yeast-derived MOS on performance has been suggested to be due to differences in the source and concentration of the yeast products, duration of the trial, presence, and type of challenge used, and the experiential condition of the studies (Alizadeh et al., 2016).

To maintain maximum digestive and absorptive capability in broiler chickens, a large luminal surface area with optimal enterocyte functional maturity is important. This is because the inadequacies of the intestine may reduce the growth and extend the time broilers reach market weight. Increased villus height and short crypt depth, are associated

with a healthy intestinal morphology which, ensures greater digestion and absorption of nutrients (Gao et al., 2008). In this study, DEX, coccidia vaccine, and a combination of DEX and coccidia vaccine-challenge reduced the length of the villi compared to the control, on d 21. Coupled with the longer crypt depth observed in this study, the coccidia vaccine challenge negatively influenced the morphology of the intestine (d 21 and 28). Other studies have reported a longer CD (Reisinger et al., 2012; Tan et al., 2014) which, indicates an increase in the migration of epithelial, and an increased rate of apoptosis to get rid of infected enterocytes The literature illustrates that the sporozoites of *Eimeria sp* infect the cells of the intestinal lining causing tissue damage and trauma to the intestinal mucosa and submucosa (Lillehoj and Trout, 1996; Lillehoj and Lillehoj, 2000). The epithelial damage can reduce the nutrient absorption, which can explain the reduction in the total tract utilization of nutrients observed in the Cocci challenge group in this study. Similarly, exogenous CORT or its analog causes a decrease in the jejunal villus height (Hu and Guo, 2008; Li et al., 2009; Carvalho et al., 2018; Barekatain et al., 2019) with varying reports on their effect on the CD. A shorter CD is associated with a healthy proliferation of enterocytes, and the ratio of villus height to crypt depth reflects the comprehensive ability for intestinal nutrient absorption and function, which is what is observed in the DEX and CocciDex challenge group. However, this does not translate to the performance results. This means the results (short villus height and CD) might be a reflection of the reduction in intestinal size associated with smaller body size rather than the absorptive function of the jejunum.

Dietary prebiotics like MOS, can improve the integrity of the intestinal mucosa by binding and inhibiting pathogenic and opportunistic pathogenic bacteria from attaching to

the intestinal mucosa. Jejunal morphology analysis on d 21 revealed that Natustat™ supplementation decreased the VH compared to the control. The reason for the decrease in VH is unknown but on d 28, the supplement increased the VH of birds challenged with the coccidia vaccine compared to the Control (without the supplement) and the DEX and CocciDex groups (with the supplement). A study conducted by Baurhoo et al. (2007) revealed that dietary MOS (0.2% of the starter diet and 0.1% of the grower diet) did not have a significant effect on the VH of the jejunum on d 14 however, a significant increase was observed on day 28 and 42 compared to virginiamycin and the control. Others have reported no significant differences in VH on d 21 (Yitbarek et al. 2012) or d 42 (Lea et al., 2013). The possible reason for Natustat supplementation to increase the VH in the Cocci group compared to the DEX and CocciDex on the same diet could be that the supplement was able to reduce the growth of the pathogenic intestinal bacteria associated with coccidia. On the other hand, Zhang et al. (2005), Yitbarek et al. (2012), observed that supplementing the diet with MOS did not affect CD in birds, as seen in this study on both d 21 and 28.

General malabsorption of nutrients in poultry during the acute phase of intestinal coccidiosis is well documented (Ruff, 1978; Persia et al., 2006; Adedokun et al., 2016). In this study, we did not observe any significant deleterious effect of the coccidia vaccine challenge on AID of DM, N, EN, Ca, and P, and DE on d 21 and 28. This is expected based on the performance data from this study but contrary to reported data from other studies (Persia et al., 2006; Adedokun and Adeola, 2017; Adedokun et al., 2016; Rochell, et al., 2017). In contrast, the TTR of DM, EN, Ca, P, and AME, and AMEn on d 21, and DM, N, EN, AME, and AMEn on d 28 in the Cocci group, were impaired compared to the Control group. With the help of specific enzymes expressed on the brush border membrane,

epithelial cells further serve to digest luminal nutrients. The digested end products are then absorbed by the epithelial cells in the intestine through the action of nutrient transporters located on the brush border membrane (Gilbert et al., 2008). Based on our result, we speculate that while the birds were able to digest and absorb the nutrients, conditions of the gut increased nutrient drainage from the body to promote endogenous catabolism and mucus secretion as part of the maintenance activity that occurs during an infection, preventing the proper utilization of those nutrients.

Furthermore, the DEX challenge impaired only the Ca and P ileal digestibilities while the CocciDex challenge significantly impaired ileal DM, EN, Ca, and P digestibility, and AME on d 21 with a significant improvement observed with DEX challenge on ileal Ca and P digestibility by d 28. Similarly, N, Ca, and P were poorly utilized with DEX and DM, N, EN, Ca, and P utilization, and AME and AMEn were impaired even further with CocciDex on d 21. These parameters improved post-challenge (d 28), with a complete recovery observed in the DEX challenge when compared to the control. To focus on Ca intestinal absorption, studies have shown that it can be impaired by the presence of glucocorticoids (**GCs**) through decreased active transport and inhibition of normal vesicle uptake by the brush border membrane (Kimberg et al., 1971, Kim et al., 2009). Though we do not fully understand the mechanisms responsible for glucocorticoid-induced inhibition of Ca, reports have suggested a decrease in soluble calcium-binding protein (Mitchell and Lyles, 1990; Kim et al., 2009), and an increase in the excretion of urinary Ca ions (Weiler et al., 1995). Thus, the negative balance from decreasing gastrointestinal Ca absorption, increasing bone resorption, and increasing renal Ca excretion can stimulate an increase in the release of parathyroid hormone which has been shown to induce phosphaturia in rats

(Lukert and Raisz, 1990; Mitchell and Lyles, 1990). Hence, the reduced Ca and P ileal digestibility in our study, the accompanying poor utilization at d 21, and complete recovery at d 28, reiterate the effects outlined above. This also suggests that the efficiency of P digestion and utilization is dependent on the efficiency of Ca metabolism.

As suggested above, nutrient uptake is mediated by digestive enzymes and membrane-bound transporter proteins located at the brush border membrane of intestinal epithelial cells. Intestinal transport of glucose and phosphate (**Pi)** is accomplished by Na^+-coupled glucose transporter-1 and phosphate transporter, respectively, expressed in several tissues, including the brush border membranes of the small intestinal epithelium, where these molecules are transported against a concentration gradient into the enterocytes (Feng et al., 2012). Understanding how stressors influence these transporters, SGLT-1, and NaPi-IIb is important. A previous study showed that GCs released during stress periods induced the expression of glucocorticoid regulated kinase, which enhances glucose transportation by increasing SGLT-1 abundance in the cell membrane (Garriga et al., 2006). Feng et al. (2012) observed an increase in mRNA abundance of SGLT-1 in LPS challenged chickens. Another interesting study by Dieter et al. (2004) showed that serum- and glucocorticoid-dependent kinase 1 (SGK1) whose transcription is stimulated by GCs, regulates the abundance expression of SGLT-1 in the apical cell membrane, stimulating glucose transport. All of this supports our result with the increased expression of SGLT-1 in the DEX group but also demonstrates the inhibitory effect GCs have on glucose uptake by peripheral tissues. We can theorize that while the birds in this group were able to absorb and utilize the glucose coming from the diet, as seen with comparable results of EN, DE digestibility and EN utilization, and AME, AMEn with the Control group, the muscle

"peripheral tissue" was not a priority. This also translates to the N digestibility where the DEX and CocciDex- challenged birds were able to digest and absorb the nitrogen in the diet, but due to the actions of GCs to stimulate hepatic gluconeogenesis from amino acids, increased the production of uric acid (in the case of chickens) hence, the low utilization of N in our results. Similarly, the expression of NaPi-IIb was increased with DEX and CocciDex challenge but decreased with the Cocci challenge. However, NaPi-IIb mRNA expression in the Control and Cocci-challenged birds was not different.

When considering the mechanisms associated with gut functionality, it is important to consider the biology of the pathogens and other possible stress-causing factors, to the host tissues. For example, as part of their life cycle, *Eimeria sp* localizes predominantly in the lumen of the GIT, persisting for extended periods and causing acute to chronic disease depending on several factors. In response to this, the GALT maintains functionality via a conserved elaborate communications system that transmits signals between the host's external physical barrier and the underlying cells of the host's innate and adaptive mucosal immune system (Smith et al, 2014; Fukui, 2016). The resultant effect from this involves the exploitation of host signaling pathways to cause the redistribution of TJ structural proteins and compromise barrier function. Also, infection by *Eimeria sp* induces both the innate and adaptive immune system, which culminates in the activation of several types of immune cells, including lymphocytes, dendritic cells, and macrophages that are close to the mucosal surface (Rose, 1987; Lillehoj and Trout, 1993; Lillehoj and Lillehoj, 2000; Laurent et al., 2001). In this study, epithelial permeability function was determined based on the levels of FITC-d in the serum, a molecule that does not usually leak through an intact gastrointestinal tract barrier (Vicuna et al., 2015). Because of the lack of severity

with the coccidia vaccine challenge, Cocci-challenge alone did not induce significant gut permeability on d 21 and 28 of age, but rather in conjunction with the DEX-challenge, increased the levels of FITC-d in the blood. This is supported by earlier reports, (Latorre et al., 2018; Hernandez-Patlan et al., 2019; Stefanello et al., 2020) where increased levels of FITC-d were observed in birds challenged with *Eimeria* and other infectious agents. However, Schneiders et al. (2019) reported an increase in FITC-d levels 6 days after *Eimeria* inoculation alone but at d 7 to 10 post-inoculation, the FITC-d levels were not different from the control group. As with other studies, the administration of DEX causes alterations in permeability characteristics that are consistent with glucocorticoid-induced changes, increasing the entry of FITC-d into circulation which, demonstrates a compromise to paracellular permeability rather than transcellular transport (Spitz et al., 1994; Kuttappan et al., 2015; Vicuana et al., 2015; Barekatain et al., 2019). Disruption of the mucus layer and TJ complex also increases intestinal permeability. In livestock production, impaired intestinal barrier function leads to reduced animal health and growth performance (Tan et al., 2014; Osho et al., 2019). Therefore, it is critically important to understand how the intestinal barrier function is maintained and regulated to achieve optimal animal health and productivity. The observed changes in epithelial permeability function for both the DEX and CocciDex group can also be explained by the decrease in the expression of MUC-2 on d 21. Although the activity of occludin cannot be explained in this study because, while there were no significant changes in the expression with any challenge type on d 21, its expression was significantly higher than the control on d 28.

