EVALUATION OF YEAST FERMENTATE SUPPLEMENTATION ON POULTRY STRESS SUSCEPTIBILITY AND PHYSIOLOGY

A Dissertation

by

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ABSTRACT

Reducing stress is an important goal in poultry production. The *Saccharomyces cerevisiae*-derived yeast fermentation product Original XPC (XPC, Diamond V Mills, Cedar Rapids, IA, United States) has been shown to reduce the severity of enteric infection and reduce measures of stress in poultry exposed to acute or chronic stress. However, the effect of dietary supplementation of yeast fermentate on other physiological parameters and its mode of action in reducing stress remains unclear. This work aimed to investigate the effects of supplementing XPC or its liquid equivalent, AviCare (Diamond V Mills), on measures of stress susceptibility, health and well-being in poultry exposed to acute and chronic stressors. Three consecutive experiments were conducted to evaluate the effects of yeast fermentate supplementation on measures of stress, growth and feed efficiency in Cobb 500 male broilers exposed to acute and rearing stressors. Both XPC and AviCare consistently and equally reduced measures of short- and long-term stress across all 3 experiments, although trends in body weight gain and feed efficiency were inconsistent. A fourth experiment investigated the effects of XPC and AviCare on measures of stress, plasma biochemistry, cecal microbiome and expression of stress- and immune-related genes in Cobb 500 male broilers. Both XPC and AviCare reduced stress by reducing expression of the ACTH receptor, and modulated immune activity by reducing IL10 and CYP1A2 gene expression as well as plasma IL-1β. However, cecal microbiome and antioxidative capacity were not affected after 42 d. Finally, 2 consecutive experiments were conducted to evaluate the effect of XPC and AviCare on measures of intestinal health in Cobb 500 male broilers and mixed-sex Pekin ducks exposed to cyclic heat stress during the last 14 d of growth. In both

experiments yeast fermentate attenuated the negative effects of heat stress on villus length and villus/crypt ratio but not goblet cell density. Yeast fermentate also affected metabolism but did not improve electrolyte balance. In conclusion, adding yeast fermentate to the feed or drinking water reduced stress susceptibility by reducing glucocorticoid production, supported intestinal cell survival during cyclic heat stress, and modulated inflammatory processes in poultry exposed to rearing stress but not cyclic heat stress.

DEDICATION

To my grandparents,

Diane LeBuis Chamberlain

Merritt Chamberlain

Alice James Nelson

Walter Nelson

Bernard Riley

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NOMENCLATURE

α_1-AGP	α_1-acid glycoprotein
ACTH	Adrenocorticotropic hormone
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
ASYM	Composite asymmetry
C	Celsius
CK	Creatine kinase
CRH	Corticotropin releasing hormone
CORT	Corticosterone
CYP1A2	Cytochrome P450 1A2
d	Day
DEFB1	Avian β-defensin 1
FRAP	Ferric reducing/antioxidant power
GC	Goblet cell
GH	Growth hormone
GLDH	Glutamate dehydrogenase
h	Hour(s)
H/L ratio	Heterophil/lymphocyte ratio
IL	Interleukin
min	Minute(s)
mRNA	Messenger ribonucleic acid

MC2R	Melanocortin 2 receptor
PGK1	Phosphoglycerate kinase 1
rRNA	ribosomal ribonucleic acid
ROS	Reactive oxygen species
s	Second(s)
SOD	Superoxide dismutase
TNF-α	Tumor necrosis factor-α
T3	Triiodothyronine
T4	Thyroxine
wk	Week

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CHAPTER I

INTRODUCTION [1]

Poultry are exposed to a multitude of potential stressors in modern animal agriculture, including vaccination, feed withdrawal, heat stress, high stocking density, and direct contact with feces in the litter. These factors can introduce foreign pathogens into an otherwise healthy animal, stimulate the stress response, and impact normal growth, production, and immune function. When a bird becomes stressed, stimulation of the hypothalamic-pituitary-adrenal axis results in the release of corticosterone into the bloodstream (Mormède et al., 2007; Virden and Kidd, 2009). As the primary stress hormone in birds (Scanes, 2016), corticosterone alters metabolism to increase readily available energy (Mormède et al., 2007). However, long-term stress suppresses the immune response (Beard and Mitchell, 1986), decreases growth rate (McFarlane, et al., 1989a), and can disrupt the balance of the cecal microbiome (Burkholder et al., 2008).

The effects of stress therefore manifest in a variety of situations. Exposure to high temperatures and feed withdrawal is associated with greater susceptibility to pathogen colonization of the gut (Burkholder et al., 2008). Heat stress can also result in poor feed intake, weight gain and feed conversion ratio (McFarlane et al., 1989a). Raising birds on poor quality, moist litter reduces welfare via footpad dermatitis (Thomas et al., 2004) and increases the likelihood that birds will be exposed to foreign microbes (Bessei, 2006). Overcrowding suppresses the immune system by reducing macrophage activity (Gomes et

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[1] This dissertation follows the style and format of Poultry Science.

al., 2014) and depresses growth rate (Thomas et al., 2004), which is critical when broilers are expected to reach market weight in a typical 6-wk grow-out. Finally, concurrent stressors can have an additive effect, impacting immune response or growth differently than if they were isolated incidents (Quinteiro-Filho et al., 2012). It is important to mitigate stress during the rearing period in order to minimize morbidity and improve animal well-being.

Functional metabolites in the *Saccharomyces cerevisiae*-derived yeast fermentation product Original XPC (XPC; Diamond V Mills, Cedar Rapids, IA, United States) and its liquid equivalent, AviCare (Diamond V Mills), have been shown to improve growth parameters and combat enteric infections in dairy calves (Brewer et al., 2014), swine (Kim et al., 2010), and poultry, including broiler chickens (Smith et al., 2016; Feye et al., 2016), ducks (Labib et al., 2014), laying hens (Lensing et al., 2012), and turkeys (Firman et al., 2013). Recent research also suggests that dietary inclusion of XPC can be an effective method to reduce measures of stress in heat-stressed turkeys (Bartz, 2016) or broilers (Price et al., 2018). When administered to humans, a single, acute dose of yeast fermentate has been shown to improve antioxidant capacity (Jensen et al., 2011). Even so, it remains to be seen whether XPC and AviCare have the same effect on the stress response and whether this effect is consistent when birds are exposed to acute and chronic stressors. Furthermore, the mode of action by which yeast fermentate in either form reduces stress, as well as its effects on other measures of health and well-being, require clarification.

The primary objective of this research was to determine whether, and how, XPC and AviCare reduce broiler chickens' susceptibility to physiological stress when challenged with both an acute stress event and short-term stressors typical of a 6-wk

broiler rearing period in the United States. This was accomplished by measuring traditional measures of stress as well as gene expression related to the stress and immune response. Experiments were also conducted to determine the effects of adding yeast fermentate to the feed (XPC) or drinking water (AviCare) on selected plasma biochemical parameters, immune parameters, and cecal microbiome structure during exposure to acute or chronic stressors. Finally, the effect of yeast fermentate on intestinal health in both broiler chickens and Pekin ducks exposed to cyclic heat stress was measured using ileum histomorphology as well as plasma biochemical indicators of intestinal dysfunction.

CHAPTER II

LITERATURE REVIEW

Introduction to Stress and the HPA Axis

Symptoms of stress are commonly studied in order to assess animal welfare in production situations. Outcomes of stress can be divided into 2 categories: distress, where a stress event has negative consequences (e.g. immunosuppression from high daytime environmental temperature); and eustress, moderate stress which does not inherently injure health and may ultimately yield positive results (e.g. vaccination; Shini et al., 2010; Siegel, 1995). The primary measures of stress evaluated in research settings include plasma corticosterone (CORT), heterophil/lymphocyte (H/L) ratio, physical asymmetry, antibody titer and measures of growth such as feed conversion ratio, feed intake, and body weight gain. In order to appropriately interpret these values, it is imperative to understand how environmental inputs influence the animal's physiology and thereby produce a stress response.

The stress response is a complex assembly of coordinated adaptive behavioral, physical and cognitive mechanisms which are elicited in order to counteract threats to the physiological equilibrium, or homeostasis (Karalis et al., 1997; Lara and Rostagno, 2013; Wilder, 1995). A stressor is any internal or external challenge, whether real or perceived, that evokes this response (Mormède et al., 2007). In fact, any change which causes an inconvenience to the animal's normal routine can be a cause of stress (Das et al., 2011). This includes exposure to changes in dietary protein and intake of the bacterial components β-glucan and lipopolysaccharide (Adriaansen-Tennekes et al., 2009). The stress response

begins with activation of the hypothalamic-pituitary-adrenal (HPA) axis and of the adaptive (humoral and cell-mediated) and innate (inflammatory process) immune responses (Karalis et al., 1997). These elements are not isolated responses. For example, pro-inflammatory cytokines produced during the acute phase of an immune response can stimulate release of neuro-hormones by the HPA axis, which in turn can induce lymphocyte proliferation (Karalis et al., 1997). Glucocorticoids released by the HPA axis can up-regulate the cell-mediated immune response, down-regulate the inflammatory response by innate immune cells, and decrease circulating lymphocytes (Shini et al., 2010).

Animals respond to environmental stressors in a consistent manner. Broiler chickens, for example, respond to stressors in the same way regardless of whether the stressor occurs singly or concurrently with up to 5 others (McFarlane et al., 1989a, 1989b). The behavioral stress response is characterized by Hans Selye's General Adaptation Syndrome, which describes an "alarm" response with initial perception of a stress challenge, accompanied by increased metabolic activity and attempts to flee from the stressor (Dohms and Metz, 1991; Virden and Kidd, 2009). If the stressor persists after the alarm phase, the "resistance" phase follows, which recruits above mentioned physiological coping mechanisms: chronic stress results in the "exhaustion" phase and could lead to systemic infection (Dohms and Metz, 1991). There is, however, individual variation in the magnitude of the stress response, probably attributed to genetics, access to feed and water, social interactions, and other environmental factors. Birds generally react similarly under heat stress conditions, for example, but show variation in the intensity and duration of individual responses, probably correlated with their microenvironment or variation in the heat stress event (Lara and Rostagno, 2013). Similarly, when broilers are vaccinated

against Newcastle disease virus (NDV), individuals demonstrate considerable variation in antibody production (van Boven et al., 2008).

The HPA axis is likely activated during the resistance phase for the purpose of enhancing gluconeogenesis to meet the energy demands of the animal's coping mechanisms (Mormède et al., 2007). Activation starts with production of corticotropin-releasing hormone (CRH) in the hypothalamus, which induces synthesis of adrenocorticotropic hormone (ACTH) by the anterior pituitary (Mormède et al., 2007; Virden and Kidd, 2009). Following the release of ACTH into general circulation, it travels to the adrenal cortex where it stimulates the synthesis of glucocorticoids; corticosterone is the primary stress hormone in avian species (Keunzel and Jurkevich, 2010; Scanes, 2016).

Corticosterone works to meet the energy demands of the stress response by directing hepatic gluconeogenesis and increasing feed intake, and when the stress response subsides it provides negative feedback to the HPA axis (Mormède et al., 2007). However, higher doses of CRH also activate the sympathetic nervous system, resulting in reduced appetite (Wilder, 1995). It has also been shown that the HPA axis can be reprogrammed, or habituated, to a stressor either from acute exposure early in life or after repeated exposure. The hypothalamus first demonstrates hypersensitivity to the stimulus, then adrenal activity progressively declines so that subsequent exposure to the same stimulus evokes a weaker response (Mormède et al., 2007; Zulkifli et al., 1995). Conversely, early exposure to stress which is unaccompanied by increasing plasma CORT may prevent future adaptability to stress (Zulkifli et al., 1995).

Introduction to the Immune Response

The bird's immune system is comprised of both innate and acquired immune systems. Innate immunity involves mucus membranes, leukocytes such as heterophils and natural killer cells, and blood-borne proteins called complement proteins. Acquired immunity is mediated by B lymphocytes, involved in the humoral immune response, T lymphocytes, involved in the cell-mediated immune response, and associated cytokines.

The humoral immune response begins when an antigen presenting cell such as a macrophage or dendritic cell presents an antigen peptide associated with the MHC1 complex to a T cell receptor (Mashaly et al., 1998; Yang et al., 2011). This interaction stimulates the T lymphocyte to produce cytokines, such as interleukin (IL)-6, which initiates the proliferation and differentiation of B lymphocytes into antibody-producing plasma cells (Scott, 2004). The first antibody or immunoglobulin (Ig) type produced in the humoral immune response is the pentamer IgM, whereas secondary exposure to the same antigen initiates class-switching and subsequent production of IgY. Antibodies bind to antigen and present it to B or T lymphocytes or signal complement proteins in the bloodstream to initiate antigen destruction and removal.

Humoral immunity is an important factor in poultry production because it responds to vaccination and is involved in the development of herd immunity (van Boven et al., 2008). Diseases like NDV are highly transmissible among birds with low antibody titers (van Boven et al., 2008). Insufficient herd immunity is likely to lead to increased mortality, resulting in economic losses for the producer. Stress has been shown to reduce antibody titer (Honda et al., 2015). Therefore, antibody production is a useful indicator of a bird's response to stress.

Stress, Immunity and Production

The endocrine, immune, nervous and inflammatory systems are interconnected so that a single source of stress can have negative consequences on an individual bird's overall health status, and different stressors can yield similar effects on its physiological state (Wilder, 1995). The stress response involves immune, behavioral, and metabolic changes to redistribute energy demands, allowing the bird to adapt to stressful situations (Shini et al., 2010). The rearing period presents a dynamic array of challenges which all have the potential for initiating the stress response and impacting production and animal well-being.

Heat Stress. Heat stress is a common management concern and can occur in response to high seasonal temperature, high stocking density, poor ventilation, equipment malfunction and dietary composition, among other factors. Prior studies have indicated an annual economic loss of $128 to $165 million in the poultry industry due to heat stress alone (St-Pierre et al., 2003). With recent growth in the poultry industry this number has likely increased. Heat stress occurs when an accumulation of metabolic heat cannot be eliminated by the performance of natural behaviors such as panting and raising the wings away from the body (Akbarian et al., 2016; Bessei, 2006). Broilers are more susceptible to high environmental temperatures than to cooler conditions because of their body fat, high body temperature and lack of sweat glands (Das et al., 2011). Increasing heat load results in hypotrophy of lymphoid organs which contributes to a higher H/L ratio (Mitchell and Kettlewell, 1998). Broilers exposed to acute heat stress are susceptible to inflammation and oxidative stress, resulting in changes in the gut microbiome and epithelial structure and increasing the likelihood of enteric pathogen infiltration (Burkholder et al., 2008;

Quinteiro-Filho et al., 2010). Depending on the strain of bird and the severity of exposure, heat stress may increase (Beard and Mitchell, 1986), decrease (Honda et al., 2015), or have no effect on antibody production (Santin et al., 2003). Finally, heat stress can result in poor feed intake, weight gain and feed conversion ratio (McFarlane, et al., 1989a). This is significant because reduced daily feed intake is the most reliable indicator of declining bird health (Lensing et al., 2012). Poor growth rate and feed conversion increases feed costs for growers and reduces carcass yield at slaughter, a combination which ultimately translates to reduced economic gain for the producer. Therefore, finding solutions to alleviate heat stress during rearing is of interest to producers from both a welfare and economic standpoint.

Rearing Stressors. Vaccination itself serves as an exogenous immunostimulant, resulting in immune stress (Liu et al., 2015). Vaccination initiates acute phase protein production and inflammatory processes, which stimulate corticosterone production. Although several processes influence H/L ratio, Kaab et al. (2018) attributed an increase in H/L ratio for 3 d post-intraocular vaccination to immune stress. Vaccinated and unvaccinated birds have the potential to achieve the same level of body weight gain and feed conversion, but the introduction of an additional immune stressor such as lipopolysaccharide can alter the intestinal microbiome and impair immune function (Liu et al., 2015; Yang et al., 2011). This is because the adaptive immune response initiated by vaccination takes several days to come to fruition, leaving the bird susceptible to other potential pathogens (Berghman, 2016). Additionally, activation of the innate immune response is more metabolically costly than the adaptive immune response, which includes both humoral and cell-mediated immunity (Iseri and Klasing, 2013, 2014). Vaccines that

incorporate live viruses, such as coccidiosis, are also capable of causing immune stress and reducing feed conversion and body weight gain, even when birds are not challenged with a secondary immune stressor (Lee et al., 2011).

Broilers are typically reared in direct contact with litter. This means they are likely to ingest microbes and debris in the litter while performing normal foraging behavior. Poultry litter is a reservoir for a variety of microbes, including the zoonotic pathogens *Escherichia coli*, *Salmonella enterica*, *Clostridium perfringens*, *Listeria monoctyogenes*, and *Campylobacter jejuni*, (Bolan et al., 2010). Previous research has shown that rearing birds on re-used litter without subtherapeutic antibiotic administration can pre-dispose birds to pathogenic bacteria (Kassem et al., 2010). As a result, raising birds on previously used litter can serve as a stressor itself.

Finally, feed withdrawal prior to slaughter, during transport or equipment malfunctions can stimulate the stress response. Feed withdrawal alters metabolic processes toward catabolism, resulting in glycogen depletion and reduced metabolic rate (Ali et al., 2008). Feed and water deprivation for 24 h also results in increased plasma corticosterone (Knowles et al., 1995). However, feed withdrawal during heat stress may provide short-term benefits. Özkan et al. (2003) found that removing feed for 6 h during heat stress reduced rectal temperature but did not affect mortality or production traits. The context of feed withdrawal is important in evaluating its effects on animal well-being.

Prebiotics in Poultry Production

According to Gibson et al., (2010), a prebiotic is defined as a "selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota thus conferring benefit(s) upon host health." In more broad

terms, a prebiotic is any feed additive that is non-digestible and enters the caeca-colonic junction intact. Prebiotics are directed toward changes at the genus level of the intestinal microbiome, particularly bifidobacteria and lactobacilli.

Bacteria in the gastrointestinal tract, particularly in the cecum, ferment oligosaccharides to produce short-chain fatty acids, including acetate, butyrate and propionate (Ricke, 2018). Changes in short-chain fatty acid content of the intestine likely contributes to a favorable pH environment for specific microbial populations, thereby reducing toxin production or colonization by pathogens such as *Escherichia coli* O157:H7 (Fukuda et al., 2011) and *Salmonella enterica* serovar Typhimurium (Kim et al., 2018). Other mechanisms of action include prebiotic-mediated immune stimulation, competitive exclusion and increased production of antimicrobial factors by intestinal bacteria (Pourabedin and Zhao, 2015). It is important to note that duration of exposure, source and stability of a given prebiotic can all affect the biological outcomes of supplementation (Ricke, 2018).

The most often used prebiotic in poultry production is mannanoligosaccharide (MOS). This non-digestible oligosaccharide has been implicated in reducing the negative effects of cyclic heat stress on small intestine morphology (Ashraf et al., 2013) and oxidant production (Sohail et al., 2011). Mannanoligosaccharide supplementation may also reduce plasma levels of stress hormones, cholesterol and thyroxine, and improve humoral immunity during cyclic heat stress (Sohail et al., 2010). Xylooligosaccharides (XOS) can be metabolized by both bifidobacteria and lactobacilli; XOS may stimulate immunity and mineral absorption and reduce plasma glucose and cholesterol (Samanta et al., 2015). Fructooligosaccharide (FOS) and inulin, a long chain of FOS, have been shown to

modulate intestinal bacteria (Pourabedin and Zhao, 2015) and even prevent macrophage cell death (Babu et al., 2012). Isomalto-oligosaccharides (IMO) supplemented alone or in combination with probiotic has been shown to increase cecal short chain fatty acid content, decrease cecal *Escherichia coli* colonization and increase cecal colonization by lactobacillus and bifidobacteria (Mookiah et al., 2014). Zhang et al. (2003) found that IMO improved body weight and feed conversion in broilers up to 21 d of age but had no effect on short chain fatty acid content of the cecum. Other sources of prebiotics include herbs such as dandelion (Al-Kassi and Witwit, 2010), rice bran (Kim et al., 2018), galactooligosaccharides, and soybean meal oligosaccharides (Pourabedin and Zhao, 2015).

Original XPC and AviCare

Original XPC (XPC; Diamond V Mills, Cedar Rapids, IA, United States) is a dried yeast fermentation product which can be incorporated into standard poultry diets. It is produced by a 2-phase commercial fermentation of *Saccharomyces cerevisiae* and contains 5 million CFU/g (Cortés-Coronado et al., 2016). Yeast fermentate contains both yeast and the media on which it was grown, which after a certain amount of time is dried to retain the yeast's fermentation activity (Yalçin et al., 2008). AviCare (Diamond V Mills) is the yeast culture after a single-phase fermentation and thus remains in the liquid phase. Both of these products have the same metabolite profile and contain the following: fermentation growth medium residues, yeast cell wall fragments, residual whole yeast cells, flavor and aroma substances, and functional fermentation metabolites such as peptides, amino acids, and organic acids (Öszoy and Yalçin, 2011; Shen et al., 2011; Yalçin et al., 2008). Original XPC is classified as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug

Administration (FDA) and has been tested as a prebiotic in a variety of livestock (Shen et al., 2011).

Dietary supplementation of XPC produces a broad spectrum of effects in livestock exposed to various production-related challenges. In swine, feeding XPC during gestation and lactation improved litter weight gain and reduced the number of days between weaning and next successful pregnancy in multiparous sows (Kim et al., 2010). Dairy calves fed XPC during *Salmonella* infection exhibited greater weight gain and better-developed rumens (Brewer et al., 2014). Ross broilers showed slightly higher anti-NDV antibody titer after vaccination, as well as better weight gain and feed conversion ratio (Cortés-Coronado et al., 2016). Laying hens demonstrated reduced incidence and severity of intestinal lesions during infection with *Eimeria maxima*, a parasite which causes coccidiosis (Lensing et al., 2012). Layers given XPC also produced heavier eggs with lower yolk cholesterol, with no apparent negative effects on performance or egg quality (Yalçin et al., 2008). The effects of XPC supplementation may, in some cases, be dose-dependent: during aflatoxin-challenge, broilers showed better feed intake and body weight at a higher inclusion rate (Osweiler et al., 2010). At a lower inclusion rate, however, turkey hens showed no improvement in growth characteristics or anti-NDV antibody production (Öszoy and Yalçin, 2011).

Systemic *Salmonella* infection resulting from the consumption of contaminated animal products is one of the leading causes of food-borne illness in humans (Rubinelli et al., 2016). *Salmonella enterica* serovars Enteritidis and Typhimurium are most often tested against XPC. When added to the drinking water at a rate of 1 mL/L, XPC reduced the severity of enteric lesions and symptoms of salmonellosis in ducks infected with

Salmonella enterica serovar Enteritidis and Duck Virus Enteritis (Labib et al., 2014). In broilers infected with *Salmonella enterica* serovar Typhimurium, feeding XPC reduced pre-harvest cecal counts, virulence and prevalence of *S. enterica* organisms, as well as the development of antibiotic resistance (Feye et al., 2016; Smith et al., 2016). Dairy calves infected with *S. enterica* serovar Typhimurium showed fewer instances of diarrhea and fever as well as earlier cessation of fecal shedding and were significantly less likely to show clinical signs of salmonellosis when fed formula supplemented with XPC (Brewer et al., 2014). Finally, adding XPC to an anaerobic *in vitro* mixed cecal culture taken from 42-d-old broilers resulted in a 5.62 $\log_{10}$ reduction in survival of *S. enterica* serovar Typhimurium (Rubinelli et al., 2016).

Effects of XPC on growth and immunity may be explained by the ability of yeast components to agglutinate, or bind to, pathogens and prevent them from colonizing the gut (Hajati and Rezaei, 2010). Bacteria must first attach themselves to the intestinal epithelium in order to colonize mucosal surfaces (Ganner and Schatzmayr, 2012). They do this by utilizing hair-like, proteinaceous appendages with adhesive properties called fimbriae (Ganner and Schatzmayr, 2012). However, yeast cell wall components such as mannose are also able to bind to fimbriae and prevent bacteria from adhering to host cells (Cortés-Coronado et al., 2016). Broilers fed mannanoligosaccharide and yeast culture have shown lower cecal counts of *S. enterica* serovar Typhimurium and Enteritidis expressing Type-1 fimbriae, without negatively affecting cecal pH, lactic acid, volatile fatty acid content, or normal microbiome structure (Spring et al., 2000). In addition, mannose may interact with immune cells in the gut mucosa to stimulate the host immune response (Cortés-Coronado et al., 2016).

