ABSTRACT

BARTZ, BROOKE MARIE. Effects of Induced Stress on Turkey Hens Supplemented with Yeast Derived Fermentation Products. (Under the direction of Dr. Jesse L. Grimes).

When turkeys are placed into commercial production, they have the potential to be exposed to several different types of stress. Economic losses are incurred by the US livestock industries since farm animals are raised in locations and season where effective temperature conditions venture outside their zone of thermal comfort (St-Pierre *et al.*, 2003). Livestock producers have used feed issued antibiotics to help alleviate some of these stress responses. However, as the animal production industry has continued to use fewer antibiotics in animal production, supplemented nutritional additives have become of increasing importance in their potential to effectively alleviate stress that may be experienced by animals during typical production processes.

In this body of research, management practices were evaluated on their potential to stimulate stress responses, and subsequent performance alterations due to mild stress conditions, studied in commercial turkey hens reared to 9 weeks. Two *Saccharomyces cerevisiae* derived fermentation products were evaluated on their ability to facilitate changes in performance parameters, aid birds that are coping with induced management related stress, and immune system aptness in birds that were supplemented. Since these two products were administered in different manners, with one supplemented in the water for short durations and the other in the feed continuously, design and mode of action of these supplements may vary in their ability to aid birds during stress at various levels. Therefore, a three part study was conducted to determine the effects of the two supplementation products in hen poults during the brooding process and when birds are placed under increasing degrees of mild induced stress, for less than 24 hours, at placement and at 5 weeks of age.

Hen poults were fed an industry standard ration that was unsupplemented or supplemented with a product derived during the yeast fermentation process (Original XPC™ dry, Diamond V Mills, Inc., Cedar Rapids, IA, USA) in all trials. A second proprietary product, derived during the yeast fermentation process (AviCare™ liquid, Diamond V Mills, Inc., Cedar Rapids, IA, USA), was either supplemented by itself, intermittently, from 0 to 10 days and 28 to 42 days in water, or in tandem with the dry product fed continuously in the feed throughout the trial. The sporadic supplementation of the liquid product was targeted for known stress events in commercial turkey production during movement into new facilities. Water treatments were completed using supplemental jug waterers so that consumption could be closely monitored for each treatment period. After water supplementation was complete, birds were placed onto automatic bell drinkers. Two separate induced stressors were used in part I, to first, invoke an immune response (Immucox® T, CEVA Animal Health, USA) at placement, and second, a 12 hour fasting at 35 days.

There was no difference observed between treatments for livability or water consumption, except 35 day stress, during this study. There was no stress induced mortality observed in any part of this study. Birds that were supplemented with either combination of products performed intermediately with control groups in regards to stress responses measure by serum corticosterone levels (EIA assay kit, Cayman Chemical, Ann Arbor, MI, USA) immediately following the induced fasting stress at 35 days and there were no differences in serum corticosterone between treatments observed at any other time during routine measurements. Body weights were significantly reduced in the positive control (stressed and unsupplemented), and the two supplementation groups were intermediate following the fasting stress at 35 days compared to the negative control (non-stressed and non-

supplemented). Feed conversion was improved at 35 and 36 days in supplemented treatment groups since they were similar to the negative control. Body weight gain was greatest at 63 days for hens provided both treatment supplements compared to birds in the other treatments.

In part II, hen poults were fed the same industry standard ration and the same two supplemental products were used. Similar inclusion levels of the supplements were used as seen in part I of this study, however, the liquid product was supplemented from 0 to 10 days and 29 to 42 days in the water for this portion of the study using supplemental jug waterers to monitor water consumption rates. Four separate management induced stresses were applied in the study. The first two were used to invoke an immune response (Immucox® T, CEVA Animal Health, USA) and 3 bd/pen were vaccinated with a 7% sheep red blood cell suspension in 1X PBS to be used for subsequent hemagglutination primary antibody titer quantification at 11 days of age with 0.5 mL of the solution, and at 37 with 1.0 mL of the solution. At 34 days, an 18 hour fast and cold stress were applied via removal of heat lamps from pens and turning off supplemental heaters in the facility; temperatures were reduced from 21°C to 10°C in all pens, excluding the negative control group. The negative control pens were provided with 4 heat lamps and supplemental feed and water were provided *ad libitum* during this time period.