One of the evolutionarily conserved receptors of the innate system (TLR4) that recognizes specific molecular patterns associated with microbes has been implicated in the

fight against coccidiosis. Once a TLR binds a molecule from an invading micro-organism or molecules produced by damaged tissues, the downstream signaling activated via the NFκB pathway triggers innate immune defenses such as inflammation, and the acquired immune system, secreting cytokines. Similar to this study, Zhou et al. (2013) and Tan et al. (2014) observed an increase in jejunal TLR4 expression with coccidia challenge on d 21, with the possibility that TLR4 activation might operate via the MyD88-dependent pathway. The involvement of TLR4 in host resistance to *Eimeria sp* infection can also be explained with the increased mRNA expressions of IL-1β, IFN-γ, and IL-6 observed in the jejunum. Extensive experimental evidence provides supports to the role cell-mediated immunity, predominantly mediated by antigen-specific and nonspecific activation of T lymphocytes and macrophages, play to confer protective immunity in avian coccidiosis (Rose, 1987; Lillehoj and Trout, 1993; Lillehoj and Lillehoj, 2000; Dalloul and Lillehoj, 2005). A subsequent decrease in the mRNA expression of TLR4 and NFκB in the jejeunum, on d 28 probably signifies the clearance of the infection however gradual it might be, since the mRNA expression levels of IL-1β, IFN-γ, and IL-6 are still elevated.

Antibodies are involved in the response against coccidia infection, albeit a minor role. Although we observed a reduced jejunal mRNA expression of IgA in the Cocci-challenge birds, secretory IgA antibodies are more likely to be involved in resistance with parasitic infection of the intestinal mucosa (Yun et al., 2000; Tan et al. 2014), and usually appear about a week after *Eimeria sp* infection reach a peak and decline thereafter (Rose, 1987). The spleen weight is assumed to directly correlate with the proliferation of immune cells and often, represents a compensatory response to a need for increased activity during infection and recovery (Panda and Combs, 1964). However, despite the increased spleen

weight observed in this study, it did not translate to increased expression of antibodies especially because the spleen is known to be involved in antibody production. Furthermore, the immune response observed against DEX-challenge birds in the current study was the opposite of that expressed in the Cocci-challenge birds. As supported by literature, DEX inhibits synthesis, release, and efficacy of T lymphocytes, cytokines, and other mediators that promote immune and inflammatory reactions, both in cell culture systems and several animal species (Isobe and Lillehoj, 1993; Sapolsky et al., 2002). As observed in this study, the expression of TLR4, IL-1β, IFN-γ, and IL-6 were reduced on d 21 in the DEX and CocciDex challenge birds, however, their expression in the CocciDex was exacerbated on d28 (except for IL-6). Though it is not clear what immune functions are enhanced with DEX, evidence supports that it triggers the apoptotic death in immature T and B cell precursors and mature T cells evident by the atrophy of the spleen and thymus in some studies (Sapolsky et al., 2002), and increase susceptibility to *Eimeria* infection (Isobe and Lillehoj, 1993).

Several conclusions were drawn from this study. First, the characteristic of the coccidia vaccine challenge effect in terms of performance, digestibility, and intestinal permeability were not observed in this study. Based on our previous experience, this observation may be due to variations in the vaccine batches. However, the challenge induced the expression of inflammatory mediators. Second, the DEX challenge supported the hypothesis based on the impact on performance, intestinal morphology, permeability, digestibility of Ca and P, utilization of N, Ca, and P, and suppressed the expression of inflammatory mediators. Moreover, exposure to another stress factor – like DEX, exacerbated the coccidia challenge increasing the susceptibility of the birds to coccidia

infection as regards to the reduced performance, intestinal permeability observed, and activation of the inflammatory pathway (TLR4) and subsequent expression of proinflammatory cytokines 7-days post-challenge. Third, the addition of Natustat did not mitigate the negative effect of the stressors as hypothesized based on nutrient and energy digestibility and utilization, and intestinal morphology and permeability observations. The supplementation had a tendency to increase the expression of anti-inflammatory cytokine (IL-10) 7-days post-challenge. It also increased IL-10 and decreased the mRNA expression of IL-6, 14-days post-challenge.

4.5 TABLES

Table 4.1 Ingredient composition and analyzed nutrient and energy contents of the experimental diets (g/kg, on an as-fed basis).

Description of diets Ingredients, g/kg	Starter diet (d 0-14)[1]		Grower diet (d 14 - 28)[2]	
	Natustat™ 0 g/kg	Natustat™ 1.0 g/kg	Natustat™ 0 g/kg	Natustat™ 1.0 g/kg
Corn	572.3	562.3	619.7	609.7
Soybean meal, 48%	360.0	360.0	302.0	302.0
Soybean oil	30.0	30.0	35.0	35.0
Limestone (38% Ca)	10.0	10.0	8.6	8.6
Dicalcium phosphate	16.3	16.3	15.4	15.4
NaCl	4.0	4.0	4.0	4.0
Vitamin-mineral premix[2]	2.5	2.5	2.5	2.5
DL-Methionine	2.9	2.9	3.8	3.8
L-Lysine HCL	1.5	1.5	2.9	2.9
L-Threonine	0.5	0.5	1.1	1.1
Natustat premix[3]	0.0	10.0	0.0	10.0
Titanium dioxide	0.0	0.0	5.0	5.0
Total	1,000	1,000	1,000	1,000
Analyzed analysis[4]				
Dry Matter, g/kg			882.0	
Crude protein[5], g/kg			210	
Calcium, g/kg			9.7	
Total phosphorus, g/kg			7.7	
Ca: tP[6], g/kg			1.2	
Non-phytate phosphorus[6], g/kg			4.2	
Gross energy, kcal/kg			4,098	

[1]Dexamethasone was added at 1.5 mg/kg of diet to groups challenged with DEX from d 14 -21

[2]Vitamin-mineral premix supplied per kg of diet: 11 025 IU of vitamin A; 3528 IU of vitamin D; 33 IU of vitamin E; 0.91mg of vitamin K; 2.21 mg of thiamin; 7.72 mg of riboflavin; 55 mg of niacin; 18 mg of pantothenate; 5 mg of vitamin B-6; 0.22 mg d-biotin; 1.10 mg of folic acid; 478 mg of choline; 0.03 mg of vitamin B-12; 75 mg of Zn; 40 mg of Fe; 64 mg of Mn; 10 mg of Cu; 1.85 mg of I; and 0.30 mg of Se.

[3]Natustat™ Alltech, Inc., Nicholasville, KY. The premix was added to the diets at the expense of ground corn to supply 1g of Natustat/kg of diet.

[4]Starter diets were not analyzed for nutrient composition. All the experimental diets were produced from a single basal diet; hence the average of the analyzed nutrients vales was used to calculate nutrient and energy digestibility and utilization.

[5]Nitrogen value multiplied by 6.25

[6]Calculated values

Table 4.2 Primers used in real-time quantitative PCR.

RNA target[1]	Primer sequences (5′-3′)	Accession no.	References
Markers of inflammation			
TLR4	F: AGT CTG AAA TTG CTG AGC TCA AAT	NM_001030693	Tan et al., 2014
	R: GCG ACG TTA AGC CAT GGA AG		
NF-κB	F: GTG TGA AGA AAC GGG AAC TG	NM_205129	Tan et al., 2014
	R: GGC ACG GTT GTC ATA GAT GG		
IL-1β	F GCT CTA CAT GTC GTG TGT GAT G	AJ245728	Shini and Kaiser 2009
	R TGT CGA TGT CCC GCA TGA		
IL-6	F GCT CGC CGG CTT CGA	AJ250838	Shini and Kaiser 2009
	R GGT AGG TCT GAA AGG CGA ACA G		
IFN-γ	F GTG AAG AAG GTG AAA GAT AT CAT GGA	Y07922	Shini and Kaiser 2009
	R GCT TTG CGC TGG ATT CTC A		
IL-10	F CAT GCT GCT GGG CCT GAA	AJ621614	Shini and Kaiser 2009
	R CGT CTC CTT GAT CTG CTT GAT G		
Intestinal transporter			
SGLT-1	F: CATCTTCCGAGATGCTGTCA	ENSGALG0000000672	Adedokun and Adeola 2017
	R: AGGTATCCGCACATCACACA		
NaPi-IIb	F: CTGCAGGACACTGGAGTCAA	NM_204474	Adedokun and Adeola 2017
	R: CCGCAACAGGATTAGAGAGC		
Markers of gut integrity			
MUC-2	F: CAG CAC CAA CTT CTC AGT TC	XM_421035	Tan et al., 2014

Table 4.2 continued			
	R: TCT GCA GCC ACA CAT TCT TT		
IgA	F: ACC ACG GCT CTG ACT GTA CC	S40610	Tan et al., 2014
	R: CGA TGG TCT CCT TCA CAT CA		
Occludin	F ATC AAC GAC CGC CTC AAT CA	NM_205128.1	Cowieson et al., 2017
	R TCC GCT TCA GGT CTT TGA GC		
Housekeeping gene			
GAPDH	F ATG ACC ACT GTC CAT GCC ATC CA	NM_204305.1	Cowieson et al., 2017
	R AGG GAT GAC TTT CCC TAC AGC GTT		

*F, forward primer; R, reverse primer.

[1]mRNA genes analyzed TLR4, toll-like receptor4; NFκB, Nuclear factor κB; IL-1β, Interleukin 1 beta, IL-6, Interleukin 6; IL-10, Interleukin 10; IFN- γ, Interferon γ; NaPi-IIb, sodium-dependent phosphate transporter; SGLT-1, sodium-dependent glucose transporter-1; MUC-2, Mucin-2; IgA, Immunoglobulin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Table 4.3 Effect of dexamethasone and coccidia vaccine challenge with or without dietary Natustat™ supplementation on growth performance of broilers (d 21 and d 28)[1].