Immune stress induced by vaccination, heat stress and exposure to pathogens can disrupt the balance of the cecal microbiome and impact intestinal immune function (Yang et al., 2011). The adaptive immune response typically takes effect 7 to 10 d after antigen exposure: during this response lag the host is left vulnerable to infection (Berghman, 2016). However, by functionally removing potential pathogens from the bird's system, yeast culture assists the bird's normal development and enhances the efficacy of the immune response. For example, normal gut microflora may be able to reach stable levels earlier, thus improving nutrient absorption and digestibility (Park et al., 2017b). As a result, several studies have reported improvements in growth and feed efficiency when yeast fermentate was added to the diet (Firman et al., 2013; Gao et al., 2009; Roto et al., 2017).

Yeast culture might also help control inflammation and improve antioxidant status (Lensing et al., 2012). This is particularly important when birds are exposed to heat stress, which weakens the intestinal epithelium and increases the potential of pathogens such as *S. enterica* to colonize the gut (Quinteiro-Filho et al., 2012). Heat stress also increases production of oxidants which impair cell signaling and mitochondrial function, manifesting in reduced feed intake and depressed growth rate (Akbarian et al., 2016). However, *in vitro* studies have shown that the functional fermentation metabolites in XPC behave as reductants toward oxidants, thereby mitigating cellular oxidative damage (Jensen et al., 2008). Indeed, Jensen et al. (2011) reported that a single, acute dose of yeast fermentate improved antioxidant status in humans.

Chou et al. (2017) suggested that XPC primes the immune system to better respond to threats without compromising growth. Firman et al. (2013) attributed better feed

efficiency during the later stages of growth in male turkeys to the effects of dietary XPC on the immune response. Additionally, broilers fed XPC and challenged with *Campylobacter coli* demonstrated enhanced feed efficiency d 35-42 and reduced incidence of cecal *C. coli* by d 42 (Hofacre et al., 2015). Dietary supplementation of XPC has also been shown to participate in the activation of natural killer (NK) cells and B-lymphocytes *in vitro* (Jensen et al., 2008). This may explain the improved growth observed in broilers supplemented with XPC and challenged with coccidial infection (Gao et al., 2009) or given a live coccidiosis vaccine (Roto et al., 2017).

Finally, dietary XPC has been shown to affect the stress response. Price et al. (2018) found that XPC reduced plasma corticosterone, heterophil/lymphocyte ratio and composite asymmetry scores in broilers exposed to cyclic heat stress. In addition, XPC has been shown to reduce heterophil/lymphocyte ratios in broilers during normal rearing stress (Al-Mansour et al., 2011). Reducing birds' susceptibility to environmental stress may enhance nutrient absorption and alter metabolism in favor of tissue accretion rather than turnover (Al-Mansour et al., 2011).

Stress Measures

Corticosterone. Corticosterone is a cholesterol-derived steroid (Mormède et al., 2007) which is synthesized by cells in the zona fasciculata of the adrenal cortex in response to ACTH (Scanes, 2016). Corticosterone produces many of the symptoms associated with chronic stress, such as cardiovascular disease, changes in glucose and mineral metabolism, intestinal lesions, and immune function modification (Siegel, 1995). On a larger scale, chronic stress manifests in typical "sickness behaviors," including fever, lethargy, weight loss, and low appetite and feed intake (Berghman, 2016). Although

broilers are resistant to sickness behavior, the effects of stress on gut health continues to present a welfare concern (Berghman, 2016).

Continuous ACTH infusion in broilers was shown to increase plasma CORT after 2 h and to increase H/L ratio after 2 d (Puvadolpirod and Thaxton, 2000c). An increase in plasma CORT is considered a measure of severe stress in poultry (Wodzika-Tomaszewska et al., 1982), probably because the bird's initial reaction to long-term, non-specific stress is to adapt to it rather than respond to it directly (Virden and Kidd, 2009). In diurnal species such as the chicken, synthesis of CORT follows a pulsatile rhythm with peak secretion occurring in the morning and minimal secretion occurring in the evening (Mormède et al., 2007). Its production is also influenced by feed consumption, age, and environmental temperature (Mormède et al., 2007). In consideration of this, plasma CORT may be a less reliable indicator of general stress in poultry than H/L ratio (McFarlane and Curtis, 1989c). Both measures, however, are useful in comparing groups of birds exposed to various types of stress (Gross and Siegel, 1983). Because it is a non-specific response and influences energy availability (Mormède et al., 2007), increased plasma CORT is also correlated with fluctuating asymmetry (Møller and Manning, 2003).

Heterophil/Lymphocyte Ratio. The avian heterophil is a granulocytic immune cell which serves as a first responder in the innate immune response to foreign microbes, which it eliminates via phagocytosis, release of its granule contents, and other microbicidal mechanisms (Genovese et al., 2013). These leukocytes predominate in the acute inflammatory response of the chicken—particularly in the lungs and air sacs, where they are primarily phagocytic (Harmon, 1998). Heterophils are also important in the early

immune response to infection by pathogens such as Salmonella enteritidis (Maxwell and Robertson, 1998).

The relative count of heterophils to lymphocytes (H/L ratio) in a total of 100 cells on a blood smear is a statistically accurate method of identifying physiologically stressed animals in a given population: a higher number is considered the most recognizable symptom of stress in poultry (Burton and Guion, 1968; Virden and Kidd, 2009). The H/L ratio indicates changes in an animal's physiological homeostasis and therefore is best used as a reflection of long-term environmental changes (Gross and Siegel, 1983). Heterophil/lymphocyte ratio has also shown potential for selection for better production performance, adaptability to stressors and general disease resistance (Al-Murrani et al., 2006). Heterophils are functionally deficient for the first 14 d of life in chickens, and the number of heterophils in the blood increases with age (Berghman, 2016; Genovese et al., 2013; Maxwell and Robertson, 1998).

Corticosterone secretion induces regression of lymphoid tissue, resulting in a reduction of circulating lymphocytes and a higher ratio of heterophils to lymphocytes (Virden and Kidd, 2009). Thus, H/L ratio responds to a variety of factors, including short-term feed restriction (Maxwell and Robertson, 1998), exposure to exogenous or endogenous corticosterone (Gross and Siegel, 1983), as well as malnutrition, water deprivation, extreme temperatures, overcrowding, injury and poor housing (Shini et al., 2008). For example, H/L ratio increased from 0.24 to 0.42 in heat-stressed broilers (Altan et al., 2003; McFarlane et al., 1989b). Broilers exposed to multiple concurrent stressors also showed an increase in H/L ratio from 0.53 to 0.86 (McFarlane and Curtis, 1989c).

Maxwell and Robertson (1998) suggest reference values of 0.20 (low), 0.50 (optimal) and 0.80 (high), though other values may be of comparable significance.

Physical Asymmetry. Animal welfare assessment requires concrete measurements of an animal's physiological status and observable changes in behavior. One such technique is based on the theory of bilateral asymmetry: that is, small, randomly directed deviations in bilateral symmetry away from the ideal phenotype which would have developed under perfect conditions (Knierim et al., 2007). There are 3 types of bilateral asymmetry: fluctuating asymmetry, directional asymmetry, and anti-symmetry (Van Poucke et al., 2007). Directional symmetry and anti-symmetry, however, may have a genetic basis, and therefore are generally not interpreted as indicators of developmental instability (Van Poucke et al., 2007).

Developmental stability is the ability to control development under given conditions, whereas developmental instability occurs when genetically predetermined development of a symmetric bilateral trait is impeded by environment or genetics (Møller, 1998; Møller and Manning, 2003; Van Nuffel et al., 2007). Because the right and left sides of a bilateral trait tend to develop under identical environmental conditions, the signed difference of right- minus left-side measurements reflects developmental instability (Møller, 1998; Van Nuffel et al., 2007). One can find the relative asymmetry of a given trait by taking the ratio of the 2 sides: this offers a standardized measurement of that particular trait (Knierim et al., 2007). Measuring more than 1 physical trait may better reflect an individual animal's ability or inability to buffer environmental change (Møller and Manning, 2003).

Fluctuating asymmetry is considered a non-specific response and is affected by corticosterone (Møller and Manning, 2003), heat stress and overcrowding (Van Nuffel et al., 2007). It is increasingly utilized as a measure of animal welfare, as it is a rather non-invasive method of stress measurement, may indicate long-term effects of stressful conditions that might otherwise not be detected, and serves as a reflection of how an animal dealt with the sum challenges faced during its development (Knierim et al., 2007; Van Nuffel et al., 2007; Van Poucke et al., 2007). It is expected that individuals who struggled to meet energy demands under conditions of high environmental stress will demonstrate greater developmental instability and therefore greater asymmetry for a given bilateral trait (Van Poucke et al., 2007).

Plasma Chemistry

Circulating levels of proteins, enzymes, minerals and electrolytes are commonly used as a clinical tool in determining bird health. Varying levels can indicate hydration level, metabolic changes, immune activity, muscle damage and antioxidant status.

Packed Cell Volume. Also called hematocrit, packed cell volume refers to the proportion or percent of red blood cells in a sample of whole blood. Heat stress results in dehydration, thereby increasing the proportionate volume of cells, including red blood cells as well as white blood cells in the buffy coat, to liquid in the blood. A dehydrated bird will have a higher packed cell volume percentage. Average hematocrit value in chickens and wild birds is 29.2% to 44% (Scanes, 2015).

Total Protein. Plasma protein concentration, or total protein, increases with age, dehydration, and exposure to corticosterone (Iheukwumere and Herbert, 2003; Meluzzi et al., 1992; Puvadolpirod and Thaxton, 2000a). Inflammatory processes induce hepatic

synthesis of acute phase proteins which function to enhance and direct the immune response and wound healing; meanwhile, reduced hepatic synthesis and increased excretion lead to reduced plasma albumin, the most abundant plasma protein (Dibner and Ivey, 1990; Gruys et al., 2005; O'Reilly and Eckersall, 2014). Total protein concentration in broilers may range from 2.58 to 5.22 g/100 mL; albumin accounts for 1.17 to 2.74 g/100 mL (Meluzzi et al., 1992).

Uric Acid. Uric acid is the primary end-product of nitrogen metabolism in birds; its plasma concentration can be used as a measure of protein synthesis and degradation and reflects dietary effects (Machín et al., 2004; Swennen et al., 2006). For example, circulating levels increase in birds supplemented with yeast extract (Huff et al., 2010) and in dehydrated or heat stressed birds (Arad and Marder, 1983; Iheukwumere and Herbert, 2003), but decrease during feed withdrawal (Delezie et al., 2007). Uric acid concentrations also increase during corticosterone-induced proteolysis (Lin et al., 2004). Because uric acid can function to scavenge reactive oxygen species in a non-enzymatic fashion, increased concentrations may help alleviate severe oxidative stress early in the stress response (Lin et al., 2004). Uric acid concentration ranges from 0.46 to 0.54 mM/L (Scanes, 2015).

Cholesterol. Cholesterol is an important component of cell membranes and endogenous substances such as steroids. Circulating levels vary between age, sex, and strain in chickens, with higher levels in males and lower levels in older birds (Meluzzi et al., 1992). Metabolic changes induced by corticosterone also increase blood cholesterol (Puvadolpirod and Thaxton, 2000a). Total cholesterol may range from 87 to 192 mg/100 mL, with free cholesterol contributing 27 to 119 mg/100 mL (Meluzzi et al., 1992).

Glucose. Glucose is important for energy production via glycolysis and oxidative respiration. Plasma glucose increases during the stress response due to enhanced gluconeogenesis (Lin et al., 2004; Puvadolpirod and Thaxton, 2000a, 2000b). This can be triggered by dehydration and heat exposure and is likely owing to the effects of CORT on insulin-mediated glucose uptake by skeletal muscle (Arad and Marder, 1983; Zhao et al., 2009). As a result, the bird becomes insulin-resistant and muscle development falters (Zhao et al., 2009). Blood glucose concentrations range between 15.08 to 15.72 mM/L (Scanes, 2015).

Electrolytes. Sodium, chloride, and potassium ions are important for protein synthesis, osmotic balance, acid-base homeostasis, proper nerve and enzyme function, and maintenance of cell membrane electrical potential (Olanrewaju et al., 2007). Heat stress and dehydration increase circulating chloride and reduce sodium and potassium through excretion (Iheukwumere and Herbert, 2003). While mild heat exposure may reduce plasma potassium due to cell uptake, severe heat stress compromises cell membrane integrity, leading to leakage and an increase in plasma concentrations (Ait-Boulahsen et al., 1989). Corticosterone reduces plasma sodium, potassium and chloride (Olanrewaju et al., 2006). Heat stress would therefore be expected to decrease sodium/potassium ratio. Typically, concentrations of electrolytes in circulation range from 151.37 to 153.67 mEq/L for sodium, 111.32 to 113.88 mEq/L for chloride, and 3.02 to 3.4 mEq/L for potassium (Scanes, 2015).

Calcium. Calcium is crucial for building bone, nerve excitability and muscle contraction. Blood calcium levels are increased through the action of parathyroid hormone, which increases intestinal absorption from the diet and retention by the kidneys and

stimulates release from bone by increasing osteoclast size and number. Alternately, calcitonin acts to reduce available calcium by stimulating cellular uptake. Calcium concentrations in broilers may vary between 4.7 to 16.25 mg/100mL (Meluzzi et al., 1992).

Phosphorus. Phosphorus is an intracellular anion that is critical to proper growth, energy metabolism and bone development (Parmer et al., 1987). Circulating inorganic phosphorus may decrease during heat stress and dehydration due to uptake by cells for use in glycolysis (Ait-Boulahsen et al., 1989; Arad and Marder, 1983). Phosphorus deficiency is followed by increased plasma calcium (Parmer et al., 1987). Phosphorus concentrations may range from 3.59 to 11.40 mg/100 mL (Meluzzi et al., 1992).

Growth Hormone. Growth hormone is released by the pituitary and plays a crucial role in somatic cell growth and development (Harvey et al., 1979). Plasma growth hormone concentration varies by genetic strain but is generally increased at 7 to 21 d of age in broilers selected for fast growth, even during feed restriction (Goddard et al., 1988; Gonzales et al., 1998). Additionally, growth hormone levels are typically high in young birds fed ad libitum, and decrease with age (McMurtry et al., 1988). Decreased levels are seen during phosphorus deficiency and may lead to the suppression of antibody production and T-lymphocyte responses to immune challenge (Mössner and Lesch, 2006; Parmer et al., 1987). Plasma concentrations of growth hormone in broilers may range from 131 to 141 ng/mL (Harvey et al., 1979).

Prolactin. Prolactin is released by the pituitary in response to stress and in birds it acts to balance the negative effects of corticosterone on immune function, including B- and T-lymphocyte function (Mössner and Lesch, 2006). It also exerts anabolic effects on tissue

growth, and decreases with age and weight (Harvey et al., 1979). Prolactin has several isoforms which may aggregate to form larger molecules that serve different biological functions: ratios of these isoforms may also change during physiological stress (Edens, 2011). Typically, plasma concentrations in broilers range from 6.26 to 7.04 ng/mL (Edens, 2011).

Serotonin. Serotonin is a neurotransmitter involved in communication between the nervous system via the hippocampus and the immune system via receptors on lymphocytes and macrophages (Mössner and Lesch, 1998). It can be stimulated by pro-inflammatory cytokines and in turn activates accessory T cells and NK cells (Mössner and Lesch, 1998). Serotonin also plays a role in modulating HPA axis activity as well as behaviors such as feed intake, sleep, mood and fear by modulating corticosterone release and neuropeptide Y activity (Linthorst and Reul, 2008). Severity and type (psychological versus physical) of stressor determine the level of serotonin stimulation and so influence the degree of neuroendocrine and behavioral changes which occur during the stress response (Linthorst and Reul, 2008).

Thyroid Hormones. Triiodothyronine (T3) and thyroxine (T4) are synthesized in the thyroid gland from iodine and thyroglobulin and are involved in metabolism and thermoregulation. Both hormones increase basal metabolic rate and subsequent heat production by increasing respiratory rate and mitochondrial mass (Lin et al., 2006b). Thyroid hormones also contribute to muscle cell homeostasis: T4 stimulates protein synthesis and differentiation, whereas T3 is responsible for inhibition of cell proliferation, apoptosis, and protein degradation (Nakashima et al., 1998). Corticosterone administration can significantly decrease T3 after just 3 d, although its effect on T4 is less pronounced

(Lin et al., 2004). Triiodothyronine also decreases during metabolic adaptations to feed withdrawal and heat stress in order to reduce heat production, and in response to TNF-α production during inflammation (Ait-Boulahsen et al., 1989; Gehad et al., 2002; Lin et al., 2006a). Thyroxine increases during feed withdrawal (Delezie et al., 2007; Gonzales et al., 1998). Chickens experience a rise in T3 and a reduction in T4 when they regain access to feed after a withdrawal period (Swennen et al., 2006). During severe stress, both T3 and T4 are reduced because of a reduction in hypothalamic release of thyroid-stimulating hormone (Wodzicka-Tomaszewska et al., 1982). In general, however, T3 concentrations remain stable throughout life in the broiler chicken; T4 increases with age (Goddard et al., 1988).

Plasma Antioxidant Status

Antioxidant status is commonly quantified using superoxide dismutase (SOD) and ferric reducing/antioxidant power (FRAP) values. However, there are also a variety of acute phase proteins and other plasma constituents involved in scavenging radical oxygen species (ROS). During normal aerobic respiration, free electrons may leak from the mitochondrial electron transport chain and react with molecular oxygen to create a superoxide radical, which can negatively affect functional biological components (Bowler et al., 1992; Imlay, 2003; Meyer et al., 2006; Mujahid et al., 2005). Called the Haber-Weiss reaction, singlet oxygen, also called superoxide, and hydrogen peroxide interact to form hydroxyl radicals. Superoxide, hydrogen peroxide, and hydroxyl radicals can all have damaging effects on cellular components, including lipid peroxidation, mutations in DNA, and denaturation of proteins (Bowler et al., 1992; Mujahid et al., 2005). Studies in plants have shown that oxygen radicals react with unsaturated fatty acids to cause peroxidation of

membrane lipids: the resulting leakage of cell contents leads to cell death (Scandalios, 1993). In animals, ROS can damage polyunsaturated fatty acids in the membrane of red blood cells (Sohail et al., 2011).

Oxidative stress occurs when formation of oxidants and subsequent cellular damage is greater than reduction of radicals by enzymatic (e.g. SOD, glutathione peroxidase, and catalase) and non-enzymatic (e.g. ascorbic acid, uric acid, and vitamin E) antioxidants (Lin et al., 2004; Machín et al., 2004; Meyer et al., 2006). In 5-wk-old broilers, acute heat exposure for as little as 3 h can induce oxidative stress, as evidenced by reduced muscle membrane integrity (Lin et al., 2006a). Reasons for this may be elevated body temperature or an increase in plasma thyroid hormones, both of which can enhance basal metabolic rate: the resulting increase in aerobic respiration can thus increase production of ROS (Lin et al., 2006a). In another study, excess ROS were produced during the initial stages of long-term corticosterone administration, resulting in increased lipid peroxidation (Lin et al., 2004). Acute heat stress-induced oxidative damage of enterocyte membranes and increased permeability of tight junctions can lead to leaky gut syndrome (Lambert, 2009). However, superoxide is often quickly metabolized by superoxide dismutase (SOD) and other antioxidants to the less damaging hydrogen peroxide (Mujahid et al., 2005).

In addition to the actions of SOD and other antioxidants, the effects of oxygen radicals may be mitigated via avian uncoupling protein (avUCP), which is up-regulated during feed deprivation and whose role is to uncouple respiration from ATP synthesis and to dissipate the proton gradient in the mitochondrial inner membrane (Mujahid et al., 2006). Down-regulation of avUCP during acute heat stress can result in excess production

of superoxide radicals during oxidative phosphorylation (Mujahid et al., 2005, 2006; Wallimann et al., 2011). Blood proteins such as the acute phase protein ceruloplasmin also prevent iron-induced oxidative stress (O'Reilly and Eckersall, 2014; Sohail et al., 2011). Finally, uric acid, the end-product of nitrogen metabolism in birds, may scavenge ROS in a non-enzymatic fashion and mitigate severe oxidative stress (Lin et al., 2004; Machín et al., 2004).

Selection for fast-growing meat type chickens has been associated with greater susceptibility to heat stress, which increases the potential for onset of oxidative stress (Mujahid et al., 2005). However, supplementation of the yeast cell wall component mannanoligosaccharide has shown potential in reducing oxidative damage in heat-stressed broilers (Sohail et al., 2011). In addition, supplementation of a single, acute dose of yeast fermentate has been shown to improve plasma antioxidative capacity in humans (Jensen et al., 2011).

Plasma Enzymes

Creatine kinase (CK), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and glutamate dehydrogenase (GLDH) are all enzymes whose plasma levels can vary as a result of physiological stress. Creatine kinase primarily exists in skeletal and cardiac muscle and catalyzes the phosphorylation of creatine to the higher energy product phosphocreatine using ATP (Szasz et al., 1976; Wallimann et al., 2011). Creatine kinase also functions to recycle ADP in muscle mitochondria and prevent oxidative stress through enzymatic reduction of reactive oxygen species (Meyer et al., 2006). Plasma CK increases with age, muscle mass and body weight and in response to heat stress (Hocking et al., 1993, 1998; Sandercock et al., 2001; Wilson et al., 1990).

Alkaline phosphatase is a membrane-bound glycoprotein which catalyzes the hydrolysis of monophosphate esters at high pH (Weiss et al., 1986). It is ubiquitous among vertebrate tissues but is primarily known for its role in bone mineralization (Golub and Boesze-Battaglia, 2007). Elevated serum levels of ALP are indicative of calcium and vitamin D deficiency and bone degradation induced by parathyroid hormone and corticosterone (Hurwitz and Griminger, 1961). Alkaline phosphatase decreases with age due to concurrent reduction in osteoblast activity (Meluzzi et al., 1992). It is apparently unaffected by water restriction (Iheukwumere and Herbert, 2003). Plasma ALP concentrations may range from 568 to 8,831 U/L (Meluzzi et al., 1992)

Aspartate aminotransferase catalyzes the transfer of an amino group from L-aspartate to 2-oxoglutarate to form glutamate, thus allowing utilization of the remaining carbon atoms to be used for glucose synthesis (Sookoian and Pirola, 2012). Plasma AST increases with muscle mass, body weight, age, and heat exposure, and is indicative of increased cell lysis and muscle damage and increased cell membrane permeability (Bollinger et al., 1989; Hocking et al., 1993; Meluzzi et al., 1992). Plasma AST does not appear to be affected by yeast cell wall supplementation (Sohail et al., 2011); concentrations in broilers may range from 70 to 220 U/L (Meluzzi et al., 1992).

Glutamate dehydrogenase is an enzyme responsible for catalyzing the reversible conversion of glutamate to α-ketoglutarate. It is primarily produced by hepatocytes, as well as in the kidneys and cardiac muscle, and increases as a result of damage to the liver caused by inflammatory or disease processes (Washington and Van Hoosier, 2012).

Plasma Cytokines

Alpha-1-Acid Glycoprotein. Alpha-1-acid glycoprotein (α1-AGP) is considered a major positive acute phase protein. Its concentration in the blood increases with inflammatory and disease states, although it may not be as effective in indicating a relatively healthier animal compared to levels of some other acute phase proteins (Chambers et al., 1987). Although its true function remains unclear, it is known to participate in the binding and transport of basic and neutral lipophilic drugs of either exogenous or endogenous origin—including heparin, serotonin, histamine, and steroid hormones—and in immunomodulation (Fournier et al., 2000). Expression of the α1-AGP gene is regulated by glucocorticoids and various cytokines, including interleukin (IL)-1β, IL-6, and TNF-α (Fournier et al., 2000). Its concentration has been shown to increase in 28-d-old broilers experiencing gut barrier failure (Chen et al., 2015).

Interleukin-1, Interleukin-6, and Tumor Necrosis Factor-Alpha. The pro-inflammatory cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNF- α) most notably produce changes in metabolic processes which preferentially support immune function rather than tissue accretion. For example, IL-1 may induce an insulin-resistant state in skeletal muscle, and TNF-α prevents differentiation of adipose and muscle cells. In addition, IL-1 and TNF-α may work synergistically to increase protein degradation and reduce protein synthesis, inhibit lipoprotein lipase activity to reduce uptake of circulating fatty acids from the bloodstream, and increase bone resorption and blood calcium levels by stimulating parathyroid hormone and osteoclast activity and desensitizing bone to vitamin D (Klasing and Johnstone, 1991). Interleukin-6 complements IL-1 activity by increasing body temperature and synthesis of acute phase proteins such as metallothionein, fibrinogen

and fibronectin, which each in turn modulate some aspect of the immune response (Klasing, 1994). Both TNF-α and IL-6 stimulate production of growth hormone and prolactin (Mashaly et al., 1998). Finally, the HPA axis interacts with these cytokines in a feedback loop: IL-1 stimulates production of corticotropic releasing hormone in the hypothalamus and adrenocorticotropic hormone by corticotrophs in the anterior pituitary, ultimately increasing circulating glucocorticoid levels; meanwhile, glucocorticoids such as corticosterone inhibit the production of IL-1, IL-6, and TNF-α by circulating macrophages (Klasing and Johnstone, 1991; Shini and Kaiser, 2009).