Similar results to part I were seen with no difference observed between treatments for livability or water consumption, except 34 day stress, during this study and there was no stress induced mortality. Birds that were in the supplemented treatment groups performed intermediately with the control groups in regards to stress responses measured by the serum corticosterone immediately following the cold and fasting stress on 34 days. Body weights were significantly reduced in the positive control, and the two supplementation groups were

intermediate following the fasting stress at 35 days compared to the negative control. Feed intake was significantly higher in birds fed the combination of products throughout the entirety of this study. Body weight gain was greatest for hens provided the combination of supplements at 63 days compared to birds in the other treatment groups.

In part III, hen poults were fed the same industry standard ration and the two supplemented products at the same inclusion levels as part I and II of this study. The liquid supplement was provided from 0 to 10 days and 29 to 42 days, in the water, using supplemental jug waterers. Four separate management induced stresses were used in the study. The first two were used to invoke an immune response (Immucox® T, 2X concentration of dose, CEVA Animal Health, USA) and 3 bd/pen were vaccinated with a 7% sheep red blood cell suspension in 1X PBS at 14 days with 1.0 mL of the solution, and at 1.0 mL at 38 days to be used for subsequent hemagglutination primary antibody titers. At 34 days, an overnight, 18 hour fast was applied, excluding the negative control. At 35 days, a heat and crowding stress were applied, except for negative control, to represent cooping and transport between brooding and grow-out facilities. This was accomplished by reducing pen size to 0.028 m^2/bd from 0.45m^2/bd and a heat lamp was placed over the top of the reduced sized pen for 6 hours.

There was no difference observed between treatments for livability or water consumption, except 35 day stress, during this study and no stress induced mortality was observed at any time in this trial. Birds in either supplemented treatment groups performed intermediately with the control groups with regards to stress, measured by plasma corticosterone immediately following the fasting, heat, and crowding stress at 35 days. Period body weight gain from 0 to 34 days was similar between the negative control and

birds given the combination of supplementation products compared to the other treatment groups. From 0 to 62 days, feed intake was significantly higher in negative control birds compared to the other three treatment groups. An analysis of contrasts revealed that the addition of the dry product, Original XPC^{TM}, to either supplemented treatments, compared to the controls, resulted in significantly lower cumulative FCR cumulatively from 0 to 62 days.

In this research, it was interpreted that stress is a pertinent issue in commercial turkey production and can lead to decreased bird performance. Using *S. cerevisiae* fermentation derived products as a nutritional supplementation to birds placed under mild stress conditions were able to evoke a response in bird performance in this study. However, additional research will need to be completed for long term feeding of products and to determine the best possible inclusion levels to be both economically favorable while complying with bird maintenance requirements. This is also true for birds placed under chronic stress conditions in which acquired resistance to stressful situations may be lost after long term stress.

Effects of Induced Stress on Turkey Hens Supplemented with Yeast Derived Fermentation Products.

by
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BIOGRAPHY

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INTRODUCTION

When turkeys are placed into commercial production, they have the potential to be exposed to several different types of stress. The poultry industry has become important to the economy in many countries and birds exposed to stressful conditions, problems related to diseases, and deterioration of environmental conditions may result in serious economic losses (Kabir, 2009). It has been estimated that of the $1.7 billion dollars in annual losses seen in dairy, beef, swine, and poultry industries, $128 million is directly from poultry industries due specifically to heat stress (St-Pierre *et al*., 2003). Stress is a complex concept that is a major component in all organisms and is necessary for survival. However, if too much stress is experienced at any one specific time, detrimental effects may be observed. It is widely accepted that individual animals differ in their reactivity to stress and fearfulness (Erasmus *et al*., 2015). These effects can be described as physiological or behavioral changes, such as decreased appetite and lethargy.

Under chronic stress, birds have the potential to have decreased immune function which may alter their ability to fight off diseases. Pathogens come in several different forms, which require the bird to have a robust, multifaceted immune system that is capable of adapting to new scenarios. A common disease that has a negative impact on early turkey growth and feed conversion is poult enteritis mortality syndrome (PEMS). PEMS can affect birds up to 4 weeks of age and can be defined by mortality profiles, diarrhea, and immunosuppression (Schultz-Cherry *et al*., 1999). Having poults exposed to stress early in life may increase their chances of contracting PEMS, given its suspected multifactorial disease status and can be characterized by decreased primary antibody response, down

regulated macrophage functions, and altered lymphocyte subpopulations resulting in a decreased lymphocyte mutagenic response (Schultz-Cherry *et al*., 1999).