Interaction effect		d 0 - 14				14 - 21				21 - 28			
		BW 14 (g)	Gain (g/bird)	FI (g/bird)	G:F (g/kg)	BW 21 (g)	Gain (g/bird)	FI (g/bird)	G:F (g/kg)	BW 28 (g)	Gain (g/bird)	FI (g/bird)	G:F (g/kg)
Challenge effect	Natustat™												
Control	No	-	-	-	-	908	456	596	766	1564	643	920	705
Cocci	No	-	-	-	-	877	443	584	757	1537	643	918	670
DEX	No	-	-	-	-	580	151	511	295	986	402	540	746
CocciDex	No	-	-	-	-	556	119	462	241	946	363	517	703
Control	Yes	-	-	-	-	895	449	592	758	1541	623	920	698
Cocci	Yes	-	-	-	-	869	433	577	750	1512	623	896	695
DEX	Yes	-	-	-	-	578	145	491	296	969	393	527	745
CocciDex	Yes	-	-	-	-	567	121	482	252	963	387	528	732
Challenge effect													
Control		-	-	-	-	902[a]	453[a]	594[a]	762[a]	1553[a]	644[a]	920[a]	701[b]
Cocci		-	-	-	-	873[a]	438[a]	581[a]	756[a]	1524[a]	633[a]	907[a]	697[b]
DEX		-	-	-	-	579[b]	148[b]	501[b]	293[b]	978[b]	398[b]	533[b]	745[a]
CocciDex		-	-	-	-	561[b]	117[c]	472[c]	247[c]	955[b]	375[b]	523[b]	717[ab]
	Natustat™												
	No	434	390	481	811	730	290	538	515	1258	514	724	713
	Yes	439	395	494	803	727	287	536	514	1246	511	718	717
SEM		22.6	21.9	31.2	40.3	13.3	8.7	9.2	9.5	21.4	15.4	17.8	15.1
P-value													
Challenge effect		-	-	-	-	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.102
Natustat™		0.457	0.3240	0.121	0.427	0.759	0.622	0.693	0.912	0.437	0.764	0.658	0.707
Challenge x Natustat™		-	-	-	-	0.815	0.692	0.201	0.729	0.729	0.522	0.791	0.602

[a–c] Means with different superscripts within the same column differ significantly (P < 0.05).

[1]Values represent the means of 7 replicate cages per treatment.

[2]Control = non-challenge; Cocci = 20x coccidia vaccine; DEX = dexamethasone; CocciDex = 20x coccidia vaccine + dexamethasone.

Table 4.4 Effect of dexamethasone and coccidia vaccine challenge with or without dietary Natustat™ supplementation on organ weights relative to body weight in broiler chickens (d 21 and d 28)[1].

Treatment effect[2]		Day 21		Day 28	
		Relative Liver weight (%)	Relative Spleen weight (%)	Relative Liver weight (%)	Relative Spleen weight (%)
Challenge effect	Natustat™				
Control	No	3.03^{x}	0.13	2.35	0.09^{x}
Cocci	No	3.52	0.15	2.62^{x}	0.12
DEX	No	5.93^{x}	0.06	2.44	0.13
CocciDex	No	5.44^{x}	0.09^{x}	2.71	0.17
Control	Yes	3.12	0.13	2.28	0.12
Cocci	Yes	3.31	0.16	2.46	0.10^{x}
DEX	Yes	5.84	0.07^{x}	2.63	0.12^{x}
CocciDex	Yes	5.05^{x}	0.10^{x}	2.70^{x}	0.15^{x}
Challenge effect					
Control		3.13^{c}	0.13^{b}	2.31^{c}	0.11^{b}
Cocci		3.38^{c}	0.16^{a}	2.54^{ab}	0.11^{b}
DEX		5.89^{a}	0.07^{c}	2.53^{ab}	0.13^{ab}
CocciDex		5.25^{b}	0.09^{c}	2.71^{a}	0.15^{a}
	Natustat™				
	No	4.48	0.11	2.53	0.12
	Yes	4.33	0.11	2.52	0.12
Pooled SD[3]		0.51	0.03	0.30	0.03
P-value					
Challenge effect		<.0001	<.0001	0.013	0.001
Natustat™		0.287	0.249	0.881	0.918
Challenge x Natustat™		0.692	0.985	0.467	0.216

$^{a–c}$ Means with different superscripts within the same column differ significantly ($P < 0.05$).

[1]Values represent the means of 7 replicate cages per treatment except for mean values with superscripts x where the number of replicates was 6.

[2]Control = non-challenge; Cocci = 20x coccidia vaccine; DEX = dexamethasone; CocciDex = 20x coccidia vaccine + dexamethasone.

[3]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 4.5 Effect of dexamethasone and coccidia vaccine challenge with or without dietary Natustat™ supplementation on jejunal morphology in 21 and 28-day broiler chickens[1].

Interaction effect		Jejunal Morphology (d 21)			Jejunal Morphology (d 28)		
		Villus Height (µm)	Crypt Depth (µm)	VH: CD	Villus Height (µm)	Crypt Depth (µm)	VH: CD
Challenge effect	Natustat™						
Control	No	1262	153^{x}	9.9	1207^{b}	140	8.7
Cocci	No	1095	236	9.0^{x}	1290$^{ab, x}$	198	6.9^{x}
DEX	No	1078^{x}	144	12.0	1272^{ab}	129^{x}	9.2^{x}
CocciDex	No	1068	164	9.8	1245$^{ab, x}$	183	6.7
Control	Yes	1180^{x}	138	10.7^{x}	1301$^{ab, x}$	146	8.8
Cocci	Yes	1079^{x}	219^{x}	8.2	1364$^{a, x}$	199	7.2
DEX	Yes	1069^{x}	132	10.7^{x}	1159b$^{, x}$	142^{x}	8.3^{x}
CocciDex	Yes	888	148^{x}	8.1	1210$^{b, x}$	180	7.0
Challenge effect							
Control		1221^{a}	145^{b}	10.3^{ab}	1254^{ab}	143^{b}	8.7^{a}
Cocci		1087^{b}	227^{a}	8.6^{c}	1327^{a}	194^{a}	7.0^{b}
DEX		1073^{b}	138^{b}	11.3^{a}	1228^{b}	136^{b}	8.7^{a}
CocciDex		978^{b}	156^{b}	9.0^{bc}	1215^{b}	182^{a}	6.9^{b}
	Natustat™						
	No	1126	174	10.2	1253	161	7.9
	Yes	1054	159	9.4	1258	167	7.8
Pooled SD[3]		114.9	33.5	1.56	82	25.3	1.03
P-value							
Challenge effect		<.0001	<.0001	0.0001	0.008	<.0001	<.0001
Natustat™		0.030	0.114	0.078	0.831	0.381	0.809
Challenge x Natustat		0.203	0.997	0.224	0.008	0.856	0.369

$^{a–c}$ Means with different superscripts within the same row differ significantly ($P < 0.05$).

[1]Values represent means of 7 replicate cages per treatment except for mean values with superscripts x where the number of replicates was 6.

[2]Control = non-challenge; Cocci = 20x coccidia vaccine; DEX = dexamethasone; CocciDex = 20x coccidia vaccine + dexamethasone.

[3]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 4.6 Effect of dexamethasone and coccidia vaccine challenge with or without dietary Natustat™ supplementation on apparent ileal digestibility of dry matter, nitrogen, energy, calcium, phosphorus, and digestible energy in 21 and 28-day broiler chickens[1].

Interaction effect		Apparent ileal digestibility d 21 (%)[3]						Apparent ileal digestibility d 28 (%)[3]					
		DM	N	EN	Ca	P	DE kcal/kg	DM	N	EN	Ca	P	DE kcal/kg
Challenge effect	Natustat™												
Control	No	72.3	83.8^{x}	76.3^{x}	33.6	51.9	3543^{x}	70.7	84.6^{x}	76.2^{x}	41.7^{x}	56.3	3562^{x}
Cocci	No	71.0^{x}	82.7^{x}	73.9	36.0	51.7	3428	69.6	84.0	74.3	38.1	50.1	3487
DEX	No	69.7	83.6^{y}	74.0^{x}	29.1	45.6	3434^{x}	70.5^{x}	84.7	75.8^{x}	51.5^{x}	53.1	3551^{x}
CocciDex	No	67.9	83.6^{y}	71.3	16.7	41.0	3315	70.1	84.1^{x}	75.6	43.0^{x}	49.9	3530
Control	Yes	72.2^{x}	84.3^{x}	75.3x	46.7	54.5^{x}	3497^{x}	69.7	83.0	74.4	48.0^{x}	55.0^{x}	3424
Cocci	Yes	70.3^{x}	82.0	74.2^{x}	43.0	50.7	3445^{x}	69.0	82.1^{x}	72.7	39.9	49.9	3377
DEX	Yes	69.1	82.3^{z}	72.9	31.1	44.7	3379	71.0^{x}	83.5	76.0	58.7^{x}	56.2^{x}	3508
CocciDex	Yes	69.3^{x}	81.4	72.5^{x}	30.3	38.1	3369^{x}	69.5^{x}	82.6^{y}	73.4	47.1	49.0^{x}	3383
Challenge effect													
Control		72.3^{a}	84.1	75.8^{a}	40.1^{a}	53.2^{a}	3520^{a}	70.2	83.8	75.3	44.8^{b}	55.6^{a}	3493
Cocci		70.7^{ab}	82.3	74.0^{ab}	39.5^{a}	51.2^{a}	3437^{ab}	69.3	83.1	73.5	39.0^{b}	54.7^{a}	3432
DEX		69.4^{ab}	82.9	73.4^{ab}	30.1^{b}	45.1^{b}	3407^{ab}	70.8	84.1	75.9	55.1^{a}	50.0^{b}	3530
Cocci/Dex		68.6^{b}	82.5	71.9^{b}	23.5^{b}	39.6^{c}	3342^{b}	69.8	83.4	74.5	45.1^{b}	49.4^{b}	3456
	Natustat™												
	No	70.2	83.4	73.9	28.9	47.5	3430	70.2	84.3	75.5	48.4	52.4	3533
	Yes	70.2	82.5	73.7	37.8	47.0	3423	69.8	82.8	74.1	43.6	52.5	3423
Pooled SD[4]		2.86	2.10	2.50	8.93	4.63	116.6	2.12	1.33	2.13	6.44	2.99	100.3
P-value													
Challenge effect		0.014	0.184	0.003	<.0001	<.0001	0.005	0.356	0.242	0.073	<.0001	<.0001	0.076
Natustat™		0.970	0.152	0.826	0.464	0.681	0.824	0.467	0.253	0.503	0.643	0.748	0.434
Challenge x Natustat™		0.762	0.479	0.591	0.271	0.491	0.595	0.831	0.684	0.613	0.520	0.226	0.546

[a–c] Means with different superscripts within the same row differ significantly ($P < 0.05$).