Interleukin-2 and Interleukin-10. Interlukin-2 and IL-10 are associated with lymphocyte growth and development (Chou et al., 2017). Interleukin-2 is produced by activated T lymphocytes and plays a critical role in stimulating the growth and development of CD4+CD25+ regulatory T (Treg) lymphocytes (Malek, 2003). Meanwhile, glucocorticoids inhibit IL-2 and subsequent lymphocyte proliferation (Isobe and Lillehoj, 1992). Interleukin-10 is a regulatory cytokine which is produced by immune cells involved in both innate and adaptive immunity. In addition to participating in an active feedback mechanism to upregulate its own production, IL-10 functions to regulate immune responses to focus them on invading pathogens and thus prevent damage to host tissues, such as is produced during inflammation (Saraiva and O'Garra, 2010). As such, IL-10 acts on antigen-presenting cells to down-regulate their expression of co-stimulatory molecules and thus reduce T-lymphocyte activation (Donnelly et al., 1999).

Interleukin-8. Interleukin-8 is a cytokine expressed by macrophages, fibroblasts, hepatocytes, and epithelial cells in response to inflammation and bacterial invasion (Baggiolini et al., 1989). The pro-inflammatory cytokines IL-1α, IL-1β and TNF-α

stimulate transcription and gene expression of other cytokines, including IL-8 (Ito et al., 2004). Reactive oxygen species and glucocorticoids may also increase IL-8 gene expression (Baggiolini et al., 1989; Shini and Kaiser, 2009). Despite increased IL-8 gene expression in the small intestine, a decrease in plasma concentrations in response to heat stress has been observed (Varasteh et al., 2015). Upon release, IL-8 stimulates an immediate but short-lived increase in intracellular free calcium, and prolonged chemotaxis of lymphocytes and neutrophils to the site of inflammation (Baggiolini et al., 1989).

Interleukin-12. Interleukin-12 is a pro-inflammatory cytokine which is produced by dendritic cells, macrophages and B lymphocytes and functions to stimulate differentiation of T lymphocytes in response to a pathogen (Vignali and Kuchroo, 2012). Heat stress has been shown to increase expression of IL-12 in broiler chickens (Ohtsu et al., 2015).

Intestinal Health and Histomorphology

The small intestine is primarily composed of columnar epithelial cells which protrude into the intestinal lumen via structures called villi. These villi function to increase the surface area of the gut and aid absorption of digested food. Epithelial cells originate from progenitor stem cells in the crypt, or base, of the villus and differentiate into mature cells as they migrate toward the tip of the villus. Epithelial cells are joined to each other by protein complexes called tight junctions, which regulate intercellular permeability. After several days, mature epithelial cells separate from the villus and are shed. This disruption of interactions between the epithelial cell and surrounding cells and extracellular matrix, which are held together by adhesion molecules called integrins (e.g. E-cadherin, claudins, occludin), induces apoptosis; this particular process of cell death following exfoliation is

referred to as anoikis (Fouquet et al., 2004; Zhang et al., 2015). The length of the villus and of the depth of space between the base of the villus and the basement membrane, called the crypt, are widely used as an indicator of intestinal health. Inflammatory cytokines, oxidative stress, and pathogenic microbes and their toxins may disrupt tight junction integrity and increase gut permeability, thereby facilitating systemic infection and inflammation (Awad et al., 2017; Zhang et al., 2015). Stress and inflammation are also capable of stimulating apoptosis of mature epithelial cells and progenitor cells. Crypt depth is correlated with epithelial cell proliferation, and changes in crypt depth and villus height may correspond to the presence of toxins such as those produced by microbes (Zhang et al., 2005). Goblet cells are distributed along the length of the villus and secrete water-soluble glycoproteins, called mucins, into the lumen. Mucus secretion occurs in response to external stimuli including acetylcholine, histamine, and prostaglandin E_2, and is further regulated by IL-10 (Birchenough et al., 2015). Mucus protects epithelial cells from physical damage caused by digested food and debris and forms an antibacterial barrier to push microbes away from enterocytes and toward the intestinal lumen (Birchenough et al., 2015). Goblet cells also arise from progenitor cells in the crypt; their number might be expected to decrease with a reduction in progenitor cell number during pathological conditions. Indeed, 28-d-old male broilers exposed to gut barrier failure exhibited reduced mRNA levels of occludin and mucin 2 (Chen et al., 2015).

Intestinal health and morphology have been studied in relation to dietary administration of probiotics, antibiotics, exogenous enzymes, and whole yeast cells or yeast cell components, however there is little information on changes in gut morphology due to administration of yeast fermentation metabolites. Heat stress is a major cause of

dysfunction and dysregulation of intestinal epithelial function. In quail, heat stress has been shown to reduce ileal villus height and goblet cell count, yet treatment with active whole yeast cells was not shown to ameliorate this effect (Sandikci et al., 2004). Non-heat stressed turkey poults fed yeast cells incorporated into the diet at 0.2% demonstrated decreased crypt depth and goblet cell count, yet villus height was unaffected (Bradley et al., 1994). In another study, turkey poults supplemented with a mannanoligosaccharide and β-glucan mixture at 1kg/metric ton feed showed increased ileal villus height and crypt depth and increased density of certain goblet cell types (Solis de los Santos et al., 2007). Moreover, glucomannoprotein complex supplemented in combination with soluble mannan increased goblet cell density and size in 15-d-old broiler chicks, yet there were no effects seen in villus size (Brümmer et al., 2010).

Cecal Microbiome

The microbiome of the chicken gastrointestinal tract is primarily made up of bacteria from *Firmicutes* and *Bacteroidetes*. *Firmicutes* makes up 50 to 90% of all taxa in the cecum and is composed of low guanine + cytosine, Gram-positive bacteria (de Vienne, 2016; Yeoman et al., 2012). Further broken down, *Firmicutes* includes class Clostridia, which is the most abundant class of *Firmicutes* bacteria in the chicken intestinal tract and constitutes the families *Ruminococcaceae*, *Christensenellaceae*, *Lachnospiraceae*, *Eubacteriaceae*, *Peptostreptococcaceae*, *Streptococcaceae*, *Lactobacillaceae*, *Acidaminococcaceae*, and *Clostridiaceae*, among others (de Vienne, 2016). Meanwhile, phylum *Bacteroidetes* includes the families *Burkholderiaceae*, *Bacteroidaceae*, *Rikenellaceae*, *Marinifilaceae*, *Barnesiellaceae*, and *Tannerellaceae* (de Vienne, 2016).

Streptococcus, represented by the family *Streptococcaceae*, utilize uric acid via aerobic or anaerobic conditions to produce acetic acid, ammonia, and carbon dioxide (Mead, 1974). The *Ruminococcaceae* family is composed entirely of obligate anaerobes which are diverse in shape and typically ferment carbohydrates to produce a variety of compounds, including acetate, succinate, butyrate, lactate, formate, ethanol, hydrogen, and carbon dioxide (Rainey, 2009). Some members of *Bifidobacterium*, *Ruminococcaceae*, and *Bacteroides* are also capable of fermenting substances produced by the host itself, including glycoproteins in mucus and products of polysaccharides such as chondroitin sulfate (Gibson and Roberfroid, 1995). Of low abundance in the chicken ceca (2% or less of the sequenced bacteria) are *Bacteroides*, *Bacillus*, *Lactobacillus*, *Actinomyces*, *Bifidobacterium*, and *Pseudomonas*—which are capable of anaerobic respiration using nitrate or nitrite (Zhu et al., 2002).

The chicken ceca microbial community is dominated by *Lachnospiraceae* and *Ruminococcaceae* (Danzeisen et al., 2011). Short-chain fatty acids, also called volatile fatty acids, produced by bacteria residing in the intestine can serve as an energy source for the host itself. Indeed, butyrate, which is primarily produced by *Lachnospiraceae* and *Ruminococcaceae*, is the preferred energy source of enterocytes, and its utilization affects smooth muscle proliferation in the intestine by stimulating protein synthesis and actin and myosin expression in myocytes (Le Blay et al., 2000; Rinttilä and Apajalahti, 2013). Short-chain fatty acids also affect microbial growth. *Enterobacteriaceae* are dominant in the young broiler ceca but decrease in number as the bird ages and the ceca environment begins to favor anaerobic bacteria (Van der Wielen et al., 2000). At the same time, levels of butyrate, acetate and propionate increase substantially: taken together with the results of

in vitro experiments, Van der Wielen et al. (2000) concluded that volatile fatty acids are partly responsible for the reduction of cecal *Enterobacteriaceae* in the growing broiler. This can be attributed to the reduction in pH caused by short-chain fatty acid synthesis, which can inhibit acid-sensitive bacteria such as *Enterobacteriaceae* (Rinttilä and Apajalahti, 2013). Short-chain fatty acids also stimulate electrolyte and fluid uptake in humans and ruminants (Yeoman et al., 2012). It is important to consider, however, that dietary changes, dietary fiber content, and intake of low-digestible protein considerably affect microbial richness and prevalence (Rinttilä and Apajalahti, 2013). Other factors unrelated to diet such as bird age and genetics, stress, immune challenge, and litter source can also affect microbiome structure (Torok et al., 2009).

The complexity of the ceca microbiome increases as the bird ages (Danzeisen et al., 2011). Moreover, Zhu et al. (2002) found that even in broilers reared under similar environmental conditions cecal microbial communities differed quantitatively and qualitatively between individual birds. This between-bird variability becomes more obvious where flocks demonstrate a history of low feed conversion efficiency and poor growth (Rinttilä and Apajalahti, 2013). Interestingly, the amount of guanine + cytosine in sequenced bacteria appear to affect bird performance, where flocks with more efficient feed conversion showed an average of 45% guanine + cytosine, whereas low-performing flocks showed a profile either below (37%) or above (65%) this (Rinttilä and Apajalahti, 2013). Keeping in mind that *Firmicutes* has a low guanine + cytosine %, the ability of the enterocytes to efficiently derive energy from carbohydrate fermentation appears to reflect the balance of *Firmicutes* to *Bacteroidetes*, or at least the relative abundance of *Firmicutes*. Stanley et al. (2012) postulated that microbes which inhibit enterocyte-mediated nutrient

uptake are likely to be more prevalent in low-performing birds as opposed to more efficient bacteria being present in high-performing birds.

Probiotics and prebiotics are increasingly being added to poultry diets: prebiotics are defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria" (Gibson and Roberfroid, 1995). Yeast fermentate may lend a prebiotic effect on microbiome structure: for example, EpiCor, a *Saccharomyces cerevisiae*-derived fermentation product, has been shown to quantitatively increase lactobacilli and qualitatively modulate bifidobacteria, and to increase butyrate production in the human colon, although no effect was seen on overall numbers of *Firmicutes* and *Bacteroidetes* (Possemiers et al., 2013). When cecal contents were cultured with XPC, another yeast fermentate, the abundance of *Bacteroides* increased over the following 48 h, with a peak at 12 h of incubation, whereas *Lactobacillus* and *Fecaelibacterium*, a butyrate-producing bacteria, remained at levels similar to the control (Park et al., 2017a). In both the negative control and the culture supplemented with XPC, total numbers of *Firmicutes* were highest at 0 h of incubation whereas *Bacteroidetes* was highest at 6 h (Park et al., 2017a).

Stress-Related Gene Expression

Cytochrome P450 1A2. The cytochrome P450 (CYP) family constitutes a set of enzymes which are responsible for catalyzing the oxidative breakdown of various substances, including drugs, steroids and aflatoxins (Reed et al., 2007). Its expression is modified by cytokines, hormones, age, disease states such as inflammation, diet, and genetic polymorphisms (Zanger and Schwab, 2013). In humans, the enzyme produced by the CYP1A2 gene is primarily responsible for the activation of aflatoxin B1 to its epoxide

form, an intermediate metabolite which has mutagenic and carcinogenic effects on the liver (Gallagher et al., 1996). Activity of the CYP1A2 enzyme has also been demonstrated in the liver of chickens and quail (Diaz et al., 2010).

Melanocortin 2 Receptor. The melanocortin 2 receptor (MC2R) is expressed in the zona reticularis and zona fasciculata of the adrenal cortex and binds exclusively to ACTH in order to modulate steroid production (Gantz and Fong, 2003). The MC2R is a membrane-bound G-protein coupled receptor which, when activated by binding ACTH, recruits cytoplasmic Gαs proteins which bind to adenylate cyclase to stimulate protein kinase A, and thereby stimulate glucocorticoid synthesis (Clark et al., 2003; Fridmanis et al., 2010). In humans, alteration of the MC2R gene results in deficient cortisol production and excess circulating ACTH, which manifests in low blood sugar and excess skin pigmentation (Clark et al., 2003; Fridmanis et al., 2010).

Immune-Related Gene Expression

Avian Beta Defensin 1. Gallinacins, now referred to as avian β-defensins, are antimicrobial peptides secreted by heterophils, macrophages and epithelial cells which induce microbial cell lysis by disrupting cell membrane integrity (Pan and Yu, 2014). Avian β-defensins have shown microbicidal activity against mycoplasma, Candida albicans, and Gram-positive and Gram-negative bacteria, although no such action was seen against infectious bronchitis virus (van Dijk et al., 2008). Pro-inflammatory cytokines such as IL-1β are known to up-regulate β-defensin expression, particularly during Salmonella infection, although the administration of probiotics has been shown to counteract this effect (Hong et al., 2012; Akbari et al., 2008). Avian beta defensin 1 (DEFB1) expression also increases in mucosal surfaces after exposure to lipopolysaccharide (Abdel Mageed et

al., 2008). On the other hand, DEFB1 mRNA expression in the spleen is down regulated during necrotic enteritis challenge in broilers infected with Eimeria maxima and Clostridium perfringens (Hong et al., 2012).

Interleukin-10. Interleukin-10 is a regulatory cytokine which directs the immune response toward foreign microbes and away from host tissue (Saraiva and O'Garra, 2010). Its expression in the spleen can be detected on d 12 of embryonic growth; although its expression fluctuates during the remaining period of embryonic growth and the early post-hatch period, peaks have been observed on embryonic d1 6, as well as 7- and 10-d post-hatch (Abdul-Careem et al., 2007). Expression of IL-10 by splenic macrophages may align with T-lymphocyte colonization of the spleen, which begins on embryonic d 15 (Abdul-Careem et al., 2007). Additionally, Original XPC has been shown to increase IL-10 gene expression in broilers after 28 d of growth (Chou et al., 2017). Unlike DEFB1, IL-10 has been shown to reduce inflammation by down-regulating IL-1β (Plunkett et al., 2001).

CHAPTER III

REDUCING STRESS SUSCEPTIBILITY OF BROILER CHICKENS BY SUPPLEMENTING A YEAST FERMENTATION PRODUCT IN THE FEED OR DRINKING WATER*

INTRODUCTION

Poultry are exposed to a multitude of environmental stressors in modern animal breeding, including vaccination, feed withdrawal, heat stress, high stocking density, and direct contact with feces in the litter. These factors can trigger the stress response, impair immunity and introduce foreign pathogens into an otherwise healthy animal, thereby impacting normal growth and production. When a bird becomes stressed, the hypothalamic-pituitary-adrenal axis is activated, ultimately resulting in the secretion of corticosterone (CORT) into the bloodstream (Mormède et al., 2007; Virden and Kidd, 2009). As the primary stress hormone in birds, CORT threatens bird health by suppressing immune responses (Beard and Mitchell, 1987), altering metabolism to increase readily available energy (Mormède et al., 2007), slowing growth rate (McFarlane et al., 1989a), and disrupting cecal microflora (Burkholder et al., 2008). A variety of commercial growing practices can induce stress in poultry. Exposure to high temperatures and feed withdrawal is associated with greater susceptibility to pathogen colonization of the gut (Burkholder et al., 2008). Heat stress can also result in poor feed intake, weight gain and

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feed conversion ratio (McFarlane et al., 1989a). Moist litter also reduces welfare status through the development of footpad dermatitis (Thomas et al., 2004) and increases the likelihood that birds will be exposed to foreign microbes (Bessei, 2006). Overcrowding suppresses the immune system by reducing macrophage activity (Gomes et al., 2014) and depresses growth rate (Thomas et al., 2004), which is critical when broilers are expected to reach market weight in a typical 42 d rearing period. Finally, concomitant stressors can have an additive effect, impacting immune response or growth differently than if they were isolated incidents (Quinteiro-Filho et al., 2012). Thus, it is essential to mitigate stress during rearing in order to minimize morbidity and improve both production performance and animal welfare.

When added to the feed, the *Saccharomyces cerevisiae* fermentation product Original XPC (XPC; Diamond V, Cedar Rapids, IA, United States) has demonstrated effectiveness in improving growth performance and combating enteric infections in dairy calves (Brewer et al., 2014), swine (Kim et al., 2010), and poultry—including laying hens (Lensing et al., 2012), ducks (Labib et al., 2014), turkeys (Firman et al., 2013) and broilers (Feye et al., 2016; Smith et al., 2016). The functional metabolites of the fermentation process, which are contained within XPC, can also be incorporated into the drinking water with the liquid product AviCare (AviCare, Diamond V, Cedar Rapids, IA, United States). Recent research has also shown that use of XPC can reduce physiological stress indicators in turkeys placed under short-term heat stress (Bartz, 2016) and in broilers under long-term heat stress (Price et al., 2018). For example, Al-Mansour et al. showed that XPC significantly decreased heterophil/lymphocyte (H/L) ratio in broilers (Al-Mansour et al., 2011). In addition to the improvements in the immune and stress response, XPC has also

been shown to have a positive impact on weight gain, feed conversion and mortality in broilers (Gao et al., 2008; Gao et al., 2009). This was even seen in broilers that were challenged with ingestion of used litter during a heat stress period (Teeter, 1993).

The objective of this study was to determine whether XPC and AviCare reduce broiler chickens' susceptibility to physiological stress when challenged with both an acute stress event and short-term stressors typical of a commercial 42 d broiler rearing program in the United States. It is hypothesized that supplementing broiler feed with XPC or adding AviCare to the drinking water will decrease the stress susceptibility of broiler chickens subjected to acute stress and stress associated with management procedures during rearing.

MATERIALS AND METHODS

Animal Husbandry

All procedures were carried out in accordance with the guidelines established by the Texas A&M Institutional Animal Care and Use Committee (AUP # 2016-0257) and the birds were managed according to the guidelines described in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). In total, 3 consecutive experiments were conducted. Experimental design was similar in all 3 experiments, and only the type of acute stress event varied among experiments. In each experiment, 1,200 healthy-looking 1-d-old Cobb 500 male broilers were randomly assigned to 1 of 4 treatments, each with 12 replicates, for a total of 300 birds/treatment. Treatments consisted of: non-stressed control (CNS), stressed control (CS), stressed and treated with AviCare (AVI) at a rate of 160mL/100L drinking water continuously from d 0 to 42, and stressed and treated with XPC (XPC) at a rate of 1.25 kg/metric ton (MT) of feed continuously from d 0 to 42. Pens measured 0.91 x 1.83 m, allowing 0.067 m^2 of floor

space/bird from d 0 to 19 (25 birds/pen)—when 5 birds/pen were sacrificed for blood sampling—and 0.083 m^2/bird from d 20 to 42 (20 birds/pen). Pens were lined with 8 to 10 cm of pine shaving substrate, which was sourced from previous broiler grow-out experiments. Building temperature was maintained at 31°C d 0 to 7, then at 29°C d 8-14, and subsequently reduced by 2.8°C each week thereafter until an ambient temperature of approximately 23°C was reached. Photoperiod consisted of 24 h of light for the first 3 d, and 20 h of light followed by 4 h of darkness for the remainder of the experiment.

One tube feeder and 1 drinker consisting of an 18.9 L bucket with 4 nipples on the bottom were hung in each pen and their heights adjusted as birds grew. Feed and water were provided *ad libitum* except during the 12 h fasting stress challenge on d 18 for CS, AVI and XPC treatment groups. Birds received standard broiler diets which were mixed at the Texas A&M Poultry Research Center Feed Mill. Birds were fed a starter diet from d 0 to 19, a grower diet from d 20 to 28 and a finisher diet from d 29 to 42. Drinkers in AVI pens were emptied and refilled from a stock solution daily, and all others were refilled as needed. All birds, feeders and drinkers for each pen were weighed at 20:00 on d 18 in each experiment. Mortality rates were also recorded.

The birds in each pen were weighed on d 0 and d 42 and body weight gain was calculated. Feed was weighed before its addition to the feeder in each pen and remaining feed was weighed on feed transition days so that total feed intake could be calculated. Feed conversion ratio was calculated by dividing the total feed intake by the total body weight gain for each pen, corrected for mortality. All birds were weighed on a per pen basis using a rolling scale (UFM-F120, UWE Scales, Cape Town, South Africa) on d 0 and

individually using a hanging scale with shackles (RPBS-1, Rotem, Petach-Tikva, Israel) on d 42, and body weight gain was calculated.

Stress Challenges

Birds in the CS, AVI, and XPC pens were exposed to an acute stress challenge on d 18 in each experiment: in Experiment 1, birds were exposed to 12 h of heat stress, which was produced by introducing a barrier to the pen in order to crowd birds and increase collective body heat, and verified every 2 h by a maintained litter temperature of approximately 32°C in these pens. The heat stress period began at 20:00 on d 18 after all birds, feeders and drinkers were weighed for each pen, and ended at 08:00 on d 19. During this time period the building lights remained on to mimic daylight conditions. At the end of the heat stress period feeders and drinkers for CNS pens were weighed. Feeders and drinkers for all treatments were then refilled and returned to the respective pen. In Experiment 2, birds were spray-vaccinated for Newcastle/Bronchitis (COMBOVAC-30, Merck, Kenilworth, NJ, United States) but birds retained free access to the entire pen. In Experiment 3, birds in the CS, AVI and XPC treatments received the Newcastle/Bronchitis vaccine and were exposed to the 12 h heat stress period as described in Experiment 1. In addition, birds in CS, AVI, and XPC pens were exposed to stressors which may occur during the rearing period in industry conditions: these included being reared on previously-used litter, spray-vaccination for coccidiosis on d 1 (COCCIVAC-B52, Merck) and 12 h without feed and water on d 18. In all 3 experiments CNS birds maintained *ad libitum* access to feed and water during the 12 h acute stress period on d 18 and were neither exposed to crowding-heat stress conditions nor did they receive vaccination for coccidiosis on d 1 or Newcastle/Bronchitis on d 18.

Stress Measures

In each experiment, blood samples were collected via exsanguination following decapitation from 5 birds/pen (n = 60/treatment) on d 19 following the 12 h acute stress challenge. Blood samples were also collected via wing vein venipuncture from 2 birds/pen (n = 24/treatment) on d 41. Approximately 1 to 2 mL of blood from each bird was transferred to a spray-coated lithium heparin and polymer separation gel vacutainer (368056, BD Medical, Franklin Lakes, NJ). Vacutainers were stored in an ice bath while remaining blood samples were collected. A drop of blood from each sample was used to make a blood smear slide. Vacutainers were spun down at 4,000 RPM for 15 min (Centrifuge 5804, Eppendorf, Hamburg, Germany). The plasma layer was then poured off into a labeled 2 mL micro-centrifuge tube and stored at -20°C; samples were thawed overnight at 4°C prior to assay. Plasma corticosterone concentration was obtained using a 96-well commercial ELISA kit (ADI-901-097, Enzo Life Sciences, Inc., Farmingdale, NY). Absorbance was measured at 450 nm using a microplate absorbance reader (Tecan Sunrise, Tecan Trading AG, Switzerland) and analyzed using the Magellan Tracker software program. Dry blood smear slides were stained with a neat stain hematology stain kit (Cat. #25034, Poly Sciences, Inc., Warrington, PA), and used to determine H/L ratio at 40x magnification using an oil immersion lens under microscopy (89404-886, VWR International, Radnor, PA).

On d 41 bilateral bone trait measurements were collected from 5 birds/pen (n = 60/treatment). Middle toe length (MTL), metatarsal length (ML) and metatarsal width (MW) in mm were obtained on the left (L) and right (R) legs using Craftsman IP54 digital

calipers (Sears Holdings, Hoffman Estates, IL). A composite asymmetry score (ASYM) for each bird was calculated using the following formula:

$$[|L\text{-}R|_{MTL} + |L\text{-}R|_{ML} + |L\text{-}R|_{MW}] \div 3.$$

Statistical Analysis

Data for stress measures, mortality, body weight and feed conversion ratio were analyzed using the General Linear Model in Minitab 17.1.0. Data was compared using one-way ANOVA, with treatment as the only effect. Mean separation was performed using the post hoc test for Least Significant Differences (LSD). A significant difference was defined as $P < 0.05$. Data was not transformed for analysis.