Animals devote considerable resources and machinery towards self-maintenance, including the immune system (Klasing, 2007) and under stressful conditions, the immune system can be compromised. Once a bird's internal defense mechanisms, such as the immune system, are jeopardized, it will be very difficult to reach peak production in those individuals. Many nutrients that are not essential for growth and reproduction regulate and modify immune responses (Koutsos and Klasing, 2014) and one way that producers have been attempting to prepare birds against several different types of stress is by using nutritional supplementation, which include yeast derived products. Commercial poultry are hatched in incubators and commercial poultry are deprived of contact with other avian adults which can alter the naturally occurring exposure to needed environmental contaminants to develop immunity (Kabir, 2009). Therefore, poultry are likely to benefit from supplementation with microbial preparations designed to restore protective gut microflora (Kabir, 2009). Yeast cell walls have the potential as immunomodulators that may serve as alternatives to antibiotics for growth promotion and disease resistance in poultry production (Huff *et al.*, 2010).

Immunomodulators have several different modes of action which can modify the immune system to reduce inflammation and suppress the immune response in some cases. Modulation of the immune system by altering the diet can decrease the incidence of some types of infectious diseases and minimize the unpleasant effects on immune responses on growth and incidence of metabolic diseases (Koutsos and Klasing, 2014). This interest in nutritional immunomodulators is meaningful for poultry production, where many stressful

factors, such as high stocking density, have a negative impact on animal health (Swiatkiewicz *et al*., 2014). Mannanoligosaccharides (MOS), derived from to inner cell wall of yeast, have been shown to have immunomodulating effects and have the ability to bind and eliminate pathogenic bacteria with type I fimbriae and to improve gut morphology and health (Huff *et al*., 2010, Swiatkiewicz *et al*., 2014). Therefore, yeast products and other metabolites harvested during the fermentation process may have a positive impact on bird performance and immune function. Nutritional supplementation is intended to provide nutrients that may not otherwise be available or consumed on a regular basis. Yeast by-products have been shown to contain high levels of protein, B vitamins, and MOS (Huff *et al.*, 2010). Nutritional immunomodulation may be defined as diet supplementation with specific nutrients or feed additives that affect some aspects of the immune function in order to achieve a goal (Korver, 2012). By supplying birds with an easily digested product that has the potential to reduce the overall stress response, affect their immune function, and allow birds to perform similar to others that are not placed under chronic stress, would be ideal.

Nutrient and non-nutrient components of the diet can impact the development, maintenance, and response of the immune system (Koutsos and Klasing, 2014). The components derived from whole yeast products or yeast cell walls have been used to improve growth and affect the physiology, morphology, and microbiology of the intestinal tract in turkeys and broiler chickens (Huff *et al.*, 2010; Ozpinar *et al*., 2012). There are several different types of products that are currently being investigated and evaluated for their efficacy and ability to properly modulate a favorable response in poultry production. It has been observed that yeast fermentation, its fractions, and other metabolites harvested during

the fermenting process, have the potential to alleviate stress and accommodate unfavorable conditions in bird production (Huff *et al.*, 2010; Merska *et al.*, 2015). Given in relatively low doses, which make these products affordable, may also have increased returns on commodities such as meat and egg production, and modulate improvements in gut health, immune function, and increase overall bird performance (Gao *et al.*, 2008).

Turkey is considered a lean meat, and therefore health conscious consumers are beginning to increase their intake (Singh *et al*., 2004), which requires producers to become more connected with their consumers' preferences and desires. This has led to a decrease in the use of antibiotics in food animal production with a steady decline as consumers become more involved in their food sources due to fear of the appearance of antibiotic resistant microbes in animal products (Swiatkiewicz *et al*., 2014). Finding alternatives to antibiotics for growth promotion, gut health, disease resistance, and increasing the bird's ability to cope with several different types of stressors, is necessary. Using supplemental nutrition products in birds placed under stress may further guide producers away from the use of antibiotics in the future and aid birds during times of known induced management stress. Nutritional supplementation is available in several different sources; however, yeast products have been well studied in poultry production.