[1]Values represent means of 7 replicate cages per treatment except for mean values with superscripts x and y where the number of replicates was 6 and 5.

[2]Control = non-challenge; Cocci = 20x coccidia vaccine; DEX = dexamethasone; CocciDex = 20x coccidia vaccine + dexamethasone.

[3]DM = dry matter; N = nitrogen; EN= energy; Ca = calcium; P = phosphorus; DE = ileal digestibile energy

[4]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 4.7 Effect of dexamethasone and coccidia vaccine challenge with or without dietary Natustat™ supplementation on total tract utilization of dry matter, nitrogen, energy, calcium, phosphorus, and metabolizable energy and nitrogen-corrected metabolizable energy in 21 and 28-day broiler chickens[1].

.		Total tract utilization d 21							Total tract utilization d 28						
Interaction effect		DM	N	EN	Ca	P	AME	AMEn	DM	N	EN	Ca	P	AME	AMEn
Challenge effect	Natustat™														
Control	No	73.9	69.0	78.3	51.6	49.3	3635	3539	73.9	71.0^{x}	78.4^{ab}	41.9	40.4	3637^{ab}	3548^{a}
Cocci	No	70.7^{x}	67.2^{x}	75.0^{x}	46.7	43.6	3483^{x}	3380^{x}	70.9	67.5^{x}	74.9^{e}	44.1	47.1	3475^{e}	3371^{e}
DEX	No	74.7^{x}	39.5^{x}	79.8^{x}	41.7^{x}	36.2^{x}	3708^{x}	3520^{x}	74.7^{x}	73.0	78.6^{a}	45.5^{x}	47.7	3650^{a}	3566^{a}
CocciDex	No	63.3^{x}	20.1	71.8^{x}	23.1	8.33^{x}	3330^{x}	3085^{x}	73.2^{x}	70.4	77.0^{bcd}	35.2^{x}	46.1	3581^{bdc}	3504^{abc,x}
Control	Yes	72.6	67.3^{x}	77.2^{x}	52.4	49.5^{x}	3579^{x}	3478^{x}	72.3	68.3^{x}	76.9^{cd}	44.3^{x}	42.3^{x}	3570^{cd}	3473^{bcd}
Cocci	Yes	70.5^{x}	67.2^{x}	75.2^{x}	40.8^{x}	40.0^{x}	3484^{x}	3381^{x}	71.3^{x}	67.6	75.5$^{de, x}$	47.6	47.7	3512$^{de, x}$	3414^{de,x}
DEX	Yes	73.7^{x}	42.8^{x}	79.5^{x}	46.7^{x}	39.3	3692^{x}	3518	73.7	72.0^{x}	78.1^{abc}	45.0	47.6	3630^{abc}	3540^{ab}
CocciDex	Yes	63.4	16.4	71.6	27.3	11.6	3328	3066	72.1	71.3	76.2$^{de, x}$	37.6	44.8^{x}	3549$^{d, x}$	3457^{cd,x}
Challenge effect															
Control		73.2^{a}	68.2^{a}	77.7^{b}	52.0^{a}	49.4^{a}	3607^{b}	3508^{a}	73.1^{ab}	69.7^{b}	77.6^{a}	43.1^{a}	41.4^{b}	3603^{ab}	3511^{b}
Cocci		70.6^{b}	67.2^{a}	75.1^{c}	43.8^{b}	41.8^{b}	3484^{c}	3381^{b}	71.1^{c}	67.5^{c}	75.2^{c}	45.9^{a}	47.4^{a}	3494^{c}	3392^{c}
DEX		74.2^{a}	41.2^{b}	79.7^{a}	44.2^{b}	37.7^{b}	3700^{a}	3519^{a}	74.2^{a}	72.5^{a}	78.4^{a}	45.3^{a}	47.6^{a}	3640^{a}	3553^{a}
CocciDex		63.4^{c}	18.3^{c}	71.7^{d}	25.2^{c}	9.9^{c}	3329^{d}	3075^{c}	72.7^{b}	70.9^{ab}	76.6^{b}	36.4^{b}	45.5^{ab}	3565^{b}	3481^{b}
	Natustat™														
	No	70.6	49.0	76.6	40.8	34.3	3539	3381	73.3	70.5	77.2	41.7	45.4	3629	3497
	Yes	70.0	48.4	75.4	41.8	35.1	3521	3361	72.4	69.8	76.7	43.6	45.6	3522	3471
Pooled SD[4]		1.15	4.19	0.84	5.56	4.09	38.07	46.9	1.06	1.76	0.89	5.39	4.51	39.1	41.0
P-value															
Challenge effect		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0001	0.003	<.0001	<.0001
Supplement		0.076	0.666	0.114	0.505	0.513	0.097	0.138	0.0110	0.168	0.029	0.195	0.836	0.057	0.026
Challenge x Supplement		0.373	0.199	0.299	0.069	0.131	0.220	0.317	0.098	0.081	0.020	0.805	0.834	0.012	0.005

[a–e] Means with different superscripts within the same row differ significantly ($P < 0.05$).

[1]Values represent means of 7 replicate cages per treatment except for mean values with superscripts x where the number of replicates was 6.

[2]Control = non-challenge; Cocci = 20x coccidia vaccine; DEX = dexamethasone; CocciDex = 20x coccidia vaccine + dexamethasone.

[3]DM = dry matter; N = nitrogen; EN= energy; Ca = calcium; P = phosphorus; AME = apparent metabolizable energy; AMEn = nitrogen-corrected apparent metabolizable energy

[4]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 4.8 Effect of dexamethasone and coccidia vaccine challenge with or without dietary Natustat™ supplementation on serum fluorescein isothiocyanate dextran (FITC-d) concentration in 21 and 28 broiler chickens[1].

Treatment effect[2]		FITC-d concentration (ng/mL)	
		Day 21	Day 28
Challenge effect	Natustat™		
Control	No	536.0[z]	903.7
Cocci	No	644.2[x]	982.6
DEX	No	1266.7	995.0[x]
CocciDex	No	2236.9	1127.7[x]
Control	Yes	471.7[x]	892.6
Cocci	Yes	516.3	836.5[x]
DEX	Yes	1318.4	1037.8[x]
CocciDex	Yes	2254.7[x]	1078.5[x]
Challenge effect			
Control		503.8[c]	898.1[b]
Cocci		580.2[c]	909.5[b]
DEX		1292.6[b]	1016.4[ab]
CocciDex		2245.8[a]	1103.1[a]
	Natustat™	1170.9	1002.2
	No	1140.3	961.4
	Yes		
Pooled SD[3]		374.5	127.3
P-value			
Challenge effect		<.0001	0.005
Natustat™		0.750	0.250
Challenge x Natustat™		0.900	0.308

[a–c] Means with different superscripts within the same row differ significantly ($P < 0.05$).

[1]Values represent means of 7 replicate cages per treatment except for mean values with superscripts x where the number of replicates was 6.

[2]Control = non-challenge; Cocci = 20x coccidia vaccine; DEX = dexamethasone; CocciDex = 20x coccidia vaccine + dexamethasone.

[3]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 4.9 Effect of dexamethasone and coccidia vaccine challenge with or without dietary Natustat™ supplementation on mRNA expression of genes analyzed from the jejunal mucosa in 21 day-old broiler chicken[1].

Treatment effect[2]		Inflammation-related genes[3]						Intestinal transporters[3]		Markers of gut integrity[3]		
		TLR4	NF-κB	IL-1	IL-6	IFN-γ	IL-10	SGLT-1	NaPi-IIb	MUC-2	Occludin	IgA
Challenge effect	Natustat™											
Control	No	0.93^{x}	1.27^{x}	1.32	1.28	1.01^{x}	0.82^{x}	1.14^{ab}	0.98	0.93^{x}	1.07^{x}	1.23^{x}
Cocci	No	2.22^{x}	1.16	2.60	1.51^{x}	3.57	1.66	0.77^{bc}	0.72^{x}	0.48	1.72	0.61
DEX	No	0.47^{x}	1.09	0.80	0.04^{x}	0.09^{x}	1.41	1.05^{bc}	1.91	0.56^{x}	1.26^{x}	1.90^{x}
CocciDex	No	0.38^{x}	0.87	1.49	0.13^{x}	1.46	1.18	0.72$^{bc, x}$	1.49	0.43^{x}	1.29^{x}	0.51^{x}
Control	Yes	0.90^{x}	0.92	1.03	1.09	0.83^{x}	1.39	1.12$^{abc, x}$	1.00^{x}	1.04	1.01^{x}	0.79^{x}
Cocci	Yes	1.74	1.02^{x}	1.91^{x}	1.95	2.45	1.91	0.56$^{c, x}$	0.68	0.53	0.92	0.60^{x}
DEX	Yes	0.47^{x}	0.85	0.71	0.28^{x}	0.28^{x}	1.30^{x}	0.97$^{bc, x}$	1.55^{x}	0.71	1.16^{x}	1.36
CocciDex	Yes	0.52^{x}	1.10^{x}	1.98^{x}	0.05^{x}	2.25^{y}	1.78	1.60$^{a, x}$	1.95^{x}	0.73	1.88^{x}	0.63^{x}
Challenge effect												
Control		0.91^{b}	1.09	1.18^{b}	1.18^{a}	0.92^{bc}	1.10	1.13^{a}	0.99^{b}	0.99^{a}	1.04	1.01^{ab}
Cocci		1.98^{a}	1.09	2.25^{a}	1.73^{a}	3.01^{a}	1.79	0.66^{b}	0.70^{b}	0.63^{b}	1.32	0.61^{b}
DEX		0.47^{b}	0.97	0.76^{b}	0.16^{b}	0.19^{c}	1.35	1.01^{a}	1.73^{a}	0.58^{b}	1.21	1.63^{a}
CocciDex		0.45^{b}	0.99	1.73^{ab}	0.09^{a}	1.86^{ab}	1.48	1.16^{a}	1.72^{a}	0.50^{b}	1.58	0.57^{b}
	Natustat™											
	No	1.00	1.10	1.55	0.74	1.53	1.27	0.92	1.28	0.60	1.33	1.06
	Yes	0.91	0.97	1.41	0.84	1.45	1.59	1.06	1.29	0.75	1.24	0.85
Pooled SD[4]		0.59	0.33	1.01	0.59	1.05	0.67	0.31	0.64	0.25	0.85	0.73
P-value												
Challenge effect		<.0001	0.646	0.002	<.0001	<.0001	0.072	0.008	0.0001	<.0001	0.470	0.002
Natustat™		0.565	0.178	0.612	0.553	0.798	0..076	0.105	0.916	0.032	0.703	0.301
Challenge x Natustat™		0.512	0.147	0.511	0.491	0.167	0.476	0.0003	0.437	0.597	0.248	0.623

[a–c] Means with different superscripts within the same row differ significantly (P < 0.05)

[1]Values represent means of 7 replicate cages per treatment except for mean values with x and y where the number of replicates was 6 and 5, respectively.