RESULTS

Plasma Corticosterone

Data for CORT in all 3 experiments are presented in Table 1. Treatment affected plasma CORT on d 19 following acute stress in all 3 experiments (Experiment 1: $F_{3,236} = 22.87$, $P < 0.001$; Experiment 2: $F_{3,236} = 3.03$, $P < 0.03$; Experiment 3: $F_{3,236} = 4.85$, $P < 0.003$), with CS birds displaying significantly higher plasma CORT values compared to the other groups (Experiment 1: $P < 0.001$; Experiment 2: $P < 0.05$; Experiment 3: $P < 0.03$). Treatment also affected plasma CORT levels on d 41 in all 3 experiments (Experiment 1: $F_{3,92} = 3.66$, $P = 0.02$; Experiment 2: $F_{3,92} = 4.77$, $P = 0.004$; Experiment 3: $F_{3,92} = 3.47$, $P = 0.02$). On d 41 both AVI (Experiment 1: $P < 0.04$; Experiment 2: $P < 0.05$; Experiment 3: $P < 0.03$) and XPC (Experiment 1: $P < 0.02$; Experiment 2: $P < 0.05$; Experiment 3: $P < 0.03$) had lower plasma CORT than CNS and CS groups in all 3 experiments. However, significant differences between AVI and XPC in CORT levels on d 41 were not observed ($P > 0.05$).

Table 1. Plasma corticosterone (pg/mL) after an acute stress challenge (d 19) and after 41 d of growth (Reprinted from Nelson et al., 2018).

	Experiment 1		Experiment 2		Experiment 3	
Treatment	**d 19[1]**	**d 41[2]**	**d 19[1]**	**d 41[2]**	**d 19[1]**	**d 41[2]**
CNS	194.3[a]	233.7[a]	1042.9[a]	5449.9[a]	580.5[a]	1684.7[a]
CS	690.9[b]	252.0[a]	1726.5[b]	5385.8[a]	973.0[b]	1625.0[a]
AVI	313.0[a]	75.0[b]	1030.7[a]	2482.4[b]	559.3[a]	468.3[b]
XPC	279.6[a]	56.3[b]	1081.7[a]	2424.1[b]	312.6[a]	518.1[b]
Pooled SEM	26.0	28.0	117.0	354.0	63.4	187.0
Treatment Main Effect *P*-value	< 0.001	0.02	0.03	0.004	0.003	0.02

[1] n = 60 birds/treatment,[2] n = 24 birds/treatment.
[a, b] Values within a column with different superscripts differ significantly at $P < 0.05$.

H/L Ratio

Data for H/L ratios for all experiments are presented in Table 2. Treatment affected H/L ratio on d 19 following acute stress in all 3 experiments (Experiment 1: $F_{3,236} = 5.85$, $P = 0.001$; Experiment 2: $F_{3,236} = 3.46$, $P = 0.02$; Experiment 3: $F_{3,236} = 3.46$, $P = 0.02$), with CS birds displaying significantly higher plasma CORT values compared to the other groups (Experiment 1: $P < 0.02$; Experiment 2: $P < 0.04$; Experiment 3: $P < 0.04$). Treatment affected H/L ratio at d 41 in all 3 experiments (Experiment 1: $F_{3,92} = 4.10$, $P = 0.009$; Experiment 2: $F_{3,92} = 2.96$, $P = 0.04$; Experiment 3: $F_{3,92} = 3.21$, $P = 0.02$). On d 41 both AVI and XPC (Experiment 1: $P < 0.04$; Experiment 2: $P < 0.05$; Experiment 3: $P < 0.03$) had lower H/L ratios than the CNS and CS groups in all 3 experiments. However, significant differences between AVI and XPC birds in H/L ratio on d 41 were not observed ($P > 0.05$).

Table 2. Heterophil/lymphocyte ratios after an acute stress challenge (d 19) and after 41 d of growth (Reprinted from Nelson et al., 2018).

	Experiment 1		Experiment 2		Experiment 3	
Treatment	**d 19[1]**	**d 41[2]**	**d 19[1]**	**d 41[2]**	**d 19[1]**	**d 41[2]**
CNS	0.10[a]	0.25[a]	0.09[a]	0.21[a]	0.09[a]	0.29[a]
CS	0.21[b]	0.25[a]	0.14[b]	0.20[a]	0.14[b]	0.28[a]
AVI	0.15[a]	0.16[b]	0.10[a]	0.12[b]	0.10[a]	0.22[b]
XPC	0.13[a]	0.16[b]	0.09[a]	0.11[b]	0.09[a]	0.21[b]
Pooled SEM	0.01	0.01	0.01	0.02	0.01	0.01
Treatment Main Effect *P*-value	0.001	0.009	0.02	0.04	0.02	0.02

[1] n = 60 birds/treatment,[2] n = 24 birds/treatment.
[a, b] Values within a column with different superscripts differ significantly at $P < 0.05$.

Physical Asymmetry

Composite asymmetry scores (ASYM) for all experiments are presented in Table 3. Treatment affected composite asymmetry score in all 3 experiments (Experiment 1: $F_{3,236} = 3.78$, $P = 0.01$; Experiment 2: $F_{3,236} = 3.32$, $P = 0.02$; Experiment 3: $F_{3,236} = 5.51$, $P = 0.001$). Composite asymmetry scores were lower in AVI (Experiment 1: $P < 0.03$; Experiment 2: $P < 0.05$; Experiment 3: $P < 0.003$) and XPC (Experiment 1: $P < 0.04$; Experiment 2: $P < 0.03$; Experiment 3: $P < 0.01$) compared to CNS and CS in all 3 experiments. There was no difference in ASYM between CNS and CS ($P > 0.05$) in any experiment.

Table 3. Composite asymmetry scores (mm) after 41 d of growth[1] (Reprinted from Nelson et al., 2018).

Treatment	Experiment 1	Experiment 2	Experiment 3
CNS	2.13[a]	2.48[a]	2.94[a]
CS	2.06[a]	2.41[a]	2.90[a]
AVI	1.68[b]	2.01[b]	2.23[b]
XPC	1.70[b]	1.98[b]	2.33[b]
Pooled SEM	0.06	0.07	0.08
Treatment Main Effect *P*-value	0.01	0.02	0.001

[1] n = 60 birds/treatment.
[a, b] Values within a column with different superscripts differ significantly at $P < 0.05$.

Mortality

There was no effect of treatment on mortality in all 3 experiments ($P > 0.05$). Average cumulative mortality was 6.7%, 2.1% and 4.9% in Experiments 1, 2, and 3, respectively.

Body Weight

Body weight data for all experiments are presented in Table 4. Body weight at d 42 did not significantly differ ($F_{3,44} = 1.21$, $P = 0.32$) between treatments in Experiment 1, although there was a trend ($P = 0.08$) toward heavier birds in the XPC group when compared to the CS group. In Experiment 2, treatment affected d 42 body weight ($F_{3,44} = 3.22$, $P = 0.03$). Both AVI ($P = 0.01$) and XPC ($P = 0.01$) had heavier d 42 body weights than CNS, while CS remained intermediate between them. In Experiment 3, treatment affected d 42 body weight ($F_{3,44} = 15.76$, $P < 0.001$). However, the only effect of treatment on d 42 body weight was seen in CS birds, which weighed less than all other treatments ($P < 0.001$).

Feed Conversion Ratio

Feed conversion ratio data for all experiments are presented in Table 4. Feed conversion did not significantly differ among treatments ($F_{3,44} = 1.35$, $P = 0.27$) in Experiment 1, although there was a trend ($P = 0.09$) toward lower FCR in XPC compared to CS. In Experiment 2, treatment affected FCR ($F_{3,44} = 2.89$, $P = 0.046$). The XPC treatment had lower ($P < 0.02$) feed conversion than all other treatments. In Experiment 3, treatment affected FCR ($F_{3,44} = 4.07$, $P = 0.01$). The AVI treatment had lower feed conversion compared to CNS ($P = 0.003$) and CS ($P < 0.007$), while XPC did not significantly differ ($P > 0.05$) from the other treatments.

Table 4. Average individual body weight (kg) from all birds at 42 d of age and cumulative feed conversion ratio d 0 to 42 (Reprinted from Nelson et al., 2018).

	d 42 Body Weight (kg)			Feed Conversion Ratio[1]		
Treatment	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Exp. 1**	**Exp. 2**	**Exp. 3**
CNS	2.41	2.65[a]	2.85[a]	1.757	1.533[a]	1.693[a]
CS	2.36	2.70[ab]	2.61[b]	1.745	1.528[a]	1.680[a]
AVI	2.44	2.76[b]	2.83[a]	1.702	1.528[a]	1.547[b]
XPC	2.47	2.76[b]	2.81[a]	1.679	1.449[b]	1.617[ab]
Pooled SEM	0.02	0.02	0.02	0.02	0.01	0.02
Treatment Main Effect *P*-value	0.32	0.03	<0.001	0.27	0.046	0.01

[1] Cumulative feed conversion ratio adjusted for mortality.
[a, b] Values within a column with different superscripts differ significantly at $P < 0.05$.

DISCUSSION

Plasma corticosterone and H/L ratio are generally used for stress assessment in birds (Al-Mansour et al., 2011; Price et al., 2018). In this study, birds in the CS group had

significantly elevated CORT and H/L ratio compared to CNS birds as a result of the imposed acute stress challenge on d 18 in all 3 experiments. In addition, XPC and AVI birds had lower H/L ratios and CORT levels than CS birds following the imposed acute stress on d 18. These results are in line with Price et al. (2018), who showed that XPC supplementation reduced CORT levels, H/L ratios and ASYM score in broilers during cyclic heat stress. Composite asymmetry score can be used to measure an animal's ability to cope with stress and direct energy toward normal growth over the long-term period (Archer and Mench, 2013). Both XPC and AVI birds had lower composite asymmetry scores after 41 d of growth than CNS and CS birds in all 3 experiments. Smaller relative ASYM scores indicate equal growth of bilateral traits over time and, therefore, an enhanced ability to shuttle energy toward growth in spite of exposure to a variety of environmental stressors. Both XPC and AVI birds also had lower CORT levels and H/L ratios than CNS and CS birds on d 41, which is in agreement with a previous study showing that XPC reduced H/L ratios in broilers during normal rearing stress (Al-Mansour et al., 2011). Birds in the AVI and XPC groups were less susceptible to physiological stress than untreated birds under the same conditions over the course of the grow-out period, and as a result presented more symmetrical growth of bilateral bone traits at 42 d of age. A proposed explanation for this is a possible immunomodulatory effect of yeast fermentation metabolites on the intestine (Firman et al., 2013). Future research could further explore the effects of ingestion of yeast fermentation metabolites on intestinal health and immune response and its relation to stress susceptibility in poultry. Plasma CORT levels fluctuated numerically within treatments across experiments: this may be

attributed to environmental conditions out of the control of the researchers and did not affect statistical differences between treatments.

Birds supplemented with XPC had improved FCR than all other treatments in Experiment 2, and in Experiment 3 those supplemented with AviCare had lower FCR than all other treatments whereas XPC birds were statistically intermediate between the control treatments and AVI. Previous experiments have demonstrated better feed conversion in male turkeys raised on used litter (Firman et al., 2013) and improved FCR and weight gain in broilers (Cortés-Coronado et al., 2016) when XPC is administered continuously in the diet. Birds that are less susceptible to environmental stress may experience improved nutrient absorption and may be more inclined to shuttle energy toward tissue accretion rather than tissue turnover (Al-Mansour et al., 2011). It has also been shown that the product of *S. cerevisiae* fermentation can help control inflammation and improve antioxidant status, both results of acute heat stress (Lensing et al., 2012). Both AVI and XPC birds had consistently lower CORT and H/L ratios after exposure to acute heat stress in this study, which does not explain why differences in FCR among treatments were not consistent across all 3 experiments. Although there is evidence that XPC improves feed efficiency in broilers exposed to acute and rearing stressors, a larger-scale investigation may clarify any trends in feed conversion when birds are supplemented with XPC compared to AviCare.

All stressed birds in this study received a live coccidiosis vaccine at the beginning of each experiment. Previous studies have shown that XPC improves growth in broilers challenged with coccidial infection (Gao et al., 2009) or administered a live coccidiosis vaccine (Roto et al., 2017). This agrees with some results regarding measures of growth in

this study: for example, XPC and AVI exhibited similar d 42 body weights to CNS birds in Experiment 3, and XPC birds tended to have a greater d 42 body weight than all other treatments in Experiment 1. However, when birds were challenged with an additional Newcastle/Bronchitis vaccination in Experiment 2 in this study, there was no effect of treatment on d 42 body weight. Although XPC and AVI birds showed improved growth of bilateral traits compared to CS and CNS birds in Experiment 2, this improvement in metabolic homeostasis was not reflected in a significantly greater overall gain.

Both XPC and AviCare reduced stress equally across all 3 experiments, as evidenced by H/L ratios, CORT levels, and ASYM score. Differences in the effects of these products on feed conversion and d 42 body weight could be elucidated by future studies, including exploration of the relationship between growth performance and inclusion level or mode of administration. The effects of XPC have been broadly demonstrated in other livestock species: this study provides information on its effects on additional measures of stress susceptibility in broiler chickens and explores the effects of AviCare when administered continuously in the drinking water during the entire rearing period. To conclude, inclusion of either Original XPC in the feed or AviCare in the drinking water during the entire rearing period reduces broiler susceptibility to acute stress events and stress induced by management practices during rearing.

CHAPTER IV

EFFECTS OF SUPPLEMENTING YEAST FERMENTATE IN THE FEED OR DRINKING WATER ON STRESS SUCSCEPTIBILITY, PLASMA CHEMISTRY, CYTOKINE LEVELS, ANTIOXIDANT STATUS, AND STRESS- AND IMMUNE-RELATED GENE EXPRESSION OF BROILER CHICKENS[*]

INTRODUCTION

Poultry are exposed to a variety of stressors associated with modern rearing practices, including vaccination, handling, heat stress, and feed withdrawal. Whether these stressors occur singly or simultaneously, they have the potential to stimulate the stress response and negatively affect bird growth and well-being. The stress response begins when the hypothalamic-pituitary-adrenal (HPA) axis is stimulated to release ACTH, which then binds to the melanocortin 2 receptor (MC2R) in the adrenal cortex and signals the production of the stress hormone corticosterone (CORT) (Mormède *et al.*, 2007; Virden and Kidd, 2009). Corticosterone regulates the immune response and shifts metabolic processes toward catabolism to increase readily available energy (Beard and Mitchell, 1987; Mormède *et al.*, 2007). However, chronic stimulation of the HPA axis can slow growth rate and suppress the immune response, thereby increasing susceptibility to infection (Bessei, 2006; Burkholder et al., 2008; McFarlane *et al*., 1989). Reducing environmental stressors is therefore an important goal in broiler production.

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[*] Reprinted from "Effects of supplementing yeast fermentate in the feed or drinking water on stress susceptibility, plasma chemistry, cytokine levels, antioxidant status, and stress- and immune-related gene expression of broiler chickens" by J.R. Nelson, E.B. Sobotik, G. Athrey, and G.S. Archer, 2020. Poultry Science, doi: 10.1016/j.psj.2020.03.037.

Previous research has demonstrated a variety of beneficial outcomes to feeding *Saccharomyces cerevisiae*-derived yeast fermentation products to livestock, including improving reproductive performance in multiparous sows (Kim et al., 2010). When added to the formula, yeast fermentate improved weight gain and intestinal development and reduced symptoms of *Salmonella enterica* infection in dairy calves (Brewer et al., 2014). In poultry, dietary yeast fermentate has been shown to improve growth performance as well as increase antibody production after vaccination (Cortés-Coronado et al., 2016) and reduce the severity and incidence of intestinal lesions in ducks (Labib et al., 2014) and laying hens (Lensing et al., 2012). There is also evidence that adding yeast fermentate to either the feed or drinking water improves small intestine histomorphology in Pekin ducks exposed to cyclic heat stress (Nelson and Archer, 2019). In addition, yeast fermentate has been shown to reduce measures of short- and long-term stress in heat-stressed turkeys (Bartz, 2016) and broiler chickens (Price et al., 2018), and in broilers exposed to acute or rearing stress (Nelson et al., 2018). It has been proposed that the metabolites in yeast fermentation products may help balance the immune and stress responses (Firman et al., 2013). Yeast fermentation metabolites may also improve antioxidant status by acting as reductants toward free radical (Jensen et al., 2008).

However, there is insufficient research on the effects of adding yeast fermentate to the feed or drinking water on other physiological parameters in broilers exposed to acute and chronic stress. Furthermore, the mode of action by which yeast fermentate reduces measures of stress is poorly understood. Therefore, the objective of this study was to elucidate the mechanism of action by which supplementing yeast fermentate in the feed or drinking water reduces measures of stress in broilers exposed to acute or chronic stressors.

MATERIALS AND METHODS

Animal Husbandry

All procedures were carried out in accordance with the guidelines established by Texas A&M Institutional Animal Care and Use Committee (AUP #2016-0004). There were 3 treatments: stressed and un-supplemented control (CS), stressed and supplemented with AviCare (Diamond V Mills, Cedar Rapids, IA, USA; 160 mL/100 L drinking water, d 0 to 43; AVI), and stressed and supplemented with Original XPC (Diamond V Mills, Cedar Rapids, IA, United States; 1.25 kg/metric ton feed, d 0 to 43; XPC). Birds were housed at the Texas A&M University Poultry Science Teaching, Research, and Extension Center. Pens were assigned to a given treatment using randomized complete block design. On d 0, 25 male 1-d-old Cobb 500 broilers were placed in each pen (n = 300/treatment). Pens measured 0.91 x 1.83 m and were lined with 4 to 6 cm of re-used pine shavings. Building temperature was maintained at 31°C on d 0 to 7, reduced to 29°C on d 8 to 14, and then allowed to decrease 2.8°C each week until ambient temperature was reached. Birds were provided 24 h of light d 0 to 3, and 20 h of light followed by 4 h of darkness d 4 to 43.

One tube feeder and 1 waterer consisting of an 18.93 L bucket with 4 nipples on the bottom were hung in each pen and their heights adjusted as birds grew. Feed and water were provided *ad libitum* except during the 12 h acute stress challenge on d 18. Birds received standard broiler diets which were mixed at the Texas A&M Poultry Research Center Feed Mill. Birds were fed a crumbled starter diet d 0 to 18, a pelleted grower diet d 19 to 27, and a pelleted finisher diet d 28 to 42. Birds in the AVI treatment received fresh

AviCare from a stock solution daily; all other treatments received fresh water without yeast fermentate as needed.

Stress Challenges

All birds were raised on re-used litter and were spray-vaccinated for coccidiosis (COCCIVAC-B52, Merck, Kenilworth, NJ, United States) on d 0. On d 18 all treatments were spray-vaccinated for Newcastle/Bronchitis (COMBOVAC-30, Merck) and then exposed to a 12 h period of heat stress and feed/water withdrawal (20:00 on d 18 to 08:00 on d 19). Wire barriers were placed in each pen to crowd birds and produce enough collective body heat to increase litter temperatures to 32 to 34°C. Building lights remained on to mimic daylight conditions.

Stress Measures

On d 38, 12 birds/treatment received an injection of ACTH in the thigh muscle: after 1 h, blood was collected via wing vein venipuncture and stored in a heparin vacutainer (367884, BD Medical, Franklin Lakes, NJ, United States) for analysis of plasma CORT. Blood was collected again on d 40 from a separate group of 5 birds/pen (n = 60/treatment) and analyzed for CORT. A drop of blood from each sample was also used to make a blood smear to determine heterophil/lymphocyte (H/L) ratio. Vacutainers were stored in an ice bath while remaining blood samples were collected and then centrifuged (Centrifuge 5804, Eppendorf, Hamburg, Germany) at 4,000 RPM for 15 min. The plasma layer was then poured off into a labeled 2 mL microcentrifuge tube and stored at -20°C until analysis. Samples were thawed at 4°C overnight before analysis. Plasma CORT concentration was obtained using a 96-well commercial ELISA kit (ADI-901-097, Enzo Life Sciences, Inc., Farmingdale, NY, United States); absorbance was read at 450 nm

using a microplate absorbance reader (Tecan Sunrise, Tecan Trading AG, Switzerland) and analyzed using the Magellan Tracker software program (Tecan Trading AG). Dry blood smear slides were stained with a neat stain hematology stain kit (Cat. #25034, Poly Sciences, Inc., Warrington, PA, United States). Heterophil/lymphocyte ratio was determined by individually counting heterophils and lymphocytes up to a total of 100 cells/slide at 40x magnification using an oil immersion lens under microscopy (89404-886, VWR International, Radnor, PA, United States).

On d 41 physical asymmetry measurements were collected from 5 birds/pen (n = 60 birds/treatment). Middle toe length (MTL), metatarsal length (ML) and metatarsal width (MW) in mm were obtained on the left (L) and right (R) legs using Craftsman IP54 digital calipers (Sears Holdings, Hoffman Estates, IL). A composite asymmetry score (ASYM) for each bird was calculated using the following formula:

$$(|L\text{-}R|_{MTL} + |L\text{-}R|_{ML} + |L\text{-}R|_{MW}) \div 3.$$

Additional Blood Parameters

On d 38 blood was collected from 12 birds/treatment and separated into 2 heparin vacutainers. Half of the sample from each bird was sent to Texas A&M Veterinary Medical Diagnostic Laboratory (College Station, TX, United States) for a plasma chemistry panel with electrolytes, which measured the following components: total protein (TP), glucose (Glu), alkaline phosphatase (ALP), creatine kinase activity (CK), aspartate aminotransferase (AST), uric acid (UA), cholesterol, calcium (Ca), phosphorus (P), sodium (Na), chloride (Cl), and sodium/potassium ratio (Na/K). The other half of the sample was used to analyze growth hormone (GH; Advanced BioChemicals, Lawrenceville, GA, United States). On d 39, blood samples from 12 birds/treatment were collected to

determine serum serotonin. Building lights were turned off and noise kept to a minimum in order to mitigate increased serotonin secretion due to handling stress. Samples were stored in clot-activator serum separation vacutainers (367981, BD Medical), allowed to clot for 24 h, and then spun down at 4,000 RPM for 15 min. Serum was then transferred to microcentrifuge tubes and stored at -20°C until analysis using a commercial ELISA kit (900-175, Enzo Life Sciences, Farmingdale, NY, United States).

On d 39 blood samples were collected to determine the plasma level of various enzymes and hormones from 12 birds/treatment using individual ELISA kits. These included prolactin (PRL; Ch1686, Advanced BioChemicals), total triiodothyronine (T_3; 74C5DC, Genway Biotech, Inc., San Diego, CA, United States), total thyroxine (T_4; 511F19, Genway Biotech Inc.), creatine kinase activity (CK; MAK116, Sigma-Aldrich, St. Louis, MO, United States), superoxide dismutase activity (SOD; EIASODC, ThermoFisher Scientific, Waltham, MA, United States) and ferric-reducing antioxidant power (FRAP; EIAFECL2, ThermoFisher Scientific). In addition, samples were assayed for the cytokines interleukin(IL)-1α (Ch1767, Advanced BioChemicals), IL-1β (Ch0539, Advanced BioChemicals), IL-2 (Ch0120, Advanced BioChemicals), IL-6 (Ch0228, Advanced BioChemicals) , IL-10 (Ch1621, Advanced BioChemicals), IL-12 (Ch1651, Advanced BioChemicals) and tumor necrosis factor-α (TNF-α; Ch0215, Advanced BioChemicals).

Blood samples used to determine FRAP were stored in spray-coated heparin vacutainers (367884, BD Medical); those used to determine IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-12, TNF-α, T3, T4, SOD, GH, PRL and CK were stored in spray-coated EDTA vacutainers (367861, BD Medical). All whole blood samples were centrifuged at 4,000 RPM for 15 min. Plasma was then transferred to a microcentrifuge tube and stored at -

20°C until analysis. Samples assigned to SOD assay had a slightly different procedure: after the initial centrifugation, the red blood cell pellet was isolated and re-suspended in 5x volume of ice-cold distilled water. The sample was centrifuged at 10,000 RPM for 10 min, and the resulting supernatant was transferred to a microcentrifuge tube and stored at -20°C until analysis. All samples were thawed overnight at 4°C prior to assay.