Induced stress associated with general management practices in turkey production poses an ethical debate on how much stress birds can handle while maintaining proper quality of life. Stress can arise from several different sources such as environmental changes, handling procedures, and transporting between facilities (Huff *et al.*, 2007; Huff *et al.*, 2009; Huff *et al.*, 2010; Huff *et al.*, 2011; Huff *et al.*, 2013a). The addition of a probiotic, such as

Saccharomyces cerevisiae, has the potential to reduce enteric disease in poultry and subsequent contamination of poultry products (Konca *et al.*, 2009). During times of stress, birds are more susceptible to diseases since they have an impaired immune system. (Graczyk *et al.*, 2006). Antibiotics have been used in animal production to decrease the prevalence of disease in the past (Konca *et al.*, 2009). However, there has been little research regarding the ability of yeast derived products ability to aid birds in their ability to cope with different types of stress they may face in production and may be beneficial as an alternative to antibiotic use in commercial bird production. Given that products can be delivered in several different forms and contain various metabolites, the mechanism of action for each product may have an alternative way of decreasing the impact of stress in birds. Therefore, it is necessary to determine effective and practical alternatives for aiding birds that are coping with stress responses associated in production to improve health, food safety, and animal welfare.

LITERATURE REVIEW

A. The HPA Axis and GAS Approach

Stress is commonly defined as a state of real or perceived threat to homeostasis (Smith and Vale, 2006; Koutsos and Klasing, 2014). Stress is a complex concept that is highly dynamic, and birds must display an array of changes in their stress response based on the degree of stress applied. When birds are exposed to stressful situations, there are two responses that will occur. The first is the Fight-or-Flight response which is driven by catecholamines such as epinephrine, norepinephrine, and dopamine (Koutsos and Klasing, 2014). The second is the activation of the hypothalamic-pituitary-adrenal (HPA) axis which results in the secretion of glucocorticoids from the adrenal cortex (Koutsos and Klasing, 2014).

Stress is comprised of both general and specific stress responses. General stress can be seen as non-specific effects, such as decreased physiological performance in response to a stressor. These non-specific effects that occur, regardless of the stressor, have been documented by Siegel (1995). Furthermore, indirect changes can occur due to hormone-induced cytokine production by other cell types in the tissues where leukocytes reside (Koutsos and Klasing, 2014). In contrast, specific stress occurs in direct relation to a known stressor. Examples of specific stress have been documented by Siegel (1995) and have been further described by Koutsos and Klasing (2014) as behavioral changes, regulation of body temperature, and stress hormones initiating a series of behavioral, physiological, metabolic, and immunological adjustments that redistribute resources. Coping with either type of stress

through the stress response, is a necessary mechanism in order to maintain homeostasis in an individual and involves the endocrine, nervous, and immune systems (Smith and Vale, 2006). The activation of the stress response initiates a number of behavioral and physiological changes that improve an individual's chance of survival when faced with homeostatic challenges (Smith and Vale, 2006). Examples of physiological adaptations during stressful events include increased cardiovascular tone, respiratory rate, and intermediate metabolism, along with inhibition of general vegetative functions such as feeding, digestion, growth, reproduction, and immunity (Smith and Vale, 2006). Therefore a well-developed and tightly regulated system is necessary to cope with stressful events.

The HPA axis is comprised of anatomical structures found in both the central nervous system and the peripheral tissues (Smith and Vale, 2006). Located within the paraventricular nucleus of the hypothalamus are high densities of corticotropin-releasing factor (CRF), which serve as the primary regulator of the HPA axis (Smith and Vale, 2006). Once stress is perceived by the individual, CRF is released into the hypophysial portal system to the anterior pituitary where it binds it's receptors on pituitary corticotropes (Smith and Vale, 2006). Binding induces the release of adrenocorticotropic hormone (ACTH) into the systemic circulation and targets the adrenal cortex located in the adrenal gland (Smith and Vale, 2006). ACTH stimulates the release of glucocorticoids from the zona fasciculata and regulates physiological changes through ubiquitously distributed intracellular receptors (Smith and Vale, 2006).