[2] Control = non-challenge; Cocci = 20x coccidia vaccine; DEX = dexamethasone; CocciDex = 20x coccidia vaccine + dexamethasone.

[3] TLR4, toll-like receptor 4; IL-1β, Interleukin 1 beta, IL-6, Interleukin 6; IL-10, Interleukin 10; IFN- γ, Interferon γ; NaPi-IIb, sodium-dependent phosphate transporter; SGLT-1, sodium-dependent glucose transporter-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MUC-2, mucin-2; IgA, immunoglobulin A

[4]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

Table 4.10 Effect of dexamethasone and coccidia vaccine challenge with or without dietary Natustat™ supplementation on mRNA expression of genes analyzed from the jejunal mucosa in 28-day-old broiler chicken[1].

Treatment effect[2]		Inflammation-related genes[3]						Intestinal transporters[3]		Markers of gut integrity[3]		
		TLR4	NF-κB	IL-1β	IL-6	IFN-γ	IL-10	SGLT-1	NaPi-IIb	MUC-2	Occludin	IgA
Challenge effect	Natustat™											
Control	No	1.14	1.27^{x}	1.41^{x}	$1.44^{bc, x}$	1.35	1.16^{bc}	0.97	0.89^{x}	1.27	0.99	1.09^{x}
Cocci	No	0.80^{x}	1.19	0.86	3.40^{a}	1.67	1.29^{bc}	0.90	1.25	1.26^{x}	1.18	0.93
DEX	No	1.45	1.13	0.68^{x}	$1.72^{b, x}$	0.90	$0.91^{c, x}$	1.36^{x}	1.87	1.15	1.32	0.98^{x}
CocciDex	No	1.56^{x}	2.48	2.06^{y}	$1.25^{bc, x}$	1.45^{x}	$1.70^{abc, y}$	2.36^{x}	2.50	1.04	1.48^{x}	1.31^{y}
Control	Yes	0.93^{x}	0.99^{x}	0.90	$0.74^{c, x}$	0.81^{x}	1.02^{c}	1.22^{x}	1.15	0.99	0.99^{x}	1.91
Cocci	Yes	1.09^{x}	1.58^{x}	1.40^{x}	$1.56^{bc, x}$	1.79	1.89^{b}	1.55	1.44^{x}	1.38	1.50	0.95^{x}
DEX	Yes	1.60	1.07^{x}	0.40^{x}	$1.50^{bc, x}$	0.97	1.01^{c}	1.56	2.20	1.55	1.31^{x}	1.00
CocciDex	Yes	1.07^{x}	2.35	1.54^{x}	1.49^{bc}	1.48	$2.54^{a, x}$	2.39	2.55^{x}	0.85	1.44	2.13
Challenge effect												
Control		1.03^{b}	1.13^{b}	1.15^{ab}	1.09^{b}	1.08^{bc}	1.09^{c}	1.10^{b}	1.12^{b}	1.13	0.99^{b}	1.50
Cocci		0.94^{b}	1.38^{b}	1.13^{ab}	2.48^{a}	1.73^{a}	1.59^{b}	1.23^{b}	1.34^{b}	1.32	1.34^{a}	0.94
DEX		1.52^{a}	1.10^{b}	0.54^{b}	1.61^{ab}	0.94^{bc}	0.96^{c}	1.46^{b}	2.04^{a}	1.35	1.32^{a}	0.99
CocciDex		1.32^{ab}	2.41^{a}	1.80^{a}	1.37^{b}	1.46^{ab}	2.12^{a}	2.37^{a}	2.52^{a}	0.95	1.46^{a}	1.72
	Natustat™											
	No	1.24	1.52	1.25	1.95	1.34	1.27	1.4	1.63	1.18	1.25	1.08
	Yes	1.17	1.5	1.06	1.33	1.26	1.62	1.68	1.83	1.19	1.31	1.5
Pooled SD		0.47	0.55	0.63	0.83	0.56	0.47	0.48	0.57	0.47	0.34	1.15
P-value												
Challenge effect		0.011	<.0001	0.0004	0.001	0.002	<.0001	<.0001	<.0001	0.104	0.007	0.261
Natustat™		0.614	0.893	0.302	0.011	0.595	0.011	0.037	0.195	0.918	0.492	0.201
Challenge x Natustat™		0.164	0.467	0.138	0.021	0.403	0.048	0.402	0.930	0.226	0.476	0.678

[a–c] Means with different superscripts within the same row differ significantly ($P < 0.05$).

[1]Values represent means of 7 replicate cages per treatment except for mean values with superscripts x and y where the number of replicates was 6 and 5, respectively.

[2]Control = non-challenge; Cocci = 20x coccidia vaccine; DEX = dexamethasone; CocciDex = 20x coccidia vaccine + dexamethasone.

[3]TLR4, toll-like receptor-4; IL-1β, Interleukin 1 beta, IL-6, Interleukin 6; IL-10, Interleukin 10; IFN- γ, Interferon γ; NaPi-IIb, sodium-dependent phosphate transporter; SGLT-1, sodium-dependent glucose transporter-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MUC-2, mucin-2; IgA, immunoglobulin A.

[4]SEM can be calculated from the pooled SD: $SEM = \frac{SD}{\sqrt{n}}$

4.6 FIGURE

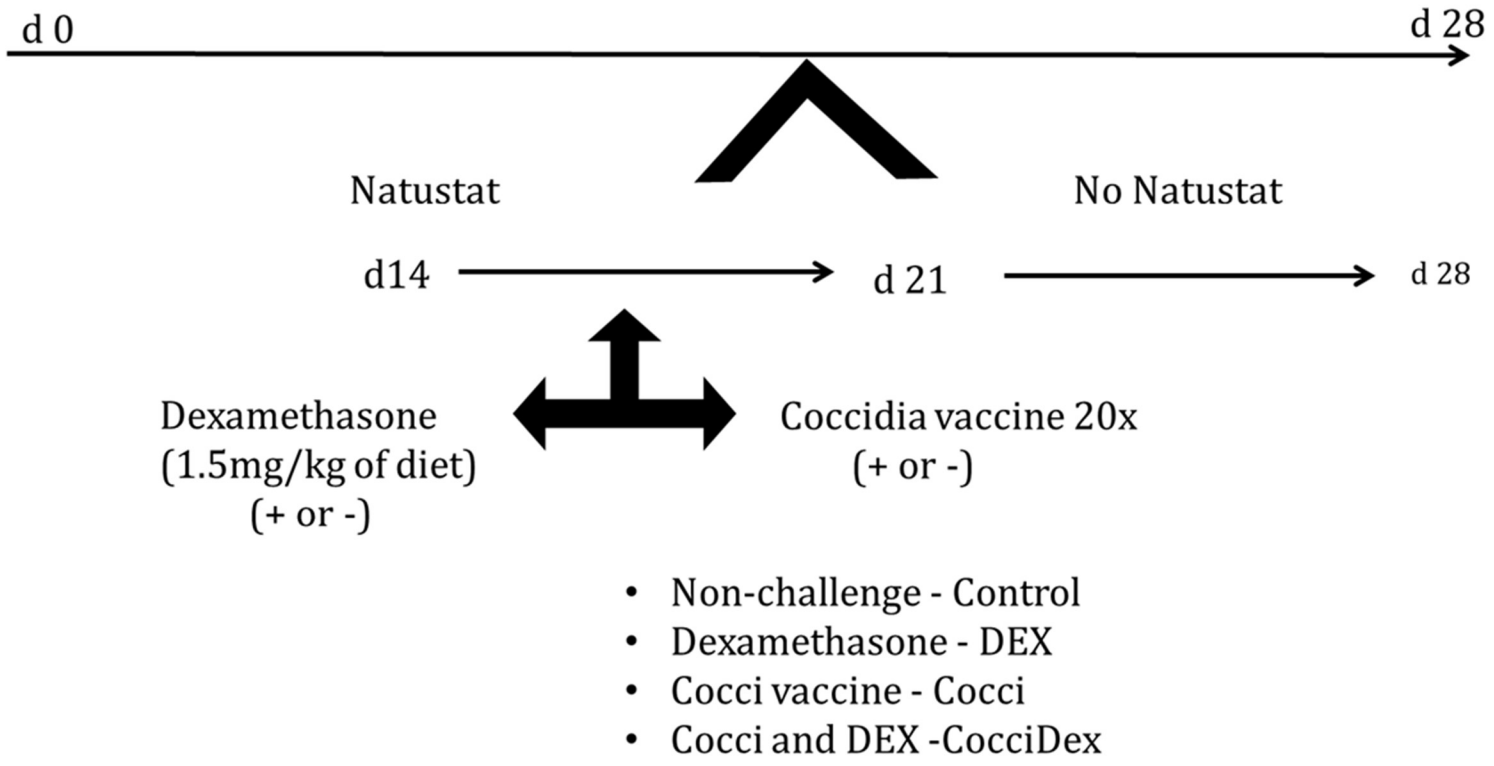

Figure 4.1 A graphical illustration of the experimental design.