Organ Collection and Gene Expression Analysis

On d 43, 20 birds from each treatment were humanely euthanized and the spleen and adrenal glands were collected and stored in 15 mL centrifuge tubes with 5 to 10 volumes RNAlater® (AM7020, ThermoFisher Scientific, Waltham, MA). Samples were kept at -20°C until further processing, at which time samples were removed from the RNAlater® and individual spleens were sliced into smaller segments. Tissue samples were then transferred to microcentrifuge tubes and stored at -80°C. Next, batches of 20 samples were thawed at 4°C overnight. We isolated total RNA from sections of spleen tissue using the TRIzol Reagent method (ThermoFisher Scientific), and samples were quantified on a Nanodrop Spectrophotometer (ThermoFisher Scientific). The quality of the RNA isolates was checked using the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, United States) with the RNA 6000 Nano Kit following the manufacturer's protocol.

We only retained samples with an RNA Integrity Number (RIN) above 7, and poor-quality samples were re-extracted until we met this threshold. We performed reverse transcription reactions using the SuperScript VILO Master Mix (ThermoFisher Scientific, Waltham, MA). We used pooled cDNA samples as templates for primer testing. We designed the primers using the NCBI Primer-BLAST online tool. We set the amplicon size to between 200 to 300bp, and only primers spanning exon-junctions were included, and

synthesized for assays (Integrated DNA Technologies, Coralville, IA, United States). Primers were tested on a pooled cDNA sample using the PowerUP SYBR Green Master Mix (ThermoFisher Scientific) as recommended by the manufacturer. Primer pairs with 90 to 110% efficiency were retained, and dissociation curves (evidence of amplification) were inspected.

The RT-qPCR reactions were performed using the PowerUP SYBR Green Master Mix protocol. We assayed expression of CYP1A2 and MC2R in adrenal tissues and IL-10 and DEFB1 expression in the spleen. The gene phosphoglycerate kinase 1 (PGK1) was used as the control locus. The expression levels of the 4 target genes and the control (housekeeping gene) were determined by amplification reactions performed on the ABI 7900 HT (Applied Biosystems, Foster City, CA) real-time PCR system. For each locus, we assayed expression in triplicate reaction tubes, and the results were used for plotting and statistical analysis using the ΔΔCT method.

Statistical Analysis

Stress and blood parameters were analyzed using one-way ANOVA in Minitab 17.1.0 (Minitab, Inc., State College, PA, United States) with treatment as the main effect and pen as a random effect, and mean separation was performed using Fisher's LSD. Gene expression data was analyzed using one-way ANOVA followed by Tukey's HSD in R (RStudio, Inc., Boston, MA, United States). A significant difference was defined as $P < 0.05$.

For the qPCR data, we calculated the log2 fold change (L2FC) of expression for each target gene using the ΔΔCT method (Livak and Schmittgen, 2001) in Microsoft Excel (Microsoft Corporation, Redmond, WA, United States). We also tested for statistical

significance of gene expression among experimental groups using a one-way ANOVA, using the R statistical platform (R Core Team, 2019), with the treatments as the independent variable and the ΔΔCT values as the dependent variable. A significant difference was defined as $P < 0.05$.

RESULTS

Stress Measures

Data for basal (serum serotonin and CORT) and chronic (H/L ratio and ASYM) stress measures are shown in Table 5. Treatment affected plasma CORT after 42 d of growth ($P = 0.029$). Plasma CORT was lower in both AVI ($P = 0.013$) and XPC ($P = 0.037$) than in CS birds. There was no difference among treatments in CORT production 1 h post-ACTH injection ($P = 0.957$). XPC birds had higher serum serotonin than CS birds ($P = 0.049$). As for measures of chronic stress, H/L ratios were lower ($P = 0.044$) in both AVI ($P = 0.026$) and XPC ($P = 0.034$) than CS birds. Composite asymmetry was not affected by treatment ($P = 0.302$).

Plasma Chemistry and Electrolytes

Data for the plasma chemistry panel with electrolytes are shown in Table 5. Phosphorus was higher ($P = 0.062$) in CS birds than AVI ($P = 0.021$). Alkaline phosphatase was higher ($P = 0.075$) in XPC than AVI ($P = 0.027$). Treatment had no effect on plasma levels of total protein ($P = 0.845$), aspartate aminotransferase ($P = 0.612$), calcium ($P = 0.478$), glucose ($P = 0.636$), uric acid ($P = 0.720$), cholesterol ($P = 0.918$), potassium ($P = 0.384$), sodium ($P = 0.251$), chloride ($P = 0.620$), or sodium/potassium ratio ($P = 0.287$).

Metabolism and Growth

Data for indicators of metabolism and growth are shown in Table 5. Treatment had no effect on T3 ($P = 0.635$), T4 ($P = 0.609$), growth hormone ($P = 0.407$), or prolactin ($P = 0.347$).

Antioxidative Capability

Data for markers of antioxidative capability are shown in Table 5. When measured in the plasma chemistry panel, CK was not affected by treatment ($P = 0.873$); this agreed with results from the independent ELISA ($P = 0.699$). There were no differences among treatments in FRAP ($P = 0.872$) or SOD ($P = 0.322$).

Cytokines

Data for cytokines are shown in Table 5. Treatment affected plasma levels of IL-1β ($P = 0.011$): IL-1 β was higher in AVI than both CS ($P = 0.009$) and XPC ($P = 0.009$). However, there were no differences among treatments in plasma concentrations of IL-1α ($P = 0.563$), IL-2 ($P = 0.299$), IL-6 ($P = 0.353$), IL-10 ($P = 0.817$), IL-12 ($P = 0.230$), or TNF- α ($P = 0.344$).

Gene Expression

Data for ΔΔCT of gene expression of each treatment compared to PGK1 is shown in Figure 1. Expression of CYP1A2 differed between treatments ($P < 0.001$) and was lower in XPC ($P < 0.01$) and AVI ($P < 0.01$) compared to CS. Gene expression of MC2R also differed by treatment ($P < 0.001$) and was lower in XPC ($P < 0.01$) and AVI ($P < 0.01$). Treatment differences were observed for IL10 ($P < 0.001$), wherein XPC ($P < 0.01$) and AVI ($P < 0.01$) were lower than CS, and XPC was lower ($P < 0.05$) than AVI. Treatments did not differ in expression of DEFB1.

Table 5. Stress measures and blood data after 42 d of growth in male broiler chickens (Reprinted from Nelson et al., 2020).

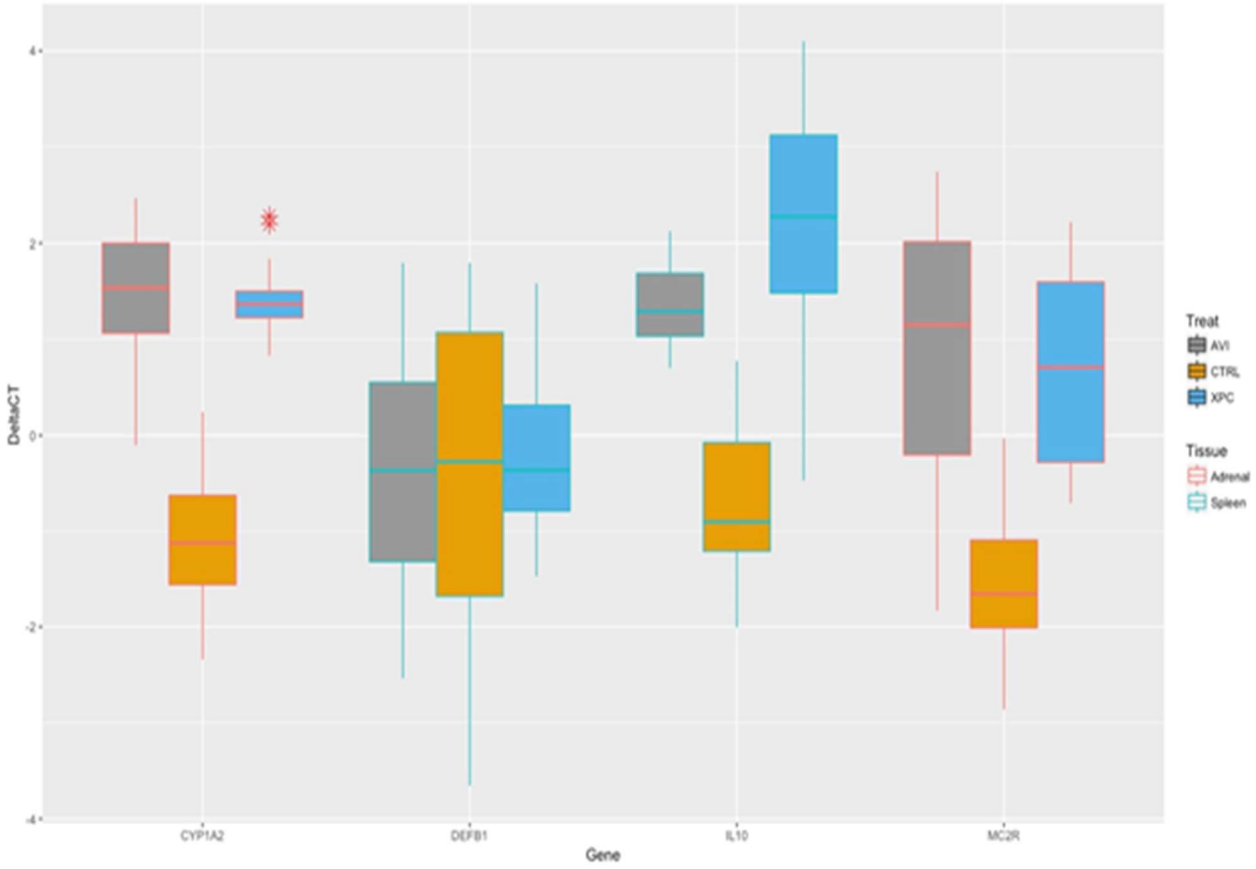
		Units	CS	AVI[1]	XPC[2]	Pooled SEM
Adrenal Exhaustion Test	CORT 1 h after ACTH injection	pg/mL	25896.94	24677.8	25151.42	2899.48
Acute Stress	CORT	pg/mL	1807.68[a]	1156.58[b]	1273.2[b]	172.15
	Serotonin	ng/mL	8512.31[a]	9668.62[ab]	11392.95[b]	983.42
Chronic Stress	H/L Ratio		0.68[a]	0.5[b]	0.51[b]	0.053
	ASYM		1.44	1.13	1.05	0.17
Plasma Chemistry	Phosphorus	mg/dL	6.72[a]	6.21[b]	6.55[ab]	0.14
	Total Protein	g/dL	3.47	3.42	3.35	0.14
	Calcium	mg/dL	12.24	10.18	9.88	0.94
	Glucose	mg/dL	268.67	262.92	270.17	5.65
	Uric Acid	mg/dL	5.37	5.9	5.81	0.46
	Cholesterol	mg/dL	136.42	139.25	138.55	5.01
	Potassium	mEq/L	7.13	6.29	7.06	0.44
	Sodium	mEq/L	150.58	152.83	151.17	0.95
	Chloride	mEq/L	112.17	113.83	113.17	1.09
	Na/K Ratio		21.92	24.68	22.82	1.21
Growth & Metabolism	T3	ng/mL	0.79	0.79	0.78	0.003
	T4	µg/mL	0.01	0.01	0.01	0
	GH	ng/mL	0.63	0.71	1.02	0.2
	Prolactin	ng/mL	52.98	67.74	59.09	6.55
	ALP	U/L	2641.92[ab]	3526.08[a]	2243.83[b]	386.9
	AST	U/L	479	498.5	426.27	50.02
Antioxidative Capability	CK	U/L	31657.83	30196.33	32346	2918.74
	FRAP	µM Fe^{2+}	76.38	74.1	77.42	4.3
	SOD	Inhibition rate (%)	45.44	49.24	54.7	4.28
Cytokine Production	IL-1α	pg/mL	174.25	211.08	179.09	25.42
	IL-1β	pg/mL	974.87[a]	1142.01[b]	975.58[a]	40.38
	IL-2	pg/mL	88.19	54.27	94.31	17.71
	IL-6	pg/mL	60.96	120.05	63.5	20.65
	IL-10	pg/mL	66.53	61.7	53.33	14.61
	IL-12	pg/mL	78.61	87.07	190.23	31.76
	TNF-α	pg/mL	214.41	187.04	158.95	22.81

[a,b] Values with different superscripts within the same row indicate a significant difference at $P < 0.05$.

[1] Received XPC in the feed (1.25 kg/metric ton) d 0 to 42.

[2] Received AviCare in the drinking water (160 mL/100 L) d 0 to 42.

Figure 1. Expression values (ΔΔCT) of CYP1A2 and MC2R in the adrenal gland and IL10 and DEFB1 in the spleen of broiler chickens after 43 d of growth. Higher (more positive) values represent lower abundance of gene expression (Reprinted from Nelson et al., 2020).



DISCUSSION

The objective of this study was to determine how adding yeast fermentate to the feed (XPC) or drinking water (AVI) would affect measures of stress, growth, immune function and antioxidant capacity in broilers exposed to acute and chronic stressors over a 42 d rearing period. Moreover, this research aimed to clarify the mechanism by which

yeast fermentate has been shown to reduce measures of stress in poultry exposed to acute and chronic stress. Primary findings suggest that both modes of administration reduce measures of acute and chronic stress by reducing gene expression of the ACTH receptor on adrenocortical cells, whereas supplementing yeast fermentate in the feed rather than in the drinking water may be more effective in modulating measures of immune activity.

In order to confirm that broilers supplemented with yeast fermentate retained their capacity to produce corticosterone in response to stress, a subset of birds from each treatment were injected with ACTH and blood samples were collected 1 h post-injection. Indeed, results showed that there were no treatment differences in plasma corticosterone 1 h post-ACTH injection, indicating that adrenal exhaustion was not of concern. After 38 d of growth, however, both XPC and AVI had lower plasma CORT compared with CS. Both XPC and AVI also had lower H/L ratios than the CS treatment. This suggests that dietary inclusion of yeast fermentate reduces measures of both short- and long-term stress. Similar results have also been reported in heat-stressed broilers (Price et al., 2018). Previous studies reported lower ASYM in broilers supplemented with yeast fermentate in the feed or drinking water and exposed to many of the same stressors used in this experiment (Nelson et al., 2018). However, in this study there were no treatment differences in ASYM. Plasma corticosterone and H/L ratio may be more reliable measures of stress than composite asymmetry.

Alkaline phosphatase (ALP) is involved in bone mineralization, where its primary role is to catalyze the hydrolysis of monophosphate esters at high pH (Weiss et al., 1986; Golub and Boesze-Battaglia, 2007). Its activity may increase in states of calcium and vitamin D deficiency and bone degradation induced by parathyroid hormone and

corticosterone (Hurwitz and Griminger, 1961). Plasma CORT was lower in both XPC and AVI than CS. However, ALP was higher in AVI than XPC, and phosphorus was lower in AVI than CS. Adding yeast fermentate to the drinking water may have affected bone deposition and phosphorus metabolism differently than including yeast fermentate in the feed.

The DEFB1 gene encodes avian β-defensin 1, an antimicrobial peptide which induces microbial cell lysis by disrupting cell membrane integrity (Pan and Yu, 2014). Probiotic administration (Akbari et al., 2008) and necrotic enteritis (Hong et al., 2012) have been shown to reduce DEFB1 expression in the spleen. On the other hand, pro-inflammatory cytokines such as IL-1β are known to up-regulate β-defensin expression (Hong et al., 2012). Birds supplemented with yeast fermentate in the feed had lower plasma IL-1β and IL10 gene expression than birds in the CS treatment. Alternatively, IL-10 has been shown to reduce inflammation by down-regulating IL-1β (Plunkett et al., 2001). In this study expression of IL-10 was lower in XPC and AVI compared to CS; furthermore, IL10 gene expression was lower in XPC than in AVI. Although plasma IL-10 did not differ between treatments, XPC had numerically lower IL-10 than both CS and AVI. This differs from previous research showing that XPC increased IL-10 gene expression in 28 d-old broilers (Chou et al., 2017). Additionally, plasma concentrations of IL-1β were higher in AVI than XPC. Therefore, XPC may be more effective than AVI in regulating the immune response during exposure to normal rearing stressors. Serotonin serves as a link between the hippocampus and immune cells, thereby playing a role in regulation of both the stress response and the immune response. In this study serum serotonin was higher in XPC than CS, whereas AVI was intermediate. Perhaps XPC

consumption played a role in regulating the immune response by increasing serotonin levels.

The cytochrome P450 (CYP) enzymes are responsible for catalyzing oxidative metabolism of various drugs and steroids (Reed et al., 2007). Increased CYP1A2 gene expression has been associated with carcinogenic and mutagenic effects in the liver (Gallagher et al., 1996), as well as oxidative stress and inflammation (Hussain et al., 2014). Although measures of antioxidative capacity did not differ between treatments, adding yeast fermentate to the feed or drinking water appeared to reduce CYP1A2 expression, indicating positive effects on regulation of inflammatory processes and production of reactive oxygen species. CYP1A2 is also involved in metabolism of aflatoxin B_1 to its epoxide form, a carcinogenic intermediary metabolite (Gallagher et al., 1996). Perhaps fermentation metabolite supplementation could alleviate the negative effects of mycotoxin consumption in poultry. Expression of MC2R in the adrenal glands was also reduced in both AVI and XPC compared to CS. The MC2R gene encodes the melanocortin 2 receptor, which is expressed in the adrenal cortex and binds exclusively to ACTH, whereupon it stimulates glucocorticoid production (Gantz and Fong, 2003). Reduced plasma CORT in birds supplemented with yeast fermentate in either the feed or drinking water is therefore a direct result of reduced MC2R gene expression.

Adding yeast fermentate to either the feed or drinking water consistently reduces measures of short- and long-term stress in broiler chickens by reducing MC2R expression. However, the different routes of administration yielded variable results regarding cytokine production and phosphorus utilization. Birds consuming XPC may have responded better to chronic stress than AVI as indicated by increased serotonin, reduced plasma IL-1β, and

reduced IL10 expression. Antioxidative capacity was not significantly affected, but CYP1A2 expression was reduced with supplementation in either the feed or drinking water. In conclusion, while both routes of administration reduced susceptibility to acute and chronic stress, supplementing yeast fermentate in the feed may be more effective than supplementation via the drinking water in regulating inflammatory processes.

CHAPTER V

EFFECT OF YEAST FERMENTATE SUPPLEMENTATION ON INTESTINAL HEALTH AND PLASMA BIOCHEMISTRY IN HEAT-STRESSED PEKIN DUCKS*

INTRODUCTION

Heat stress is a common problem encountered in commercial poultry houses, particularly in the summer months. Chronic and acute heat stress can compromise intestinal function and negatively impact nutrient absorption, growth rate, and feed efficiency (Awad et al., 2017). This is primarily attributed to inflammation, oxidative stress, and the effects of microbial toxins and stress hormones (Awad et al., 2017; Zhang et al., 2015). With gut barrier integrity compromised, pathogenic microbes and other debris from the intestinal tract are more likely to enter circulation and cause systemic infection (Awad et al., 2017). These factors can increase mortality and decrease yield at slaughter. It is important to investigate the mechanisms of heat stress on intestinal barrier function and to find ways to ameliorate or prevent gut barrier failure. Intestinal morphology is frequently used as an indicator of gut health. Germ cells reside in the crypt between the base of the villus and the basement membrane, and give rise to epithelial cells, which differentiate as they migrate toward the tip of the villus (Fouquet et al., 2004). Mature epithelial cells are continually exfoliated and regenerated. Disruption of the adhesion proteins which connect the enterocyte to nearby cells and the extracellular matrix causes

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*Reprinted from "Effect of yeast fermentate supplementation on intestinal health and plasma biochemistry in heat-stressed Pekin ducks" by J.R. Nelson and G.S. Archer, 2019. Animals, 9, 790, doi: 10.3390/ani9100790.

the cell to separate and begin apoptosis: this particular process of induced cell death is called anoikis (Awad et al., 2017; Fouquet et al., 2004). Anoikis is necessary for the maintenance of healthy epithelial cells that line the gastrointestinal tract; however, intestinal integrity can become compromised when stress, inflammation, and other factors stimulate the apoptosis of both germ cells and mature epithelial cells (Fouquet et al., 2004). For example, microbial toxins have been shown to disturb cell proliferation, resulting in reduced crypt depth and villus length (Zhang et al., 2005).

Goblet cells, which also originate from germ cells in the crypt, are distributed along the length of the villus and produce water-soluble glycoproteins that make up the mucus in the intestinal lumen (Birchenough et al., 2015). This acts as a physical barrier against microbes and debris and offers protection against physical damage to the epithelium (Zhang et al., 2005). Mucus secretion is thought to be stimulated by histamine, acetylcholine, and prostaglandin E2, and is further regulated by interleukin (IL)-10 (Birchenough et al., 2015). However, goblet cell density and mucus secretion can be negatively affected during heat stress and gut barrier failure (Chen et al., 2015; Sandikci et al., 2004). Several cytokines have also been used to measure intestinal health and inflammatory processes (Chen et al., 2015). Stress hormones, IL-1α, and reactive oxygen species stimulate the transcription of IL-8, which directs immune cells to the site of inflammation (Baggiolini et al., 1989; Ito et al., 2004; Shini et al., 2009). Alpha-1-acid glycoprotein (α_1-AGP), which is partially regulated by IL-1α, is a major positive acute phase protein which binds and transports substances of either endogenous or exogenous origin and modulates the immune response (Chambers et al., 1987; Fournier et al., 2000).

Concentrations of α_1-AGP have been shown to increase during gut barrier failure (Chen et al., 2015).

Dietary supplementation of whole yeast cells and yeast cell wall components have been tested in poultry. Mannan-oligosaccharides and β-glucan have been shown to increase the density of certain goblet cell types, as well as villus length (Solis de los Santos et al., 2007). The metabolites contained in the *Saccharomyces cerevisiae*-derived yeast fermentation product Original XPC and its liquid equivalent, AviCare, have demonstrated the ability to reduce measures of stress in broilers during cyclic heat stress (Price et al., 2018) and rearing stress (Al-Mansour et al., 2011). Supplementation of XPC has also been shown to mitigate enteric lesions in ducks infected with duck viral enteritis (Labib et al., 2011) and intestinal damage in laying hens infected with *Eimeria maxima* (Lensing et al., 2012). Dairy calves fed XPC during *Salmonella* infection also exhibited greater weight gain and more developed rumens (Brewer et al., 2014). While there is abundant research available on the effects of whole yeast cells or cell wall components on intestinal health, little exists on the effects of metabolites produced by yeast fermentation. The purpose of this research is to compare measures of intestinal health and plasma biochemistry of mixed-sex White Pekin ducks exposed to either cyclic heat stress or thermoneutral conditions when supplemented with XPC in the feed or AviCare in the drinking water.

MATERIALS AND METHODS

General Husbandry

All procedures were carried out in accordance with the guidelines established by Texas A&M Institutional Animal Care and Use Committee (AUP#2018-0135) and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS,

2010). Birds were housed at the Texas A&M University Poultry Science Teaching, Research, and Extension Center, and all diets were mixed on site. One experiment was conducted in which 144 1-d-old mixed-sex White Pekin ducks were randomly assigned to 1 of 6 treatments: stressed control (CS); stressed and supplemented with XPC (Original XPC, Diamond V Mills, Cedar Rapids, IA, United States) d 0 to 35 (XS); stressed and supplemented with AviCare (Diamond V Mills) d 0 to 35 (AS); non-stressed control (CN); non-stressed and supplemented with XPC d 0 to 35 (XN); and non-stressed and supplemented with AviCare d 0 to 35 (AN). Original XPC was added to the feed (1.25 kg/metric ton feed), and AviCare was mixed into the drinking water (160 mL/100 L drinking water), according to the manufacturer's recommended dose.

There were 24 birds in each treatment. Stressed and non-stressed treatments were separated into 2 identical rooms in order to control environmental conditions during the cyclic heat stress period. Pens measured 0.91 x 1.83 m and were lined with 3 to 5 cm of fresh pine shavings. Each pen was equipped with 1 tube feeder and 1 waterer consisting of an 18.93 L plastic bucket with 4 nipples on the bottom. Birds in each treatment were separated into 2 pens, with 12 birds/pen. Pens were arranged in a repeating pattern of Control-XPC-AviCare in each room, so that pens from a given treatment were not placed next to each other. Birds were fed a crumbled starter diet d 0 to 14 and a pelleted grower diet d 15 to 35. The ingredient composition of both diets is shown in Table 6, and nutrient information for both diets is presented in Table 7. Birds had *ad libitum* access to feed and water for the duration of the experiment, and feeders and waterers were raised as birds grew. Water in the AviCare pens (AS and AN) was emptied and then refilled from a stock solution daily; all other waterers were refilled as needed.

Table 6. Ingredient composition (g/kg) of basal diets for each growing period[1] (Reprinted from Nelson and Archer, 2019).

Ingredient	Starter (d 0-14)	Grower (d 15-35)
Corn	433.66	552.26
Soybean meal	396.95	272.82
Wheat middlings	60.17	60.08
DL-Methionine	3.61	2.71
L-Lysine	0.10	0.80
Soybean oil	59.07	79.04
Limestone	26.68	11.84
Monocalcium phosphate	12.54	13.24
Sodium chloride	4.21	4.21
TAMU Trace Mineral Mix[2]	0.50	0.50
TAMU Vitamin Mix[3]	2.51	2.51

[1] Pellet binder (calcium lignosulfonate) was added to both the starter and grower diets at a rate of 2.72 g/kg feed.

[2] Trace mineral premix added at this rate yields the following per kg: 13.33 g manganese, 13.33 g zinc, 13.33 g iron, 1.56 g copper, 0.09 g iodine, a minimum of 1.39 g calcium and a maximum of 1.93 g calcium. Calcium carbonate was used as a carrier.

[3] Vitamin premix added at this rate yields the following per kg: 36741.67 IU vitamin A, 12860 IU vitamin D3, 151.67 IU vitamin E, 435.17 mg choline, 153 mg niacin, 67.33 mg D-pantothenic acid, 23.83 mg pyridoxine, 19.83 mg riboflavin, 9.78 mg thiamin, 5.83 mg folic acid, 1.83 mg biotin, and 0.07 mg vitamin B12.

Table 7. Nutrient composition of basal diets for each growing period (Reprinted from Nelson and Archer, 2019).

Parameter	Units	Starter (d 0-14)	Grower (d 15-35)
Dry Matter (DM)	%	89.21	89.66
Crude Protein	% of DM	23.43	20.63
Crude Fat	% of DM	8.17	10.67
Acid Detergent Fiber	% of DM	4.15	3.46
Ash	% of DM	7.14	5.13
Sulfur	% of DM	0.31	0.30
Phosphorus	% of DM	0.77	0.80
Potassium	% of DM	1.32	1.19
Magnesium	% of DM	0.20	0.20
Calcium	% of DM	1.60	1.16
Sodium	% of DM	0.22	0.20
Iron	ppm (DM)	345.25	339.06
Manganese	ppm (DM)	145.72	105.73
Copper	ppm (DM)	22.31	21.86
Zinc	ppm (DM)	103.69	94.69
Digestible Energy	kcal/kg DM	3831.13	4082.42
Metabolizable Energy	kcal/kg DM	3509.81	3738.12

Room temperature was maintained at 35°C for 3 d prior to the start of the experiment, then reduced to 31°C for the first 3 d of the experiment, and further reduced 2°C every other day for the next week. Due to high external environmental temperatures, large fans were placed in each room on d 10 to assist with the circulation of cool air from the cooling pad. Birds were provided with 24 h of light for the first 3 d, and 16 h of light (0600 h to 2200 h) followed by 8 h of darkness for the remainder of the experiment. Starting on d 21 until d 35, birds in the stressed treatments (CS, XS, and AS) were exposed to cyclic heat stress from 0800 h to 1600 h daily (8 h/d). This was induced by turning off the cooling pad and the large interior fan circulating cool air, and increasing the heater

temperature to achieve a litter temperature between 31 to 34°C. Three small vertical-facing fans were evenly spaced on the floor in the heat-stressed room in order to circulate air upwards and mitigate ammonia buildup at the bird level. Birds in the thermoneutral treatments were maintained at a litter temperature between 23 to 26°C. At the end of the heat stress period, the cooling pad and large horizontal-facing fan were turned back on to circulate cooler air throughout the room.

Blood Sampling

On d 33, 12 birds from each treatment were chosen at random, and 1 to 2 mL of blood was collected via the brachial vein and separated between a clot activator serum separation vacutainer (367981, BD Medical, Franklin Lakes, NJ, United States) and a heparin and lithium gel separation vacutainer (367884, BD Medical). One heparinized hematocrit capillary tube (505, Chase Scientific Glass, Inc., Rockwood, TN, United States) was used to collect whole blood from the heparin vacutainer for each bird, then spun down using a Haematocrit 200 centrifuge (1801, Hettich Group, Kirchlengern, Germany) at 13,000 RPM for 2 min. Tubes were then used to measure packed cell volume (PCV) as a % of the total sample in the capillary tube. Vacutainers containing whole blood were inverted 2 to 3 times and stored in an ice bath until remaining blood samples were collected. Heparin vacutainers were centrifuged (Centrifuge 5804, Eppendorf, Hamburg, Germany) at 4,000 RPM for 15 min; plasma was then poured off into a microcentrifuge tube and stored at -20°C until analysis.

Commercially available ELISA kits were used to measure plasma levels of IL-1 (Ch1767, Advanced BioChemicals, Lawrenceville, GA, United States), IL-8 (Ch1234, Advanced BioChemicals), and α_1-AGP (GWB-374Z11, GenWay Biotech, Inc., San Diego,

CA, United States). Plasma concentrations of IL-1α, IL-8, and α_1-AGP were determined by measuring absorbance at 450 nm (Tecan Sunrise, Tecan Trading AG, Switzerland). Samples were assayed in duplicate, and the average of the duplicates was used in statistical analysis. Serum vacutainers were stored horizontally at 4°C for 3 h until clotting was achieved, and were then centrifuged (Centrifuge 5804, Eppendorf, Hamburg, Germany) at 4,000 RPM for 15 min. After centrifugation, 0.5 mL of serum was transferred to a microcentrifuge tube and sent to Texas A&M Veterinary Medical Diagnostic Laboratory (College Station, TX, United States) for analysis of plasma chemistry.

Ileum Sampling and Histomorphology Measurements

On d 34, 12 birds were randomly selected from each treatment, and a 1 cm-long section of ileum was collected from the halfway point between the ileocecal junction and Meckel's diverticulum. Samples were rinsed with phosphate buffered saline and stored in 30 mL of 10% neutral buffered formalin at room temperature. Samples were sent to Histo-Scientific Research Laboratories (Mt. Jackson, VA, USA) to be processed and stained with Periodic Acid-Schiff (PAS) in combination with Alcian Blue (AB). The mounted and stained ileum sections were photographed at 4x magnification, using a Nikon Eclipse Ci-L microscope (Nikon Corporation, Tokyo, Japan). The accompanying Elements software program was used to obtain villus length, crypt depth, villus/crypt ratio, and goblet cell density (# goblet cells/100 μm villus) from 6 villi/sample.

Statistical Analysis

Plasma biochemical parameters were analyzed using the General Linear Model in Minitab 17.1.0 (Minitab, Inc., State College, PA, United States) to determine the main effects of heat (heat stress, no heat stress), diet (control, XPC, AviCare), and heat x diet

interaction. Histomorphological data did not meet assumptions for ANOVA; the main effects of heat and diet, and the heat x diet interaction were analyzed using Kruskal–Wallis, followed by pairwise comparisons using the Dwass–Steel–Critchlow–Fligner method (Hollander and Wolfe, 1999) for the main effects of heat and diet. A significant difference was defined as $P < 0.05$.

RESULTS

Plasma Biochemical Parameters and Cytokines

Data for d 33 blood measurements are shown in Tables 8 and 9. There was a main effect of diet ($P < 0.001$) on plasma phosphorus: control birds had the highest, XPC-supplemented birds had the next highest, and AviCare-supplemented birds had the lowest levels. There was also an interaction effect ($P = 0.003$): AS had higher plasma phosphorus than AN, and XS was higher than XN. There was a trend ($P = 0.059$) toward higher plasma alkaline phosphatase (ALP) in non-heat-stressed birds compared to heat-stressed birds. Temperature also affected plasma glutamate dehydrogenase (GLDH) ($P = 0.008$), which was higher in heat-stressed birds. Diet affected plasma uric acid ($P = 0.002$): both XPC- and AviCare-supplemented birds had higher uric acid than the control but did not differ from each other. There was an interaction effect on plasma sodium ($P = 0.046$), which was higher in AN and XS compared to CS. An interaction effect was also observed for plasma IL-1 α ($P = 0.021$), which showed that levels were highest in CN and lowest in AN.

Neither main effects ($P > 0.05$) nor an interaction effect ($P > 0.05$) was observed for the following plasma biochemical parameters: packed cell volume (PCV), total protein, calcium, glucose, alkaline phosphatase (ALP), creatine kinase (CK), aspartate

aminotransferase (AST), cholesterol, potassium, chloride, sodium/potassium ratio, IL-8, and α_1-AGP.

Ileum Histomorphology

Data for d 34 ileal measurements are shown in Table 10. Villus length was affected by heat ($P \leq 0.001$) and diet ($P \leq 0.001$): heat stressed birds had shorter villi, and both XPC and AviCare had longer villi compared to the control but were not different from each other. Crypt depth was affected by diet ($P \leq 0.001$): birds supplemented with XPC or AviCare had lower crypt depth compared to the control but did not differ from each other. Both heat ($P \leq 0.001$) and diet ($P \leq 0.001$) affected the villus/crypt ratio as well. The villus/crypt ratio was lower in heat-stressed birds as well as those fed the control diet. Birds fed XPC or AviCare did not differ from each other in villus/crypt ratio. Heat affected goblet cell density ($P \leq 0.001$), which was lower in heat-stressed birds.

Table 8. Blood plasma chemistry and cytokine levels in straight run White Pekin ducks after 33 d of growth[1] (Reprinted from Nelson and Archer, 2019).

	PCV	Total Protein	Calcium	Phosphorus	Glucose	ALP	CK	AST	GLDH
Units	%	g/dL	mg/dL	mg/dL	mg/dL	U/L	U/L	U/L	U/L
CS	33.42	3.00	9.98	7.98^{abc}	199.75	813.33	940.42	17.33	3.27
XS	32.42	3.00	10.03	8.18^{ab}	204.17	914.67	711.58	17.08	3.17
AS	34.00	3.00	9.93	7.77^{bc}	204.08	896.08	964.50	16.83	3.83
CN	30.00	3.01	9.72	8.43^{a}	206.67	870.42	817.83	14.08	2.75
XN	32.25	3.00	10.02	7.56^{cd}	202.25	1010.92	1028.17	15.75	2.66
AN	32.58	3.02	9.81	7.20^{d}	205.00	826.10	929.40	16.40	2.50
Pooled SEM	1.05	0.01	0.17	0.16	3.61	52.47	114.68	1.39	0.34
Main Effect Heat									
Heat	33.28	3.00	9.98	7.98	202.67	874.69	872.17	17.08	3.43^{a}
No Heat	31.66	3.01	9.85	7.76	204.62	924.88	924.88	15.35	2.64^{b}
Main Effect Diet									
Control	31.78	3.00	9.85	8.21^{a}	203.21	879.13	879.13	15.71	3.00
XPC	32.33	3.00	10.03	7.87^{b}	203.21	869.88	869.88	16.42	2.91
AviCare	33.29	3.01	9.87	7.51^{c}	204.50	948.55	948.55	16.64	3.23
***P*-value Heat**	0.063	0.143	0.364	0.087	0.507	0.531	0.610	0.147	0.008
***P*-value Diet**	0.342	0.448	0.528	≤ 0.001	0.916	0.059	0.807	0.791	0.768
***P*-value Heat x Diet**	0.325	0.448	0.778	0.003	0.454	0.292	0.184	0.591	0.418

[a, b, c, d] Values with different letters within a row indicate a significant difference at $P < 0.05$.

Table 9. Blood plasma chemistry and cytokine levels in straight run White Pekin ducks after 33 d of growth[1] (Reprinted from Nelson and Archer, 2019).

	Uric acid	Cholesterol	Sodium	Potassium	Chloride	Sodium/ Potassium Ratio	IL-1α	IL-8	α1-AGP
Units	mg/dL	mg/dL	mEq/L	mEq/L	mEq/L		pg/mL	pg/mL	ng/mL
CS	3.06	148.00	149.89[b]	5.09	106.22	29.83	301.15[ab]	460.94	3801.79
XS	3.78	155.42	157.67[a]	5.12	110.56	31.12	406.94[ab]	444.90	3773.30
AS	3.57	150.36	153.82[ab]	5.05	109.36	31.06	427.23[ab]	499.51	3815.85
CN	2.92	138.64	154.80[ab]	5.05	110.80	30.96	446.83[a]	405.52	3855.39
XN	4.47	139.09	153.64[ab]	5.21	108.73	29.95	333.40[ab]	450.79	3781.48
AN	3.69	146.50	155.38[a]	4.99	110.75	31.65	282.09[b]	492.04	3743.36
Pooled SEM	0.30	7.39	1.75	0.21	1.37	1.24	121.74	31.77	41.12
Main Effect Heat									
Heat	3.47	151.29	153.79	5.08	108.76	30.70	378.44	468.45	3796.98
No Heat	3.67	141.25	154.52	5.09	110.00	30.77	354.11	449.45	3793.41
Main Effect Diet									
Control	2.99[b]	143.52	152.47	5.07	108.63	30.42	373.99	433.23	3828.59
XPC	4.11[a]	147.61	155.45	5.17	109.55	30.48	370.17	447.85	3777.39
AviCare	3.62[a]	148.52	154.47	5.02	109.95	31.31	354.66	495.78	3779.61
***P*-value Heat**	0.371	0.138	0.578	0.985	0.224	0.858	0.576	0.791	0.923
***P*-value Diet**	0.002	0.800	0.172	0.783	0.516	0.711	0.928	0.688	0.446
***P*-value Heat x Diet**	0.377	0.742	0.046	0.935	0.073	0.623	0.021	0.889	0.375

[a, b] Values with different letters within a row indicate a significant difference at $P < 0.05$.

Table 10. Average ileal measurements in straight run white Pekin ducks after 34 d of growth. Birds were either exposed to cyclic heat stress (S) or thermoneutral conditions (N) d 21 to 35[1] (Reprinted from Nelson and Archer, 2019).

	Villus Length	Crypt Depth	Villus/ Crypt Ratio	Goblet Cell Density
Units	μm	μm		#/100μm
CS	524.57	168.78	3.36	13.02
XS	529.66	151.04	3.85	12.94
AS	519.00	222.29	3.31	12.44
CN	431.13	212.59	2.91	15.9
XN	636.24	169.14	4.55	15.85
AN	653.54	143.69	4.95	15.40
Pooled SEM	14.13	10.59	0.20	0.72
Main Effect Heat				
Heat	524.41[a]	180.70	3.50[a]	12.80[a]
No Heat	573.64[b]	175.14	4.14[b]	15.72[b]
Main Effect Diet				
Control	477.85[a]	190.68[a]	3.13[a]	14.46
XPC	582.95[b]	160.09[b]	4.20[b]	14.40
AviCare	586.27[b]	182.99[b]	4.13[b]	13.92
***P*-value Heat**	≤ 0.001	0.10	≤ 0.001	≤ 0.001
***P*-value Diet**	≤ 0.001	≤ 0.001	≤ 0.001	0.96

[a, b] Values with different letters within a row indicate a significant difference at $P < 0.05$.

[1]Means represent the average of measurements taken from 6 villi from each of 12 birds/treatment (72 measurements/treatment).

DISCUSSION

Glutamate dehydrogenase (GLDH) is an enzyme responsible for catalyzing the reversible conversion of glutamate to α-ketoglutarate, which results in the production of ammonium cations (Vittorelli et al., 2005). It is primarily produced by hepatocytes, as well as in the kidneys and cardiac muscle, and its concentration in the blood increases as a result of damage to the liver caused by inflammatory or disease processes (Washington and Van Hoosier, 2012). This experiment showed that heat-stressed ducks had higher plasma

GLDH concentrations, which has also been demonstrated in heat-stressed parrots (Xie et al., 2019). Phosphorus plays a significant role in energy metabolism and bone deposition (Parmer et al., 1987). Heat stress has been shown to reduce plasma phosphorus in ducks (Ma et al., 2014). On the other hand, dietary phosphorus depletion has been shown to reduce GLDH activity (Huber and Breves, 1999). This is because GLDH requires the coenzyme nicotinamide adenine dinucleotide phosphate (NADP) (Baghavan, 2002). Heat-stressed birds supplemented with either XPC or AviCare had higher plasma phosphorus than their non-stressed counterparts. In addition, plasma GLDH activity was higher in heat-stressed birds. There appears to be an interaction between plasma phosphorus levels in supplemented treatments and GLDH activity in heat-stressed birds. Dietary supplementation of yeast fermentate may have altered available phosphorus, and as a result, amino acid metabolism. This could explain why plasma levels of uric acid were higher in birds fed XPC or AviCare, although heat stress alone did not affect uric acid concentrations. However, because there was no interaction effect for GLDH, further research is needed to clarify whether yeast fermentate supplementation or heat stress ultimately affected amino acid metabolism.

While uric acid is a waste product of protein metabolism, it also serves to scavenge superoxide radicals. One study found that heat stress altered enzyme activities of catalase and superoxide dismutase, both of which scavenge free radicals produced by oxidative metabolism; however, the study did not assess uric acid concentrations (Zeng et al., 2013). Uric acid was higher in both XPC and AviCare-supplemented birds, suggesting that adding yeast fermentate to either the feed or the drinking water has similar effects on amino acid metabolism. Yeast fermentate has been shown to improve antioxidant capacity in humans

(Jensen et al., 2011). The increased production of uric acid in ducks supplemented with yeast fermentate may contribute to reduced oxidative damage to intestinal villi. For example, villus length and villus/crypt ratio were higher in supplemented birds compared to the control, and lower in heat-stressed birds. This implies some interaction of heat and diet, although unequal variances prevented the analysis of interactions among histomorphological parameters. However, goblet cell density was reduced in heat-stressed birds and was unaffected by diet. Further research investigating the activity of plasma antioxidative enzymes may clarify any potential relationship between diet-mediated changes in uric acid production and the effects of oxidative stress on ileal histomorphology.

Pekin ducks are thought to be more sensitive to heat stress than other duck breeds (Zeng et al., 2013). Among the inflammatory cytokines measured, only IL-1α was shown to be affected, and this was higher in CN than AN. Likewise, plasma sodium levels were lowest in CS and highest in XS and AN. Pairwise comparisons of plasma sodium concentrations between XS and XN and between AS and AN were not significant. Therefore, it is unclear whether differences in IL-1α and sodium levels can be attributed to yeast fermentate supplementation or other environmental factors. Previous studies have shown differences in plasma phosphorus, ALP enzyme activity, and IL-1α in broilers supplemented with XPC or AviCare and reared under thermoneutral conditions (Nelson et al., 2018).

In this study effects observed in plasma GLDH, phosphorus, and uric acid suggest that dietary supplementation of yeast fermentate primarily affects amino acid metabolism. The identities of the metabolites in XPC and AviCare are not currently known, and

knowledge about their mode of action is limited thus far. However, this study shows that dietary supplementation of yeast fermentate in either the feed or drinking water supports villus length, crypt depth, and villus/crypt ratio, and increases plasma uric acid and phosphorus in mixed-sex White Pekin ducks.

Heat stress reduced villus length, villus/crypt ratio, and goblet cell density. However, dietary inclusion of yeast fermentation metabolites in the feed or drinking water increased villus length and villus/crypt ratio and therefore, surface area for nutrient absorption. Yeast fermentate may have also affected amino acid metabolism during heat stress by modulating plasma phosphorus levels. Finally, interactions between heat stress and diet were observed for sodium and IL-1α. Future studies investigating plasma antioxidant capacity may clarify associations between dietary yeast fermentate supplementation and amino acid metabolism, oxidative stress, and ileal histomorphology in heat-stressed ducks.

CHAPTER VI

EFFECTS OF YEAST FERMENTATE SUPPLEMENTATION ON CECAL MICROBIOME, PLASMA BIOCHEMISTRY AND ILEAL HISTOMORPHOLOGY IN STRESSED BROILER CHICKENS

INTRODUCTION

Environmental stress is a major contributing factor to economic loss in the poultry industry (Awad et al., 2017). During the summer months, for example, broiler chickens are prone to heat stress, which increases production of reactive oxygen species (ROS): oxidative stress, in turn, can affect intestinal microflora and histological structure, thereby increasing the likelihood of pathogen infiltration (Burkholder et al., 2008; Quinteiro-Filho et al., 2010). Heat stress-induced behavioral changes and oxidative stress can lead to reduced feed intake, depressed growth rate, and impaired intestinal function (Akbarian et al., 2016; Awad et al., 2017; Zhang et al., 2015). Typically, progenitor cells in the intestine crypt generate epithelial cells which differentiate as they migrate toward the tip of the villus, where they remain for a few days and are subsequently shed through a unique apoptotic process called anoikis (Fouquet et al., 2004; Zhang et al., 2015). However, intestinal barrier dysfunction manifests during exposure to heat stress, pathogens such as those in re-used litter, as well as immune stress such as vaccination (Bolan et al., 2010; Kassem et al., 2010; Yang et al., 2011). In turn, increased apoptosis of both healthy epithelial cells and progenitor cells results in decreased villus height (Chen et al., 2015; Zhang et al., 2015).

Several dietary strategies have been proposed to reduce the effects of heat stress on growth, production and well-being, including vitamin A, vitamin E, and ascorbic acid (Lin et al., 2006b). However, because heat stress impairs the absorption of these vitamins (Sahin and Kucuk, 2003), other dietary methods may be more practical in alleviating the effects of heat stress on poultry growth and intestinal health. For example, prebiotics such as mannanoligosaccharide (MOS) may reduce the negative effects of cyclic heat stress on small intestine morphology (Ashraf et al., 2013), ROS production (Sohail et al., 2011), and humoral immunity (Sohail et al., 2010). Yeast fermentation metabolites have also been shown to reduce measures of stress in poultry exposed to heat stress (Bartz, 2016; Price et al., 2018) and rearing stress (Al-Mansour et al., 2011; Nelson et al., 2018). Further, supplementing yeast fermentate (YF) prior to heat stress has been shown to attenuate measures of intestinal barrier dysfunction (Ducray et al., 2016; Nelson and Archer, 2019). The functional metabolites in YF may behave as reductants toward free radicals, thus reducing oxidative damage (Jensen et al., 2008), toxin production or colonization by pathogenic bacteria (Fukuda et al., 2011; Kim et al., 2018). This, in turn, may increase nutrient absorption and digestibility by helping to stabilize microbial structure at an early age (Park et al., 2017a).

Previous reports have shown the effects of whole yeast cells (Bradley et al., 1994; Sandikci et al., 2004) and cell wall components (Brümmer et al., 2010; Solis de los Santos et al., 2007) on intestinal health in poultry under various conditions. However, the effect of YF on intestinal morphology, plasma biochemistry, and cecal microbiome in heat-stressed broilers remains to be seen. Therefore, 2 experiments were conducted to evaluate the effect of YF on intestinal health. The objective of Exp. 1 was to determine whether adding YF to

the feed or drinking water affects the diversity or abundance of the cecal microbiome in male broiler chickens exposed to acute and rearing stressors. The objective of Exp. 2 was to determine whether adding YF to the feed or drinking water attenuates the effects of cyclic heat stress on measures of intestinal health in male broiler chickens.

MATERIALS AND METHODS

Experiment 1: Cecal Microbiome

Animal Husbandry. All procedures were carried out in accordance with the guidelines established by Texas A&M Institutional Animal Care and Use Committee (AUP2016-0004) and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). Birds were housed at the Texas A&M University Poultry Science Teaching, Research, and Extension Center (Texas A&M University, College Station, TX, United States). This experiment consisted of 3 treatments: stressed and un-supplemented control (CS), stressed and supplemented with YF in the drinking water (AVI; AviCare, Diamond V Mills, Cedar Rapids, IA, United States), and stressed and supplemented with YF in the feed (XPC; Original XPC, Diamond V Mills, Cedar Rapids, IA, United States). Pens were assigned to a given treatment using randomized complete block design. Twenty-five 1-d-old male broiler chickens were randomly assigned to each of 12 pens/treatment (n = 300 replicate/treatment). Pens measured 0.91 m x 1.83 m and were lined with 4 to 6 cm of re-used pine shavings. Building temperature was maintained at 35°C for 7 d prior to the start of the experiment, then at 31°C for the following 8 d. Week 2 house temperature was maintained at 29°C and then allowed to decrease 2.8°C each week until ambient temperature was reached. Birds were provided 24 h of light for the first 3 d of the

experiment and 20 h of light followed by 4 h of darkness for the remainder of the experiment.

One tube feeder and 1 waterer consisting of an 18.93 L bucket with 4 nipples on the bottom were hung in each pen and their heights adjusted as birds grew. Feed and water were provided *ad libitum* except during the 12 h fasting stress challenge on d 18. Birds were fed a crumbled starter diet d 0 to 14, a pelleted grower diet d 15 to 28, and a pelleted finisher diet d 29 to 42. Ingredient composition for the basal diet for each growing phase is presented in Table 11, and nutrient analysis for each diet and growing phase is presented in Table 12. Yeast fermentate was added to the feed (1.25 kg/t feed, d 0 to 42) or drinking water (1.60 mL/L drinking water, d 0 to 42) according to the manufacturer's recommended dose. Water was measured in gallons using a P0550 water meter (P3 International, New York, NY, United States). Birds supplemented with YF in the drinking water received fresh water with YF from a stock solution daily; all other treatments received fresh water without added YF daily.