CHAPTER 5. SUMMARY AND CONCLUSION

Host response to stressors and enteric parasites are extremely complex and involve different effector mechanisms depending on the intensity and duration of the stress, prior host exposure to the parasite or infection, stage of parasite development, the nutritional status of infected chickens, and the genetic makeup of the host. Unfortunately, we cannot measure all components of the system simultaneously and must rely on measurements of only a few of them in any one study. Because a healthy intestine is necessary for adequate nutrient digestion, enteric diseases such as coccidiosis or transient stress factors on the farm that compromise the integrity of the intestinal tract, are of great importance when considering how to improve intestinal health through nutrition. While good nutrition is important, it is equally important to understand the role of nutrition in stressful conditions. The question this dissertation tried to answer is, how do certain feed additives ameliorate the impact of disease or stress conditions in poultry? The research conducted in this dissertation used stress-causing factors such as dexamethasone (oral or in-feed applications) and coccidia vaccine challenge in broilers, or heat stress in laying hens, with the addition of specific feed additives to answer the above question. In the broiler studies performance (BWG, feed intake, and feed efficiency), digestibility (ileal digestibility and total tract utilization of energy and nutrients), intestinal morphology, intestinal permeability (FITC-d marker, tight junction proteins), and immune response (cytokines, chemokines using RT-PCR) were evaluated (chapters 2 and 4). In the laying hen study, production parameters, egg quality, blood metabolites intestinal morphology, keel bone,

bone-breaking strength, and bone ash at different environmental temperatures were evaluated (chapter 3).

In the first experiment (Chapter 2), broilers challenged with DEX (via oral gavage) on alternate days (d 16, 18, and 20) had increased levels of CORT in the plasma which marks the disruption in homeostasis. The BWG and FE were also decreased, however, nutrient and energy digestibility and utilization were not impaired. This suggests that the birds were able to absorb and utilize the nutrients but because of the presence of CORT, those nutrients were diverted to other processes, glucose uptake was inhibited, energy storage was limited. Similarly, N digestibility was not impaired, but its utilization was, contributing to the diversion of glucose use and increase favor towards gluconeogenesis hence, a reduction in the utilization of nitrogenous compounds is expected. Antioxidant status (SOD and CAT), bone-breaking strength, bone ash percent, immune response, and tight junction proteins were not affected by the DEX challenge. Some contributing factors could be the route of DEX administration and the intensity of dosage (once, intermittent, or continuous exposure) which might play a role in its severity. Two sodium sources were added to the diet to help maintain acid-base balance. The GC action can increase urinary electrolyte excretion, which might lead to electrolyte deficiencies during physiological stress and affect the digestibility of nutrients. In this study, the sodium sources did not improve any of the parameters measured. A potential reason could be the absence of pH change in the digestive tract with the addition of $NaHCO_3$. Several reports have shown that digestive enzymes function differently based on the pH of the GIT, which can in part explain the lack of significance in the performance and nutrient and energy digestibility observations in this dissertation. Similarly, EcoE supplementation only decreased CORT

plasma levels and increase nutrient and energy utilization in broiler chickens in the non-challenge birds. It failed to mitigate the negative effect of the stressor.

To further understand the dynamics of stressors and the application of a feed additive on performance, ileal nutrient and energy digestibility, gut permeability, and immune response in broiler chickens, study 3 (Chapter 4) was conducted. Intestinal permeability dysfunction was determined using the FITC-d marker. The stressors applied in this study were DEX added at 1.5 mg/kg of diet (fed to the birds for 7 days) and coccidia vaccine administered at 20 times the recommended dosage for day-old chicks via oral gavage. A combination of the two stressors was also introduced (CocciDex). The coccidia vaccine challenge did not affect BWG, FI, FE, AID of DM, N, EN, Ca, and P, and DE, and intestinal permeability on d 21 and 28. This is an unusual result because one of the reasons why the vaccine is used as a challenge model in poultry nutrition research studies is because its use on the farm often results in a decline in these (BWG, FI, FE) parameters which, are unfavorable to the producer. Hence, research studies are developed using this challenge model to understand some of the biological functions impaired in the first few days of use. On the other hand, the coccidia vaccine challenge reduced the TTR DM, EN, Ca, P, AME, and AMEn on d 21, and DM, N, EN, AME, and AMEn on d 28. The challenge also increased the jejunal mRNA expression of TLR4 and the expression of other inflammatory mediators. The increase in IL-1β, IFN-γ, and IL-6 mRNA expression levels can also be attributed to TLR4 involvement in protecting the host against *Eimeria sp* infection. The immune system produces various mediators that either act directly to destroy invading microbes, or act on other cells to propagate the immune response. The expression of these inflammatory mediators suggests the birds' immune system was activated against the

Eimeria sp. However, the cost of activating these mediators was negligible, as such, did not affect performance and gut integrity. The 20X dose of the vaccine used in this study has consistently resulted in a significant reduction in performance, however, based on our experience, in about 20% of the time, 20X dose has resulted in very mild or no significant effect on performance. Although difficult to explain, this effect could be associated with variations in the batch of vaccine.

In contrast, DEX and CocciDex challenge reduced BWG, FI, and FE and this reduction is more evident in the CocciDex challenge birds on d 21. Birds are exposed to a variety of stressors in the industry, it stands to reason that the combined effect can exacerbate an infectious disease as seen in this study. The absence of DEX or a stressor, whatever the case may be, that the birds are exposed to, can improve the BWG and FI but might not be sufficient to revert to the control. Like the first study, DEX did not affect AID of DM, EN, and DE on d 21. We observed a decrease in Ca and P digestibility and no effect on N digestibility. The CocciDex challenge significantly impaired ileal DM, EN, Ca, and P digestibility, and AME on d 21 with a significant improvement observed with DEX challenge on ileal Ca and P digestibility by d 28. Similarly, N, Ca, and P were poorly utilized with DEX and DM, N, EN, Ca, and P utilization, and AME and AMEn were impaired even further with CocciDex on d 21. These parameters improved post-challenge (d 28), with a complete recovery observed in the DEX challenge when compared to the Control. By equating the effect on DEX with any type of stressor the bird might encounter in the field that affects Ca and P digestion, it is safe to say that the implications can be great. Since most of the total body Ca is stored in the bone, the negative balance from decreasing gastrointestinal Ca absorption, increasing bone resorption, and increasing renal

Ca excretion can increase skeletal disorders in poultry, and can be exacerbated by an infection. As with other studies, the administration of DEX causes alterations in permeability characteristics that are consistent with glucocorticoid-induced changes, increasing the entry of FITC-d into circulation which, demonstrates a compromise to paracellular permeability rather than transcellular transport. Therefore, it is critically important to understand how the intestinal barrier function is maintained and regulated to achieve optimal animal health and productivity. Further insults to the intestinal permeability from DEX and CocciDex challenge is evident in the decreased expression of MUC-2 on d 21.

Unlike the coccidia challenge where the expression of inflammatory mediators was enhanced, the DEX challenge reduced the mRNA expression of TLR4, IL-1β, IFN-γ, IL- 6. This predisposes the bird to insults without protection from the immune system. The proposed hypothesis was that Natustat™ supplementation would mitigate the effect of the stressor, DEX, and coccidia vaccine. However, the feed additive did not affect performance, nutrient and energy digestion and absorption, intestinal morphology, and intestinal permeability (FITC-d levels). The reason for this is unknown and limited conclusions can be drawn because of the limited information about the product. However, the supplementation of Natustat™ had a tendency to increase the expression of anti-inflammatory cytokine (IL-10) 7-days post-challenge. It also increased IL-10 and decreased the mRNA expression of IL-6, 14-days post-challenge. Overall, exposure to compounding stressors can exacerbate the negative effects the birds encounter at the farm. Stressors that induce the secretion of CORT can adversely affect the development of the

bird and render them vulnerable to other stress factors since inflammatory mediators can be depressed.

Examining another form of stress in poultry production, study 2 (Chapter 3) investigated the effect of exposing laying hens to different temperature regimen on egg quality, performance, blood metabolites, keel bone damage and bone parameters, and mRNA expression of heat shock proteins and the mitigating actions of supplementing two sodium sources (NaCl or NaCl+$NaHCO_3$) and EconomasE™. Overall, the dietary treatment did not improve performance, egg quality, intestinal morphology, keel bone, bone-breaking strength, and HSP 70 and 90 during ET1 and ET2. Exceptions to this were the increase in albumen height and Haugh unit with EcoE and NaCl+$NaHCO_3$ during TN2 and EcoE alone during ET2 regimens, which suggests that the supplementation can improve the fresh appearance of the egg during ET conditions. Similarly, NaCl+$NaHCO_3$ as the sodium source helped circumvent the effects of respiratory alkalosis by reducing Cl^- levels and increasing HCO_3^- during the ET regimen. In normal temperature conditions, EcoE and NaCl+$NaHCO_3$ diet were able to improve VH and VH: CD which suggests that morphological characteristics can be maintained with the use of these supplements and possibly improve the absorption of nutrients.

Collectively, data from these experiments illustrate that stress factors impact several metabolic and biological responses in the bird. We showed that the cost of the stress response is offset by enhanced immunosurveillance and higher biological priority for the animal to fight off the infection to survive as observed in the coccidia challenge birds. The DEX challenge model successfully suppressed growth, immune response, and increase intestinal perturbation. Coccidia challenge though had no limiting effect on performance

but activated an immune response to elicit protection. It will be relevant to quantify the oocyst in the excreta because several observations reflects that the level of the effect of coccidia vaccine challenge varies from batch to batch and from experiment to experiment. The utilization of nutrients and energy was impaired with the coccidia challenge emphasizing its economic importance, and the need for modifications during coccidia challenge to limit wastage of nutrients. Similarly, with the DEX challenge, it could be surmised that by reducing the level of protein in the diet, or including more dietary glucogenic amino acids in the diet during stressful conditions, the glucose production burden placed on skeletal muscle during stress is lessened, CORT-driven catabolism is decreased, and protein wastage is reduced which is sustainable to the environment. This is based on the reduction in N utilization and increased uric acid excretion associated with DEX challenge models. Further research should be done to understand why the DEX challenge model and the *Eimeria* challenge immune response diverge in terms of activated immune cells. This can be focused on the endocrine and immune molecular cross-talk in avian species, especially pathways by which innate and acquired cells are involved in the stress response.

It is important to note that the reports from this dissertation, illustrate some of the biological responses associated with specific stress models. In conjunction with other published studies, the results observed (DEX- suppression of inflammatory responses, depression of performance parameters, nutrient utilization, etc. or Coccidia vaccine challenge - activation of inflammatory mediators, reduction in utilization of nutrients, etc.) can serve as a road map to understanding the complexities of these stressors in the poultry management. It also highlights the specific areas nutritional strategies can target to modify

the negative effects of the stressor. Future research with EconomasE™ supplementation can incorporate different levels in the diet to understand its effects on the status of the antioxidant / free radical ratio. Also, the level of free radical production can be influenced by a variety of factors. For example, the type or concentration of an antigen may affect the antioxidant status of an animal. Hence, the benefits associated with EcoE might depend on the type and level of the antigen, and it would be desirable to evaluate the relationship between the type or/and level of the antigen, cellular free radical status, and the presence of the immunomodulatory effects. Also, more research should be done using the feed additives used in this study, especially for a longer duration post-challenge. By extending the length of the study to market age, 42, or 56 days, the birds might benefit from diets supplemented with the feed additives for longer periods. This may improve performance the absorption and utilization of nutrients, and enhance immunomodulatory actions.

APPENDICES

APPENDIX 1. Analysis of intestinal perturbation

Intestinal health is crucial for the general health and well-being of animals and humans alike. In farm animals, feed intake and the efficient absorption of nutrients are very much determined by the health status of the GIT. Currently, the most direct method to quantitatively assess the intestinal barrier function is the measurement of intestinal permeability. This is assessed noninvasively in vivo by measuring serum/plasma levels of orally administered test substances. The two protocols described below provides a specific index of intestinal permeability. We adopted the second method in one of our studies (chapter 4).

A colorimetric micro-method for d-xylose in serum

Scope: D-Xylose is a pentose sugar absorbed by the upper small intestine primarily by passive transport, like the sodium-dependent active transport of glucose and amino acids (Goodwin et al., 1984; Doerfler et al., 2000). However, it is usually excreted in the urine because the body does not metabolize it properly. Because urine collection can be difficult in chickens, change in plasma xylose levels are indicative of its absorption from the intestinal tract.

This method describes a procedure for xylose absorption test as an index of the small intestinal function

Assay protocol

Color reagent:

- 0.5g of Phloroglucinol

- 100mL of concentrated acetic acid
- 10mL of concentrated HCL

Prepare color reagent:

Dissolve 0.5g of phloroglucinol in 100ml of concentrated acetic acid (AcOH) and 10 ml of concentrated hydrochloric acid (HCL). Use caution when working with strong acids.

Xylose reagent:

a) Benzoic acid
b) Heat block
c) Spectrophotometer
d) Flow-through sampling device

Prepare d-xylose standard dilutions

Xylose assay:

a) 50µL Serum/plasma (undiluted)
b) Standard solution
c) Blank solutions

Prepare samples:

- In duplicates add 50µL serum/plasma, xylose standards, and blanks into disposable test tubes
- Add 5 ml of phloroglucinol color reagent
- Heat all tubes for exactly 4 mins at 100°C, then cool at room temperature in water
- Mix well and read the absorbances at 540nm

Serum FITC-d Assay Protocol (Duff et al. (2019)

Scope: Recent publications describing both poultry and rodent models of enteric inflammation and leaky gut have described FITC-d as a marker of enteric leakage (Brandl et al., 2009; Yan et al., 2009; Kuttappan et al., 2015; Vicuna et al., 2015; Duff et al., 2019).

Reagent and supplies:

- Black 96- well plate
- 5-50 multichannel pipette
- 50-300 multichannel pipette
- 20-200 pipette
- Pipette tip
- Plate/sample layout
- 1X PBS
- FITC-d standard curve
- Negative serum
- FITC-d serum

Prepare FITC-d standard dilutions

(Samples ran are diluted 1:4)

Standard Curve:

1. 30µL of PBS
2. 20µL of negative serum
3. 50µL of standard curve dilutions

Sample Wells:

1. 80µL of PBS
2. 20µL of FITC-d serum

Negative Control Wells:

1. 80µL of PBS
2. 20µL of negative serum

The Plate Reader

1. Create a new experiment using an existing protocol
2. Read plates using the Gain 70 and 80 protocol
3. Export "statistics" read (plate layout read additionally if desired) to Excel

Guidelines

- Always work in a low light environment
- Entering specific plate layouts and sample IDs into the GEN5 software can speed up later analysis
- Check that samples are evenly drawn up across multichannel pipettes
- Running samples in duplicate are preferred
- Warm up the bulb in the machine before loading FITC-d serum in the first plate
- Each plate ran needs to have a negative control
- If running only one sampling period (i.e. all d7 serum), one standard curve can be used for multiple plates If multiple sampling periods are running (i.e. d7 serum, d14 serum, etc), a new standard and negative control should be made for each period using the respective negative serum
- If working with multiple plates, prep with PBS and negative serum all at once then cover with respective plate ID lids until all are ready for the addition of FITC-d serum.

APPENDIX 2. Photo- characteristic effect of DEX on chicken excreta consistency

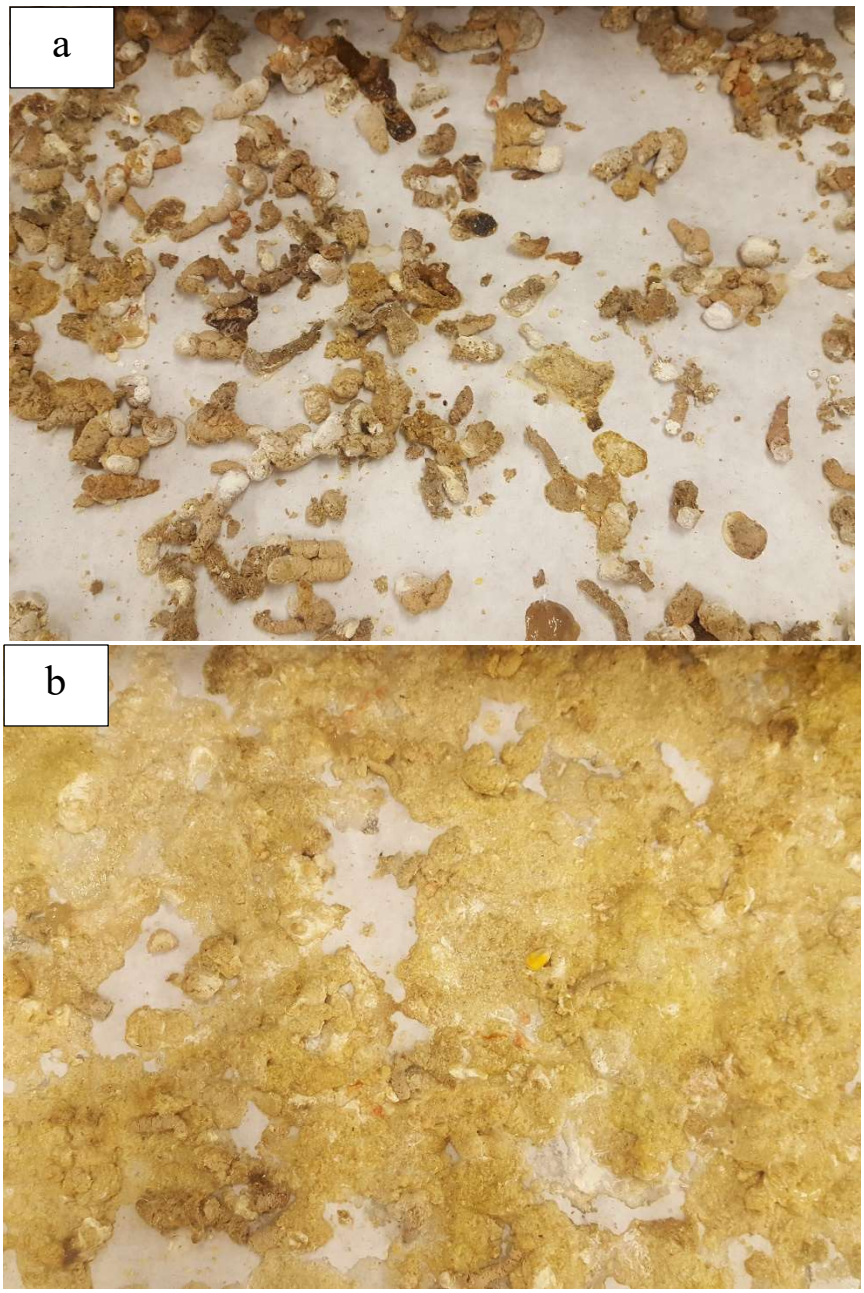

Figure A 5.1 Photo of excreta collected from birds challenged with or without dexamethasone a). birds were not challenged with dexamethasone b). birds were challenged with dexamethasone

APPENDIX 3. Analysis of layer study (Chapter 3)

The second study of this dissertation was focused on the effect of heat stress on laying hen performance among other things. We were posed with certain limitations with regards to running the experiment concurrently with birds in different rooms and exposed to different temperatures (normal room temperature and elevated room temperature). For one, there was limited room availability, and secondly, a question was raised on how we can run the experiment with birds in different rooms. Hence, the protocol was adjusted such that the experiment was conducted with the birds exposed to an environmental temperature at a time. For every 5 weeks, throughout 15 weeks, the laying hens were either exposed to normal temperature (23.8 ℃), elevated temperature (32.2 ℃), and returned to normal temperature (23.8 ℃). After a week break, the laying hens were exposed to the different environmental temperatures following the above trends. Within each temperature regimen, the effects of dietary treatments (with or without EconomasE™ and the addition of NaCl or NaCl+$NaHCO_3$ as the source of inorganic Na source) were studied. The experimental temperature regimen is as follows; Phase 1 [TN1 (23.2℃; 26-30 weeks), ET1 (32.2℃; 33 – 37 weeks), REC1 (23.2℃; 38 – 42 weeks), and Phase 2 TN2 (23.2℃; 44 - 48 weeks), ET2 (32.2℃; 49 - 53 weeks), REC2 (23.2℃; 54 -58 weeks)]. Hence, statistical analysis was conducted for all parameters within each temperature regimen as described in Chapter 3- section statistical analysis.