Stressors. The stress model in this experiment closely followed that used by Nelson et al. (2018). All birds were raised on previously used litter and were spray-vaccinated for coccidiosis (COCCIVAC-B52, Merck, Kenilworth, NJ, United States) on d 0. On d 18, all treatments were spray-vaccinated for Newcastle/Bronchitis (COMBOVAC-30, Merck), then exposed to a 12 h acute stress challenge consisting of a simultaneous feed/water withdrawal and heat stress. At 2000 h on d 18, feeders and waterers were removed and barriers were put in place to crowd birds and produce enough collective body heat to increase litter temperatures until they were at least 32°C in order to induce heat stress.

Building lights remained on to mimic daylight conditions. At 0800 h on d 19 barriers were removed and feeders and waterers were refilled and added back to the respective pen.

Table 11. Ingredient composition and nutrient analysis of basal diets for each phase of the growing period.

Ingredient	Units	Starter (d 0-14)	Grower (d 15-28)	Finisher (d 29-42)
Corn	g/kg	583.93	630.92	689.41
Soybean meal	g/kg	343.96	298.46	240.97
DL-Methionine	g/kg	3.20	2.77	2.37
L-Lysine	g/kg	1.95	2.10	2.02
L-Threonine	g/kg	0.67	0.77	0.77
Soybean oil	g/kg	28.50	28.50	30.00
Limestone	g/kg	14.35	13.55	12.45
Monocalcium phosphate	g/kg	15.60	14.45	12.95
Sodium chloride	g/kg	4.35	3.72	2.35
Sodium bicarbonate	g/kg	0.00	1.22	3.15
Trace Mineral Mix[1]	g/kg	0.50	0.50	0.50
Vitamin Mix[2]	g/kg	2.50	2.50	2.50

[1] Mineral premix fed at this level yields the following per kg: 13.33 g manganese, 13.33 g zinc, 13.33 g iron, 1.56 g copper, 0.09 g iodine, a minimum of 1.39 g calcium and a maximum of 1.93 g calcium.

[2] Vitamin premix fed at this level yields the following per kg: 36,741.67 IU vitamin A, 12,860.00 IU vitamin D_3, 151.67 IU vitamin E, 0.07 mg vitamin B_{12}, 19.83 mg riboflavin, 153.00 mg niacin, 67.33 mg D-pantothenic acid, 435.17 mg choline, 4.83 mg menadione, 5.83 mg folic acid, 23.83 mg pyridoxine, 9.78 mg thiamine, and 1.83 mg biotin.

Table 12. Nutrient analysis of basal diets for each phase of the growing period.

Ingredient	Units	Starter (d 0-14)	Grower (d 15-28)	Finisher (d 29-42)
Dry Matter	%	88.8	88.9	88.8
Crude protein	% of DM	24.4	22.6	19.4
Crude fat	% of DM	5.76	5.92	6.35
Acid detergent fiber	% of DM	2.90	2.50	2.50
Ash	% of DM	5.60	5.64	5.01
Sulfur	% of DM	0.33	0.30	0.27
Phosphorus	% of DM	0.86	0.89	0.75
Potassium	% of DM	1.25	1.16	0.95
Magnesium	% of DM	0.20	0.19	0.17
Calcium	% of DM	1.15	1.12	1.09
Sodium	% of DM	0.25	0.24	0.25
Iron	ppm (DM)	373	387	410
Manganese	ppm (DM)	106	94.5	104
Copper	ppm (DM)	16.8	11.4	19.9
Zinc	ppm (DM)	98.8	102	101
Digestible energy	kcal/kg (DM)	3,792	3,836	3,902
Metabolizable energy	kcal/kg (DM)	3,461	3,505	3,594

Sample Collection and Processing. On d 42, ceca were collected using aseptic technique from 20 randomly selected birds/treatment and placed in 15 mL centrifuge tubes with 5-10 volumes RNAlater® (AM7020, ThermoFisher Scientific, Waltham, MA, United States). Samples were stored at -20°C until further processing. Next, samples were removed from the RNAlater® and cecal contents were separated from the intestinal epithelium. Cecal contents and cecal tissue were then transferred to microcentrifuge tubes

and stored at -80°C. Batches of 20 samples were thawed at 4°C and 0.2 g of each sample was used to extract DNA using a QIAamp® PowerFecal® DNA Kit (12830-50, Qiagen, Hilden, Germany); sample absorbance was measured using Qdot (ThermoFisher Scientific, Waltham, MA, United States) to quantify sample DNA concentration.

The V4 region of the 16S rRNA gene of extracted DNA was amplified using polymerase chain reaction (PCR). First, 2 µL of sample DNA was mixed with 10 µL of NEBNext high fidelity 2X PCR MasterMix (New England Biolabs, Inc., Ipswich, MA, United States) and 1µL each of the universal primers Hyp515F (5'-GTGYCAGCMGCCGCGGTA-3') and Hyp806R (5'-GGACTACHVGGGTWTCTAAT-3') in 6µL nuclease-free water. This reaction mixture was then centrifuged briefly and submitted to initial denaturation for 30 s at 98°C followed by 15 cycles of denaturation for 10 s at 98°C, annealing for 30 s at 64°C, and extension first for 30 s and then for 5 min at 72°C. The PCR products were kept at 4°C until clean-up using Agencourt AMPure XP magnetic beads (Beckman Coulter, Inc., Indianapolis, IN, United States). Next, 250 bp paired-end reads were generated by sequencing of the 16S amplicons on the Illumina MiSeq platform. Sample sequences were processed with Mothur in order to distinguish and classify operational taxonomic units (OTUs) in each sample (Schloss et al., 2009). Sample sequences were screened for 97% similarity of matched pairs. Sequences were then filtered to remove chimeric sequences and sequences from non-prokaryote sources such as mitochondria and chloroplast. Following quality control, those samples which contained the minimum number of usable sequences were used to conduct taxonomic classification of OTUs by Order and by species. Finally, a phylogenetic tree was generated based on shared OTUs between treatments.

Statistical Analysis. Phylogenetic data from cecal content sample sequences was first analyzed in Mothur (Schloss et al., 2009) for parsimony among treatments using UniFrac. This was performed in order to determine weighted and unweighted phylogenetic differences in microbial community structure between treatments. This was followed by Metastats analysis of pairwise comparisons in Mothur. Analysis of molecular variance (AMOVA) and non-parametric multivariate analysis of variance with permutation (PERMANOVA) was carried out to compare shared OTUs between treatments, and homogeneity of molecular variance (HOMOVA) was used to compare individual samples. Next, the diversity, number and distribution of OTUs between treatments were compared using the non-parametric Wilcoxon Signed-Rank test in R (RStudio, Inc., Boston, MA, United States). This was followed by the analysis of diversity within treatments using Inverse Simpson's evenness index (InvSimpson) and Fisher's alpha-diversity, as well as analysis of beta-diversity between treatments using Chao test for species number, abundance-based coverage estimators (ACE), and Shannon-Weiner diversity index. Alpha diversity was considered significantly different at $P < 0.05$.

Experiment 2: Plasma Biochemistry and Ileal Morphology

Animal Husbandry. All procedures were carried out in accordance with the guidelines established by Texas A&M Institutional Animal Care and Use Committee (AUP#2018-0135). Birds were cared for according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). Birds were housed at the Texas A&M University Poultry Science Teaching, Research, and Extension Center (Texas A&M University, College Station, TX, United States) and all diets were mixed on-site.

One experiment was conducted in which 20 1-d-old male broiler chickens were placed in each of 12 pens (n = 40 replicates/treatment) and reared to 43 d. Birds were randomly assigned to each of 6 treatments: stressed control (CS), stressed and supplemented with YF in the feed (XS; Original XPC, Diamond V Mills), stressed and supplemented with YF in the drinking water (AS; AviCare, Diamond V Mills), non-stressed control (CN), non-stressed and supplemented with YF in the feed (XN), and non-stressed and supplemented with YF in the drinking water (AN). Yeast fermentate was added to the feed (1.25 kg/t feed, d 0 to 43) or to the drinking water (1.60 mL/L drinking water, d 0 to 43) according to the manufacturer's recommended dose.

Stressed and non-stressed treatments were separated into 2 identical rooms in order to control environmental conditions during the cyclic heat stress period. Pens measured 0.91 x 1.83 m and were lined with 4 to 6 cm of fresh pine shavings. Each pen was equipped with 1 tube feeder and 1 waterer consisting of an 18.93 L plastic bucket with 4 nipples on the bottom. Birds were fed a crumbled starter diet d 0 to 14, pelleted grower diet d 15 to 28, and pelleted finisher diet d 29 to 43. Ingredient composition for the basal diet for each growing phase is presented in Table 1, and nutrient analysis for each diet and growing phase is presented in Table 2. Birds had ad libitum access to feed and water for the duration of the trial, and feeders and waterers were raised as birds grew. Birds supplemented with YF in the drinking water (AS and AN) received fresh water with YF from a stock solution daily; all other treatments received fresh water without YF daily.

Room temperature was maintained at 35°C for 3 d prior to the start of the experiment, then reduced to 31°C for the first 3 d of the trial, and further reduced 2°C every other day for the next week. Birds were provided with 24 h of light d 0 to 3, and 20 h of light

followed by 4 h of darkness d 4 to 42. On d 28 to 42, birds in the stressed treatments were exposed to cyclic heat stress for 16 h/d. This was induced by increasing the heater temperature to 30°C. Three small vertical-facing fans were evenly spaced on the floor 1.0 m away from the pens in the heat-stressed room in order to circulate air upwards and mitigate ammonia. At the end of the heat stress period the heater temperature was reduced to 22°C and a large horizontal-facing fan inside the room was turned on to circulate cooler air from the cooling pad throughout the room.

Sample Collection and Processing. On d 42, 1 to 2 mL of blood was collected from each of 12 randomly selected birds/treatment. Blood from each bird was divided between a clot activator serum separation vacutainer (367981, BD Medical, Franklin Lakes, NJ, United States) and a heparin and lithium gel separation vacutainer (367884, BD Medical). One heparinized hematocrit capillary tube (505, Chase Scientific Glass, Inc., Rockwood, TN, United States) was used to collect whole blood from the heparin vacutainer for each bird, then spun down using a Haematocrit 200 centrifuge (1801, Hettich Group, Kirchlengern, Germany) at 13,000 RPM for 2 min. Hematocrit tubes were then used to measure packed cell volume (PCV) as a percentage of the total sample in the capillary tube. Vacutainers containing whole blood were inverted 2 to 3 times and stored in an ice bath until remaining blood samples were collected. Heparin vacutainers were centrifuged (Centrifuge 5804, Eppendorf, Hamburg, Germany) at 4,000 RPM for 15 min; plasma was then poured off into a microcentrifuge tube and stored at -20°C until analysis. Commercially available ELISA kits were used to measure plasma levels of IL-1α (Ch1767, Advanced BioChemicals, Lawrenceville, GA, United States), IL-8 (Ch1234, Advanced BioChemicals), and α1-AGP (GWB-374Z11, GenWay Biotech, Inc., San Diego,

CA, United States). Plasma concentrations of IL-1α, IL-8, and α1-AGP were determined by measuring absorbance at 450 nm (Tecan Sunrise, Tecan Trading AG, Switzerland). Serum vacutainers were stored horizontally at 4°C for 3 h until clotting was achieved and then centrifuged (Centrifuge 5804, Eppendorf, Hamburg, Germany) at 4,000 RPM for 15 min. After centrifugation serum was transferred to a microcentrifuge tube and sent to Texas A&M Veterinary Medical Diagnostic Laboratory (College Station, TX, United States) for analysis of plasma chemistry.

On d 43 a 1 cm-long section of ileum from the mid-way point between Meckel's diverticulum and the ileocecal junction was collected from 12 birds/treatment. Ceca segments were rinsed with phosphate-buffered saline and stored in 30 mL of 10% neutral buffered formalin at room temperature. Samples were sent to Histo-Scientific Research Laboratories (Mt. Jackson, VA, USA) to be processed and stained with Periodic Acid-Schiff in combination with Alcian Blue. The mounted and stained ileum sections were then analyzed at 4x magnification using a Nikon Eclipse Ci-L microscope (Nikon Corporation, Tokyo, Japan). The accompanying Elements software package was used to measure villus height, crypt depth, villus/crypt ratio, and goblet cell density (# of goblet cells/100 µm villus) from 6 villi/sample.

Statistical Analysis. Plasma biochemistry data were analyzed using the GLM procedure in Minitab 17.1.0 (Minitab, Inc., State College, PA, United States) to assess main effects of heat (heat, no heat) and diet (control, XPC, AviCare) and any interaction effect (heat x diet), with individual bird as the experimental unit. Ileal morphological data did not meet the assumptions for ANOVA. These data were analyzed for the main effects

of heat and diet using Kruskal-Wallis, followed by the Dwass-Steel-Critchlow-Fligner method (Hollander and Wolfe, 1999) to assess main effects of heat and diet. A significant difference was defined as $P < 0.05$.

RESULTS

Experiment 1: Cecal Microbiome

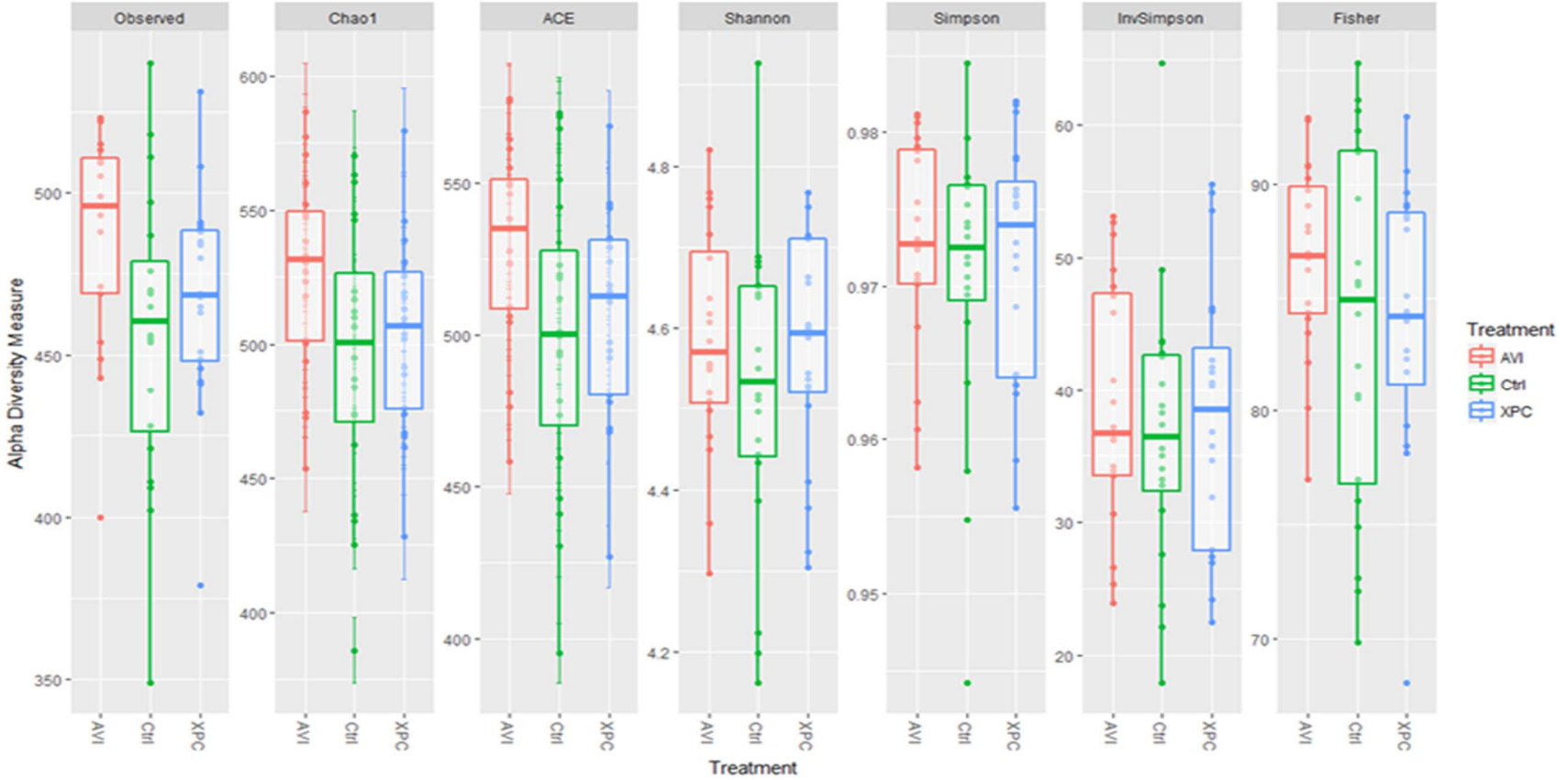
After quality control, the analyzed dataset retained an average of 10, 271 sequences. Sample sequence content was normally distributed and ranged from 11,000 to 36,000 reads/sample. Beta diversity, based on parsimony, was not significantly different between CS and AS ($P = 0.620$), between CS and XS ($P = 0.809$) or between AS and XS ($P = 0.645$). Neither the weighted nor unweighted UniFrac analyses indicated significant differences between the treatments. The unweighted analysis was not significant between CS and AS ($P = 0.838$), between CS and XS ($P = 0.780$) or between AS and XS ($P = 0.832$). Weighted UniFrac analysis was not significant between CS and AS ($P = 0.518$), between CS and XS ($P = 0.509$) and between AS and XS ($p = 0.572$). Similarly, the analysis of molecular variance (AMOVA) revealed that treatments were not significantly different ($P = 0.069$), and HOMOVA was not significant, indicating that the data had equal variances ($P = 0.358$). Metastats analysis showed no significant differences with respect to pairwise comparisons between replicates within a treatment and between treatment groups. However, PERMANOVA was significant ($P = 0.023$), suggesting that the cecal microbiome differed between treatments.

Diversity, richness and evenness of OTUs for each treatment are shown in Figure 1. Non-parametric analysis showed that OTU richness did not differ between treatments ($P =$

0.321). Microbial diversity between treatments as represented by InvSimpson was not significant ($P = 0.769$), but coverage of each treatment was significantly different ($P = 0.036$). Median values in each measure of alpha diversity showed slight treatment differences but these were not statistically significant ($P > 0.05$).

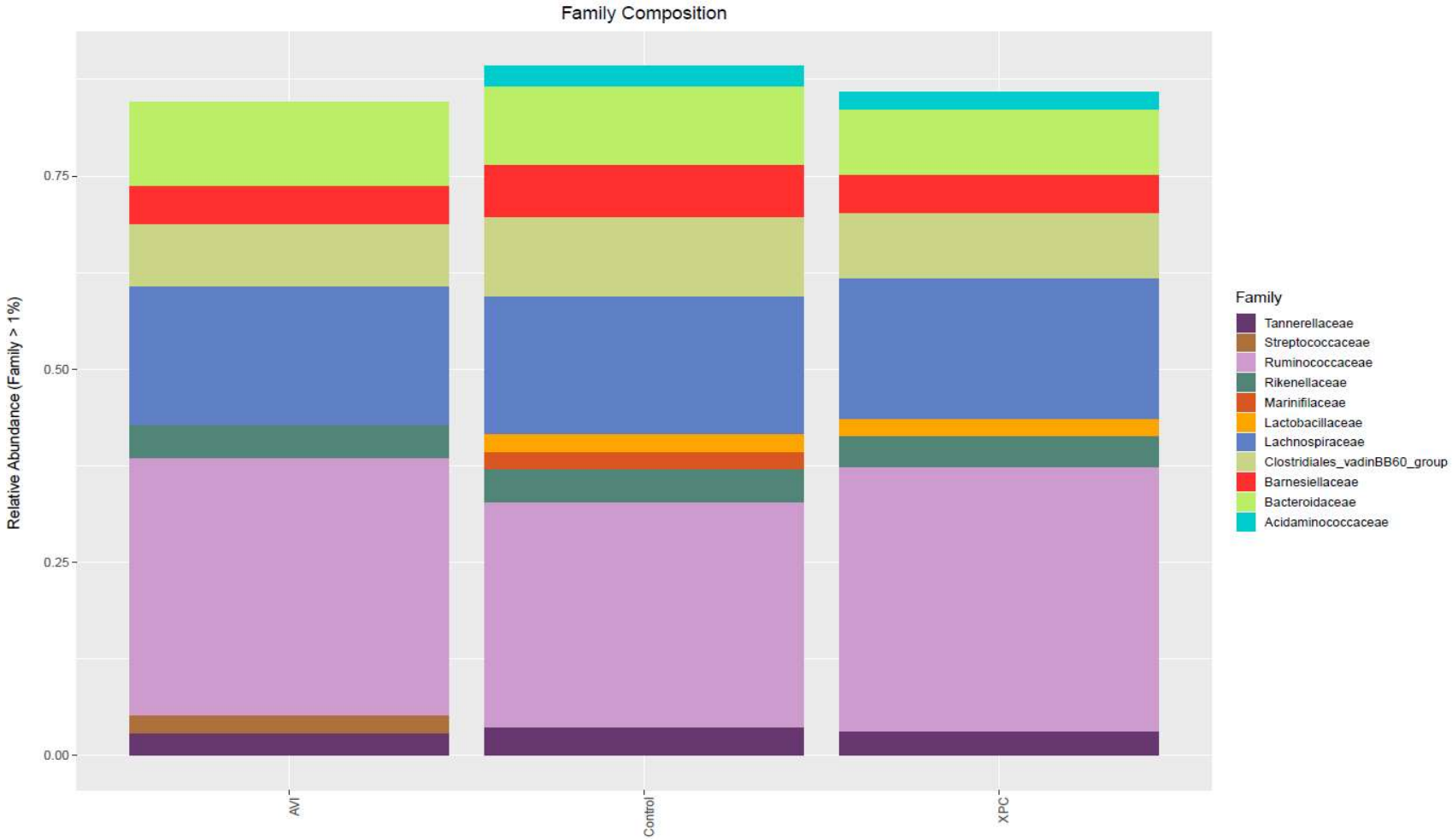
The relative abundance of microbial taxonomic families for each treatment is shown in Figure 2, and relative abundance of taxonomic families for individual samples is shown in Figure 3. Treatment-wise comparisons show that *Streptococcaceae* was present only in AVI and that *Marinifilaceae* was present only in the Control group. Likewise, the treatment-wise comparison shows that both *Acidaminococcaceae* and *Lactobacillaceae* were absent in AVI. However, the sample-wise comparison shows that each of these families were present to some degree in all 3 treatments. *Ruminococcaceae* and *Lachnospiraceae* were dominant in all samples. *Rikenellaceae* and *Bacteroidaceae* were approximately equally distributed in individual samples between the 3 treatments. *Acidamonococcaceae* was the least abundant of the families that were represented in nearly all samples. Six samples contained unclassified *Clostridiales*, 2 samples contained unclassified *Bacteria*, and 1 sample contained uncultured bacteria. Similarly, *Peptostreptococcaceae*, *Burkholderiaceae*, *Christensenellaceae*, and *Gastranaerophilales* were poorly represented across many of the samples. Furthermore, all samples were composed of approximately the same relative abundance of taxonomic families, and individual samples differed primarily in the evenness with which these families were distributed.

Figure 2. Species diversity comparison between CS, AS and XS as shown by Observed OTUs, Chao1 index of species richness, abundance-based coverage estimators (ACE), Shannon-Weiner diversity index (Shannon), Simpson's index of evenness (Simpson) and its inverse (InvSimpson), and Fisher's alpha diversity (Fisher).