Alternative statistical analysis

Another suggested statistical analysis would be to analyze the data differently. As a refresher, the experimental unit for the performance parameters (body weight, feed intake, feed conversion, egg production), and egg quality parameters was a replicate

consisting of two adjacently caged laying hens (3 adjacent cages; top and bottom tier) fed as a group, with a total of 9 replicates. While the experimental unit for the blood parameters, histology, gene expression was a single bird from 6 replicates rather than 9 replicates. Because the production and egg quality data were collected from the same group of hens over time, a MIXED model of SAS with repeated measures can be considered as another option of analysis. In this section, the production and egg quality data were analyzed as a completely randomized design using a MIXED procedure of SAS with repeated measures in a 4 x 6 factorial experiment, where diet represented one factor and temperature as the repeated factor. The method applied was based on a mixed model, where the data were fit to a model that included the effects of diet, temperature, and temperature × diet. The repeated statement indicates via sub=cage (diet) that the data are correlated on the same animal (i.e. cage(diet)). Due to the sequential nature of the data on the animals, the appropriate covariance structure that fits the model must be set. Thus, covariance structures were compared using the goodness of fit criteria including the REML log-likelihood (REML), Akaike information criterion (AIC), Schwartz Bayesian criterion (SBC), and the model with the lowest fit statistics value and the fewest number of parameters were selected. For this analysis, the best covariance structure, heterogeneous AR (1) covariance structure was chosen. The data were subjected to ANOVA in a completely randomized design with a 2 x 2 factorial arrangement of treatments using the GLM procedure of SAS software. The main effects of inorganic sodium source, EconomasE™, and their interaction within each temperature-period were tested. All statements of significance are based on a probability of <0.05. The mean values were compared using Tukey

Tables

Table A 5.1 The scoring scale of keel bone damage in laying hens fed diets with or without EconomasE™ (0 or 0.2 g/kg) supplementation and two inorganic sodium sources (NaCl or $NaCl+NaHCO_3$) during different environmental temperature regimen[1].

Curvature	Score	Fracture	Score
No curvature	0	No fracture	0
Slight curvature	1	Slight fracture	1
Severe curvature	2	Severe fracture	2

[1]Total score was the count of two hens per 36 cages.
Keel bone damage was determined at the end of each environmental temperature regimen (with the exception of REC1): (Phase 1) TN1 = Thermoneutral temperature; ET1 = Elevated temperature; (Phase 2) TN2 = Thermoneutral temperature; ET2 = Elevated temperature; REC2 = Recovery temperature.

Table A.5.2 Effects of dietary supplementation of EconomasE™ and two sodium sources on production parameters across different temperature regimen in laying hens[1].

Temperature[2]	NaCl	NaCl + EconomasE™	NaCl+ $NaHCO_3$	NaCl+ $NaHCO_3$ + EconomasE™
TN (23.8 ℃) 26 - 30 wk	$1.99^{a} \pm 0.04$	$1.96^{a} \pm 0.04$	$1.97^{a} \pm 0.04$	$1.98^{b} \pm 0.06$
ET (32.2 ℃) 33 - 37 wk	$1.92^{b} \pm 0.04$	$1.91^{b} \pm 0.03$	$1.95^{b} \pm 0.04$	$1.91^{c} \pm 0.06$
REC (23.8 ℃) 38 - 42 wk	$1.95^{b} \pm 0.03$	$1.93^{b} \pm 0.06$	$1.97^{b} \pm 0.08$	$1.95^{b} \pm 0.08$
TN1 (23.8 ℃) 44 - 48 wk	$2.01^{a} \pm 0.03$	$2.06^{a} \pm 0.05$	$2.06^{a} \pm 0.10$	$2.04^{a} \pm 0.04$
ET1 (32 ℃) 49 - 53 wk	$1.90^{c} \pm 0.04$	$1.93^{b} \pm 0.08$	$1.95^{b} \pm 0.10$	$1.90^{c} \pm 0.09$
REC1 (23.8℃) 54 - 58 wk	$1.95^{b} \pm 0.05$	$2.03^{a} \pm 0.07$	$2.07^{a} \pm 0.06$	$1.99^{a} \pm 0.06$
P-value				
Temperature	<.0001			
Diet type	0.320			
Temperature * Diet	<.0001			

[1]Values are expressed as mean ± SEM. Hens were subjected to each environmental temperature regimen for 5 consecutive weeks.

[2]Temperature: (Phase 1) TN1 = Thermoneutral temperature; ET1 = Elevated temperature; REC1 = Recovery temperature; (Phase 2) TN2 = Thermoneutral temperature; ET2 = Elevated temperature; REC2 = Recovery temperature

Table A 5.3 Effects of dietary supplementation of EconomasE™ and two sodium sources on production parameters across different temperature regimen in laying hens[1].

Temperature	Diet	Feed Intake (g/hen/d)	Feed Efficiency (feed/egg mass)	Hen-d egg production (%)
TN 23.8 ℃ (26 - 30 wk)		$105.4^{b} \pm 1.14$	$1.80^{a} \pm 0.02$	$96.84^{a} \pm 0.55$
ET 32.2 ℃ (33 - 37 wk)		$101.9^{cd} \pm 0.51$	$1.75^{b} \pm 0.01$	$95.63^{ab} \pm 0.52$
REC 23.8 ℃ (38 - 42 wk)		$100.1^{d} \pm 0.61$	$1.70^{c} \pm 0.01$	$94.44^{b} \pm 0.84$
TN1 23.8 ℃ (44 - 48 wk)		$103.7^{bc} \pm 0.79$	$1.72^{bc} \pm 0.01$	$89.97^{c} \pm 0.70$
ET1 32 ℃ (49 - 53 wk)		$95.7^{e} \pm 0.88$	$1.62^{d} \pm 0.02$	$84.09^{e} \pm 0.72$
REC1 23.8℃ (54 - 58 wk)		$110.3^{a} \pm 1.51$	$1.82^{a} \pm 0.03$	$86.94^{e} \pm 1.16$
	NaCl	$100.8^{b} \pm 0.96$	$1.70^{b} \pm 0.02$	91.67 ± 0.81
	NaCl + EcoE	$104.3^{a} \pm 0.96$	$1.76^{a} \pm 0.02$	90.74 ± 0.81
	NaCl+ $NaHCO_3$	$101.9^{ab} \pm 0.96$	$1.72^{ab} \pm 0.02$	91.06 ± 0.81
	NaCl+ $NaHCO_3$ + EcoE	$104.3^{a} \pm 0.96$	$1.75^{a} \pm 0.02$	91.81 ± 0.81
P values				
Temperature		<.0001	<.0001	<.0001
Diet		0.029	0.057	0.759
Temperature * Diet		0.994	0.993	0.964

[1]Values are expressed as mean ± SEM. Hens were subjected to each environmental temperature for 5 consecutive weeks.

[2]Temperature: (Phase 1) TN1 = Thermoneutral temperature; ET1 = Elevated temperature; REC1 = Recovery temperature; (Phase 2) TN2 = Thermoneutral temperature; ET2 = Elevated temperature; REC2 = Recovery temperature

Table A 5.4 Effects of dietary supplementation of EconomasE™ and two sodium sources on egg quality across different temperature regimen in laying hens[1].

Temperature	Diet	Average egg weight (g)	Eggshell weight (%)	Eggshell breaking strength (kg/cm^2)	Albumen height (mm)	Haugh Unit
TN (23.8 ℃) 26 - 30 wk		58.5^{bc} ± 0.22	9.79^{b} ± 0.03	4.25^{a} ± 0.03	8.81^{a} ± 0.06	93.8^{a} ± 0.27
ET (32.2 ℃) 33 - 37 wk		58.4^{c} ± 0.20	9.76^{b} ± 0.03	3.66^{bc} ± 0.03	8.10^{b} ± 0.03	90.3^{b} ± 0.19
REC (23.8 ℃) 38 - 42 wk		59.0^{b} ± 0.17	9.77^{b}± 0.03	3.61^{c} ± 0.03	7.91^{c} ± 0.03	89.2^{c} ± 0.19
TN1 (23.8 ℃) 44 - 48 wk		60.2^{a} ± 0.20	10.06^{a} ± 0.03	3.72^{b} ± 0.03	8.03b^{c} ± 0.05	89.5^{c} ± 0.25
ET1 (32 ℃) 49 - 53 wk		59.1^{b} ± 0.21	9.56^{c} ± 0.04	3.25^{d} ± 0.03	7.57^{d} ± 0.05	87.0^{d} ± 0.29
REC1 (23.8℃) 54 - 58 wk		60.5^{a} ± 0.22	10.04^{a} ± 0.04	3.63^{bc} ± 0.04	7.41^{e} ± 0.06	85.8^{e} ± 0.38
	NaCl	59.2 ± 0.23	9.85 ± 0.04	3.66 ± 0.04	8.00^{a} ± 0.05	89.5^{a} ± 0.31
	NaCl + EcoE	59.2 ± 0.23	9.79 ± 0.04	3.66 ± 0.04	7.83^{b} ± 0.05	88.4^{b} ± 0.31
	NaCl+ $NaHCO_3$	59.1 ± 0.23	9.82 ± 0.04	3.73 ± 0.04	8.02^{a} ± 0.05	89.5^{a} ± 0.31
	NaCl+ $NaHCO_3$ + EcoE	59.6 ± 0.23	9.85 ± 0.04	3.69 ± 0.04	8.04^{a} ± 0.05	89.6^{a} ± 0.31
P-values						
Temperature		<.0001	<.0001	<.0001	<.0001	<.0001
Diet		0.359	0.691	0.457	0.028	0.030
Temperature * Diet		0.177	0.611	0.743	0.722	0.575

[1]Values are expressed as mean ± SEM. Hens were subjected to each environmental temperature for 5 consecutive weeks.

[2]Temperature: (Phase 1) TN1 = Thermoneutral temperature; ET1 = Elevated temperature; REC1 = Recovery temperature; (Phase 2) TN2 = Thermoneutral temperature; ET2 = Elevated temperature; REC2 = Recovery temperature

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Book chapter

Adedokun, S. A., B. L. Bryson, **O. C. Olojede**, and A. E. Dunaway. **2019**. Animal discards in animal feed manufacture. In B. K. Simpson and A. N. A. Aryee (eds.). By-products from Agriculture and Fisheries: Adding value for food, feed, pharma, and fuels. Wiley Livestock Science. (In press).

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O. C. Olojede, M. J. Ford, T. Ao, A. J. Pescatore and S. A. Adedokun. Effect of dietary supplementation with EconomasE™ and two sodium sources on production parameters, egg quality, and blood electrolyte responses of laying hens exposed to elevated temperatures.

O. C. Olojede, T. Ao, A. J. Pescatore, and S. A. Adedokun. Evaluating the effect of dexamethasone and coccidia vaccine challenge with Natustat™ supplementation on growth performance, nutrient digestibilities and utilization, intestinal barrier integrity, and immune response in broiler chickens.