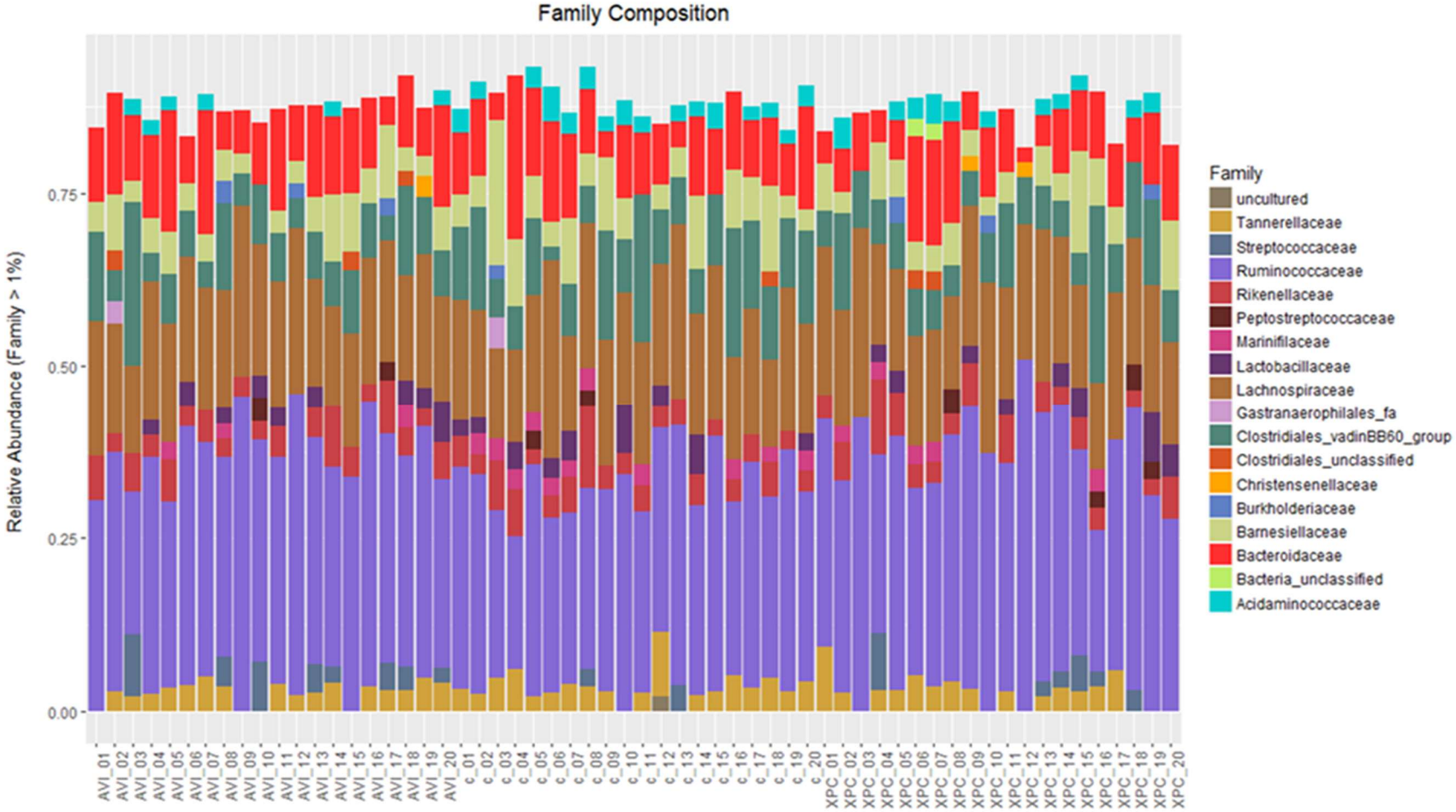
[1] Error bars represent the standard error of the mean. AVI = AS (stressed and supplemented with AviCare at a rate of 160 mL/100 L drinking water, d 0 to 42); Ctrl = CS (stressed and non-supplemented); XPC = XS (stressed and supplemented with XPC at a rate of 1.25 kg/metric ton feed, d 0 to 42).

Figure 3. The relative abundance (%) and distribution of family-level taxa in each treatment.



[1] AVI = AS (stressed and supplemented with AviCare at a rate of 160 mL/100 L drinking water, d 0 to 42); Control = CS (stressed and non-supplemented); XPC = XS (stressed and supplemented with XPC at a rate of 1.25 kg/metric ton feed, d 0 to 42).

Figure 4. The relative abundance (%) and distribution of family-level taxa in each sample grouped by treatment.



[1] AVI = AS (stressed and supplemented with AviCare at a rate of 160 mL/100 L drinking water, d 0 to 42); C = CS (stressed and non-supplemented); XPC = XS (stressed and supplemented with XPC at a rate of 1.25 kg/metric ton feed, d 0 to 42).

Experiment 2: Plasma Biochemistry and Cytokines

Data for plasma biochemical measures taken on d 42 are shown in Table 13 and Table 14. There were no main effects of heat or diet ($P > 0.05$) or a heat x diet interaction effect ($P > 0.05$) on the following plasma biochemical measures: packed cell volume, total protein, calcium, phosphorus, aspartate aminotransferase (AST), cholesterol, sodium, and chloride. There was no effect of diet ($P > 0.05$) or heat x diet interaction ($P > 0.05$) on the following measures: alkaline phosphatase (ALP), uric acid, potassium, sodium/potassium ratio, IL-1α, IL-8, and α_1-AGP.

Heat stress increased plasma creatine kinase (CK; $P < 0.001$), uric acid ($P < 0.001$), sodium/potassium ratio ($P < 0.001$), and IL-1α ($P < 0.001$), but decreased plasma ALP ($P = 0.04$), potassium ($P < 0.001$), IL-8 ($P < 0.001$), and α_1-AGP ($P = 0.048$).

An interaction effect was observed for glutamate dehydrogenase (GLDH; $P = 0.001$): plasma levels were higher in AS and CN compared to AN, CS, XS, and XN. Diet affected plasma CK ($P < 0.001$), which was higher in birds fed the control or supplemented with YF in the feed compared to birds supplemented with YF in the drinking water. Finally, there was an interaction effect on plasma CK ($P < 0.001$), where plasma levels were highest in CS and XS compared to all other treatments.

Experiment 2: Ileal Histomorphology

Data for ileal histomorphological measures are shown in Table 15. Heat stress decreased villus height ($P < 0.001$) and crypt depth ($P < 0.001$), but increased villus/crypt ratio ($P < 0.001$) and goblet cell density ($P < 0.001$). There was no effect of diet ($P > 0.05$) on villus/crypt ratio or goblet cell density. However, diet affected villus height ($P <$

0.001), which was higher in AviCare compared to control or XPC birds. On the other hand, crypt depth differed among treatments ($P = 0.036$) but *post hoc* analysis showed no clear differences between specific treatments.

Table 13. Blood plasma chemistry in male broiler chickens after 42 d of growth.

	PCV	Total Protein	Calcium	Phosphorus	Glucose	ALP	CK	AST	GLDH
Units	%	g/dL	mg/dL	mg/dL	mg/dL	U/L	U/L	U/L	U/L
CS	32.67	3.08	8.77	6.08	252.00	2307.27	62772.08^{a}	734.00	21.08^{bc}
XS	26.58	3.09	8.34	5.81	260.01	1971.40	38866.67^{b}	601.50	21.88^{bc}
AS	27.25	3.03	8.64	6.16	266.75	1591.75	1025.58^{c}	497.50	38.92^{a}
CN	26.17	3.57	7.84	6.12	242.60	2698.80	1356.64^{c}	631.91	29.73^{ab}
XN	28.67	3.15	9.13	6.43	274.17	2108.27	1699.50^{c}	775.58	24.50^{bc}
AN	26.82	3.01	8.68	6.03	262.50	2463.75	1118.08^{c}	593.17	17.42^{c}
Pooled SEM	2.15	0.12	3.97	0.18	4.98	270.47	3902.46^{c}	66.17	4.25
Main Effect Heat									
Heat	28.83	3.06	8.60	6.03	259.59	1945.30^{b}	33799.15^{a}	607.85	27.97
No Heat	27.23	3.24	15.46	6.19	260.76	2416.49^{a}	1416.40^{b}	667.54	23.67
Main Effect Diet									
Control	29.42	3.31	18.81	6.10	247.73^{b}	2493.71	33399.48^{a}	682.95	25.22
XPC	27.63	3.12	8.77	6.13	267.77^{a}	2043.10	17628.29^{a}	695.91	23.33
AviCare	27.04	3.02	8.66	6.10	264.63^{a}	2027.75	1106.83^{b}	545.33	28.17
***P*-value Heat**	0.370	0.264	0.918	0.233	0.975	0.040	< 0.001	0.318	0.331
***P*-value Diet**	0.530	0.280	0.517	0.991	0.001	0.155	< 0.001	0.060	0.514
***P*-value Heat x Diet**	0.137	0.354	0.109	0.114	0.081	0.387	< 0.001	0.126	0.001

^{a} , ^{b} Values with different letters within a row indicate a significant difference at $P < 0.05$.

Table 14. Blood plasma chemistry and cytokine levels in male broiler chickens after 42 d of growth.

	Uric Acid	Cholesterol	Sodium	Potassium	Chloride	Sodium/ Potassium Ratio	IL-1α[2]	IL-8[2]	α1-AGP[2]
Units	mg/dL	mg/dL	mEq/L	mEq/L	mEq/L		pg/mL	pg/mL	ng/mL
CS	7.78	144.25	154.83	4.56	110.92	34.68	2822.17	200.95	15364.64
XS	8.22	144.10	157.33	4.97	113.67	31.99	3089.43	154.17	11954.70
AS	7.33	135.58	154.00	5.00	111.00	31.19	3050.09	188.49	12802.52
CN	4.21	130.10	156.27	6.05	111.73	26.17	2385.91	258.38	15912.66
XN	4.97	147.09	157.90	6.09	113.30	25.99	2230.13	302.70	17432.73
AN	5.46	130.75	156.25	6.09	113.75	25.82	2172.51	324.08	18313.11
Pooled SEM	0.79	6.48	1.77	0.17	1.30	1.02	149.70	31.34	2152.31
Main Effect Heat									
Heat	7.76[a]	141.15	155.21	4.83[b]	111.70	32.68[a]	2987.23[a]	181.20[b]	13373.96[b]
No Heat	4.90[b]	136.00	156.76	6.08[a]	112.94	25.99[b]	2262.85[b]	295.05[a]	17219.50[a]
Main Effect Diet									
Control	6.07	137.82	155.52	5.27	111.30	30.61	2604.04	229.67	15638.65
XPC	6.52	145.67	157.63	5.56	113.47	28.83	2659.78	228.44	14693.72
AviCare	6.39	133.17	155.13	5.55	112.38	28.50	2611.30	256.29	15557.82
***P*-value Heat**	< 0.001	0.332	0.364	< 0.001	0.343	< 0.001	< 0.001	< 0.001	0.048
***P*-value Diet**	0.821	0.174	0.403	0.327	0.307	0.196	0.923	0.679	0.905
***P*-value Heat x Diet**	0.632	0.455	0.909	0.450	0.517	0.325	0.265	0.390	0.480

[a, b] Values with different letters within a row indicate a significant difference at $P < 0.05$.

[1] Unless otherwise noted, means represent the average of values from 12 birds/treatment.

Table 15. Ileal histomorphology and mucus-secreting cell density in broiler chickens after 43 d of growth. Birds were exposed to either cyclic heat stress or ambient temperature d 28 to 42.

	Villus Height	Crypt Depth	Villus/Crypt Ratio	Goblet Cell Density
Units	µm	µm		#/100µm
CS	730.33	137.82	5.87	19.50
XS	749.89	130.21	6.13	28.01
AS	916.82	145.64	6.69	26.30
CN	891.11	196.85	4.95	20.75
XN	903.69	172.99	5.83	17.82
AN	904.25	188.09	5.48	15.87
Pooled SEM	21.74	6.12	0.26	1.09
Main Effect Heat				
Heat	798.99[b]	137.89[b]	6.23[a]	24.61[a]
No Heat	899.57[a]	186.35[a]	5.41[b]	18.15[b]
Main Effect Diet				
Control	810.72[b]	167.33[a]	5.41	20.12
XPC	823.41[b]	150.67[b]	5.99	23.14
AviCare	910.53[a]	166.87[a]	6.08	21.08
***P*-value Heat**	< 0.001	< 0.001	< 0.001	< 0.001
***P*-value Diet**	< 0.001	0.036	0.065	0.074

[a, b] Values with different letters within a row indicate a significant difference at $P < 0.05$.

[1] Means represent the average of 6 measurements from each of 12 birds/treatment (72 measurements/treatment).

DISCUSSION

The stress model used in Experiment 1 is similar to that used by Nelson et al. (2018a), which showed that broilers given YF in the feed or drinking water had consistently reduced measures of short- and long-term stress compared to the stressed control after both an acute stressor and after 42 d of growth. Despite the evidence that YF effectively reduced

stress susceptibility, results from this experiment indicate that there were no treatment differences in microbial community structure after 43 d of growth. Data showed that *Ruminococcaceae* and *Lachnospiraceae* were the most dominant families and that Firmicutes outnumbered Bacteroidetes. These results are similar to cecal microbial structure described by previous studies, which have indicated that Firmicutes account for as much as 44 to 56% of the cecal microbiome and Bacteroidetes make up 23 to 46%, followed by Proteobacteria, Archaea, and Fungi (Oakley et al., 2014; Yeoman et al., 2012). Unclassified *Bacteria* and *Clostridiales* and uncultured bacteria were present in several of the samples, although these were not relegated to a single treatment. Indeed, despite major technological advancements in the identification of microbial diversity, it is not uncommon to find unidentified bacteria in intestinal cultures (Stanley et al., 2012). Although treatment-wise comparisons indicate differences in the presence or absence of a family, sample-wise comparisons show that all families were present to some degree across all 3 treatments. Furthermore, only PERMANOVA indicated significant differences in the distribution of the cecal microbiome between treatment groups, yet parametric and other non-parametric analyses did not support this. Therefore, supplementing YF in the feed or drinking water had a relatively small effect on cecal microbial distribution and richness in birds exposed to acute and rearing stressors.

These results may be indicative of the time lag between the acute stress event on d 18 and sample collection on d 42. Nelson et al. (2018) showed that broilers supplemented with YF in either the feed or drinking water and exposed to an acute stress on d 18 had reduced stress measures compared to a non-supplemented, stressed control after 42 d of

growth. However, in this study differences in cecal microbiome between supplemented and non-supplemented birds exposed to the same acute stress on d 18 were not observed after 42 d of growth. Some species of intestinal bacteria are only present for short periods during the bird's growth (Danzeisen et al., 2011). For example, culturing cecal contents with YF has shown that *Bacteroides* increased in abundance over 48 h and peaked at 12 h of incubation, whereas *Lactobacillus* and *Fecaelibacterium*, a butyrate-producing bacteria, remained at levels similar to the control (Park et al., 2017a). Additionally, although YF has been shown to modulate butyrate production by lactobacilli and bifidobacteria in the human colon (Possemiers et al., 2013), no effect was observed on overall numbers of Firmicutes and Bacteroidetes in this study. Even if the acute stress event on d 18 altered cecal microbial structure, this change may have been too transient to be observed in samples collected on d 42. Further study may elucidate whether YF supplementation affects cecal microbiome by comparing samples from stressed versus non-stressed birds at different time points following an acute stress.

There was an effect of heat, but not diet, on several plasma biochemical measures, including uric acid. Uric acid is the major by-product of nitrogen metabolism, and plasma levels reflect protein synthesis and degradation (Machín et al., 2004; Swennen et al., 2006). Research has shown that uric acid is also increased in dehydrated and heat stressed birds, as well as in birds supplemented with yeast extract (Huff et al., 2010) and YF (Nelson and Archer, 2019). Although there was no dietary effect on uric acid—and no effect of diet or heat stress on packed cell volume, an indicator of dehydration—plasma glucose was higher in birds supplemented with YF in either the feed or drinking water.

Previous research has proposed that changes in metabolism may be attributed to secondary effects of YF supplementation on the intestinal microbiome (Pan and Yu, 2014). However, in this study microbial structure was not affected by long-term consumption of YF. The increase in blood glucose in this experiment may be the result of a metabolic shift toward catabolism and gluconeogenesis. Unfortunately, neither feed efficiency nor body weight gain were recorded for this experiment. Further research may be able to clarify whether catabolism of muscle and fat, utilization of fermentation metabolites for energy, or increased nutrient absorption due to increased villus height altered plasma glucose levels. Overall, however, adding YF to either the feed or drinking water appears to produce similar effects on plasma biochemical measures.

Plasma ALP was higher in non-heat stressed birds. This enzyme is a membrane-bound glycoprotein which catalyzes the hydrolysis of monophosphate esters at high pH (Weiss et al., 1986). Heat stress increases the frequency of heat-dissipating behaviors such as panting, which results in respiratory alkalosis (Teeter et al., 1985). Heat-stressed birds may have had a more alkaline blood pH, which could contribute to reduced ALP activity. Plasma creatine kinase activity was increased with heat stress and in birds fed either the control or XPC diets. This difference could be a result of sampling procedure, where CS was the first group sampled, followed by XS and so on. If this is not the case, however, it appears that AviCare may have been more effective in reducing any heat-stress induced muscle damage that resulted in heightened blood CK activity compared to XPC. Plasma GLDH was affected by a heat x diet interaction and was highest in AS and lowest in AN. Glutamate dehydrogenase (GLDH) is an enzyme responsible for catalyzing the reversible

conversion of glutamate to α-ketoglutarate. It is primarily produced by hepatocytes, as well as in the kidneys and cardiac muscle, and increases as a result of damage to the liver caused by inflammatory or disease processes (Washington and Van Hoosier, 2012). Because plasma CK was higher in XS but GLDH was higher in AS, the effect of dietary YF and mode of administration on plasma enzyme activities in heat-stressed birds appears to be inconclusive at this point. Heat stress increased plasma potassium but did not affect sodium. As a result, heat-stressed birds had a higher sodium/potassium ratio. Potassium is an important electrolyte which functions in protein synthesis, nerve and enzyme function, osmotic balance, and cell membrane electrical potential for molecular transport (Olanrewaju et al., 2007). Heat stress has been shown to increase potassium and sodium excretion (Iheukwumere and Herbert, 2003). However, supplementation of YF did not alleviate changes in electrolyte balance.

Heat stress reduced plasma levels of the chemotactic cytokine IL-8 and acute phase protein α_1-AGP, but increased levels of the pro-inflammatory cytokine IL-1α. Heat stress induces changes in intestinal tight junction integrity, which could allow the passage of pathogens in the intestinal lumen to underlying tissue and increase production of pro-inflammatory cytokines (Awad et al., 2017; Zhang et al., 2015). It is not surprising, then, that heat stress increased IL-1α. Previous research has shown that acute administration of YF in humans led to decreased circulating levels of T lymphocytes and natural killer cells, probably due to cell trafficking and homing (Jensen et al., 2011). Because IL-8 is a chemotactic cytokine, plasma levels increase in response to glucocorticoids and oxidative stress (Baggiolini et al., 1989; Shini and Kaiser, 2009). Additionally, inflammation,

intestinal barrier dysfunction, and corticosteroids increase plasma levels of α_1-AGP (Chen et al., 2015; Fournier et al., 2000). However, plasma α_1-AGP and IL-8 were lower in heat-stressed birds. Yeast fermentate has been shown to reduce plasma corticosterone after acute and rearing stress (Nelson et al., 2018a), associated with reduced expression of the glucocorticoid receptor (Nelson et al., 2018b). However, Nelson et al. (2020) showed no effect of adding YF to the feed or drinking water on plasma levels of IL-1α, among other cytokines, in broilers exposed to acute or rearing stress. Similarly, this experiment showed no effect of dietary YF supplementation on IL-1α, IL-8, or α1-AGP. Despite its effect on stress susceptibility, dietary YF supplementation may not be effective in modulating plasma cytokine levels during cyclic heat stress.

There was a main effect of both heat and diet on villus height and crypt depth. Heat stress has been shown to reduce villus height, villus/crypt ratio, and goblet cell density (Deng et al., 2012; Nelson and Archer, 2019). In this experiment, villus/crypt ratio and goblet cell density were higher in heat-stressed birds and were not affected by diet. Heat stress also reduced villus height and crypt depth. However, adding YF to the drinking water increased villus height compared to birds fed the control diet or supplemented with YF in feed, and increased crypt depth compared to birds supplemented with YF in the feed. This agrees with previous research in Pekin ducks showing that adding YF to either the feed or drinking water attenuates heat stress-induced changes in villus height and villus/crypt ratio (Nelson and Archer, 2019). Mode of administration may play an important role in the ability of YF to modulate intestinal morphology. For example, heat-stressed birds consume more water and less feed (McFarlane et al., 1989). Adding AviCare

to the drinking water may be more effective than adding XPC to the feed in ameliorating heat stress-induced changes in intestinal morphology.

Adding YF to the feed or drinking water had no effect on d 42 cecal microbial structure in broilers exposed to acute or rearing stressors in this study. Analysis of the intestinal microbiome shortly after an acute stress event may be more helpful in determining time-sensitive changes in microbial structure. Neither supplement produced consistent results regarding plasma enzyme activity, nor did they alleviate the effects of heat stress on nitrogen metabolism, electrolyte balance or cytokine production. However, adding YF to the drinking water was more successful in increasing villus height and crypt depth. Mode of administration may therefore play an important role in the effects of YF supplementation on physiological outcomes during heat stress.

CHAPTER VII

CONCLUSIONS

Reducing stress is an important goal in modern poultry production because of its effects on poultry health and well-being. Dietary inclusion of *Saccharomyces cerevisiae*-derived yeast fermentate has previously shown potential in reducing measures of short- and long-term stress. However, its usefulness in improving other measures of poultry health and well-being, as well as its mode of action in reducing stress measures, have not been elucidated. Therefore, the primary objective of this research was to report the effects of dietary yeast fermentate supplementation on the stress response and other measures of bird health and production. The second objective was to clarify the mode of action by which yeast fermentate reduces measures of stress in poultry.

Primary findings indicate that when added to either the feed or drinking water, yeast fermentate reduces stress by reducing expression of MC2R, the ACTH receptor, on adrenocortical cells. Moreover, birds supplemented with yeast fermentate did not show signs of adrenal exhaustion in response to ACTH injection. Therefore, broiler chickens supplemented with yeast fermentate responded to stress but did so at a lower magnitude than non-supplemented birds. As a result of changes in MC2R expression, dietary supplementation of yeast fermentate consistently reduced measures of stress such as plasma corticosterone and heterophil/lymphocyte ratio in broilers exposed to either acute or rearing stressors, including vaccination, acute heat stress, and feed and water withdrawal. Yeast fermentate supplementation improved physical asymmetry in 3 out of 4 experiments. Plasma serotonin levels were higher when yeast fermentate was added to the

feed. However, because serotonin cannot cross the blood-brain barrier, the implications of this finding for animal welfare could be further clarified by investigating the effects of yeast fermentate on serotonin receptor activity in the hypothalamus.

Results also suggest that dietary yeast fermentate modulates the immune response during short-term stress by reducing expression of IL-10, a regulatory cytokine, and CYP1A2, an enzyme primarily involved in the metabolism of aflatoxins. This could explain why dietary inclusion of *S. cerevisiae* whole yeast cells or cell wall derivatives has been shown to improve feed conversion in birds consuming feed contaminated with aflatoxin B1 (Çelýk et al., 2003; Santin et al., 2003). However, yeast fermentate was not effective in modulating heat stress-induced changes in plasma concentrations of indicators of intestinal barrier dysfunction, including the acute phase protein α_1-AGP or the cytokines IL-1α and IL-8. In addition, although yeast fermentate attenuated some heat stress-induced changes in ileal histomorphology in both mixed sex Pekin ducks and male broilers, it did not have the same effect on goblet cell density in either species. Furthermore, supplementing yeast fermentate for the duration of the rearing period did not affect hematocrit, antioxidant capacity, thyroid hormone balance, electrolyte balance, or cecal microbiome structure in birds exposed to acute and rearing stressors. Nevertheless, adding yeast to the feed or drinking water increased villus length in both male broilers and mixed sex Pekin ducks, thereby increasing available surface area for nutrient absorption.

Yeast fermentate supplementation did not consistently affect body weight gain or feed efficiency. However, yeast fermentate supplementation influenced plasma levels of phosphorus, uric acid, and alkaline phosphatase enzyme activity, suggesting an increase in protein catabolism. An increase in plasma uric acid and glucose and a decrease in plasma

phosphorus in supplemented birds indicate changes in metabolic processes in response to yeast fermentate consumption. However, these results were not consistent across multiple experiments. Moreover, without significant differences in cecal microbiome structure between supplemented and un-supplemented birds, it is not possible to conclude whether the functional metabolites in yeast fermentate contribute either directly or indirectly to changes in metabolic activity.

Adding yeast fermentate to either the feed or drinking water effectively and consistently reduces stress susceptibility in broilers exposed to short-term rearing stressors, including vaccination, handling, feed withdrawal, acute heat stress, and crowding. Birds are commonly exposed to one or more of these environmental stressors in commercial poultry operations. Adding yeast fermentate to the feed is more practicable because it requires less time and manual labor than adding yeast fermentate to the drinking water. Therefore, adding yeast fermentate to the feed at the recommended dose for the duration of the rearing period will help to reduce stress and, potentially, improve feed conversion in broilers reared under a variety of conditions and during different seasons, including during aflatoxin exposure. Furthermore, because birds tend to consumer more water and less feed during high ambient temperatures, adding yeast fermentate to the drinking water on a daily basis, particularly in small-scale poultry operations, will attenuate the effects of heat stress on intestinal histomorphology, potentially improving nutrient absorption and feed conversion.

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