Corticosterone, the systemic circulating glucocorticoid in birds, is the most frequently studied stress hormone in poultry and early exposure to stress hormones can affect fear behavior (Dixon *et al.*, 2016) and is important in the regulation of metabolic, cardiovascular, immune, and behavioral processes (Smith and Vale, 2006). Birds that are exposed to high levels of glucocorticoids can have a negative effect on bird performance. For example, chickens exposed to UV deficient light environments displayed less exploratory behaviors and have increased basal levels of plasma corticosterone (Maddocks *et al*., 2002). Physiological effects of glucocorticoids are mediated by the glucocorticoid receptor (GR) which, after ligand binding, undergo conformational changes in the GR, resulting in the dissociation of the receptor from the heat shock protein (HSP) complex and is translocated to the nucleus (Smith and Vale, 2006). Once the GR homodimerizes and binds to specific DNA motifs, glucocorticoid response elements (GREs) in the promoter region of the glucocorticoid response genes, it regulates expression through interaction with transcription factors (Smith and Vale, 2006). It has been shown that the GR may regulate activation of target genes independent of GRE-binding through direct protein-protein interactions with transcriptions factors that include activating protein 1 (AP-1) and nuclear factor-κB (NF-κB) (Smith and Vale, 2006). NF-κB is a protein complex that controls the transcription of DNA, cytokine production and cell survival. NF-κB activity and subsequent cytokine expression can be altered based on nutrients given during production (Koutsos and Klasing, 2014).

Regulation and activation of the HPA axis involves several neuronal and endocrine systems that are tightly controlled within the individual. Glucocorticoids and mineralocorticoids regulate not only carbohydrate and mineraloid/electrolyte metabolism,

respectively, but they also exhort anti-inflammatory or pro-inflammatory effects (Szabo *et al*., 2012). Glucocorticoids play a prominent role in regulating the magnitude and duration of the HPA axis activation (Smith and Vale, 2006). The HPA axis is subjected to glucocorticoid independent regulation and after exposure to stress, glucocorticoids act to inhibit the HPA axis via negative feedback mechanisms in the hypothalamus and pituitary (Smith and Vale, 2006). Traditionally, glucocorticoids have been thought to inhibit the activation of the HPA axis through a delayed feedback system that is responsive to glucocorticoid levels and involve alterations in the genome (Smith and Vale, 2006). However, there is increasing evidence that there may be a second negative feedback mechanism which is sensitive to the rate of glucocorticoid secretion (Smith and Vale, 2006).

The HPA axis is activated as part of the general adaptation syndrome (GAS), described by Hans Selye in 1946 and subsequent years, in which he outlined and discussed how an individual responds to stressful stimuli. The GAS is considered the sum of all non-specific, systemic reactions of the body which ensue upon long continued exposure to stress (Selye, 1946). Stressors can be described as physical, chemical, or physiological in nature (Szabo *et al*., 2012). The GAS is comprised of three main phases, alarm, resistance, and exhaustion. During the alarm phase, Selye noted catabolism, hypoglycemia, gastro-intestinal erosions, discharge of secretory granules from the adrenal cortex, and haemoconcentration are manifested (Selye, 1950). However, these effects disappear or are reversed during the resistance stage of the GAS approach, but, reappear during the exhaustion phase (Selye, 1950). These initial findings aid in describing that there is a finite amount of adaptive ability in individuals. He also theorized that the mechanism in which these responses were occurring

was either through humoral or nervous impulses coming from the site of direct injury to induce the hypothalamus-hypophysis system to prepare the body for defense against a stressor (Selye, 1950).

B. Components of the Stress Response

Birds in production must cope with stress based on their external or internal housing environment, management related practices, and stress that may resonate from within the bird itself. Stressors may be external or internal stimuli, and severity, duration, novelty, and host status all affect the response to them (Koutsos and Klasing, 2014). In production, stress can be seen as either favorable or antagonistic depending on the stimulus. Stress has an equivocal response in which some can be beneficial; however, too much can be harmful (Huff *et al.*, 2009), and therefore, a balance between stress and the stress response it creates is important. It has been observed that in environments with high temperatures and humidity are detrimental to the productivity of commercial animal agriculture (St-Pierre *et al*., 2003), and stimuli that can elicit fear have been described as emotional stressors (Erasmus *et al*., 2015).

Bird performance is highly integrated with their environment. Extrinsic stress can resonate from environmental stress experienced during production and transportation of poultry and have been shown to impact immunity which is a factor in the development of poultry disease (Huff *et al.*, 2009). Ambient temperatures can have adverse effects of bird performance and reduced feed intake can be observed under elevated temperature conditions (Rozenboim *et al.*, 2004) and animals have known zones of thermal comfort that are primarily dependent on the species, the physiological status of the animal, the relative

humidity, velocity of the ambient air, and the degree of solar radiation (St-Pierre *et al*., 2003). When this zone of comfort is no longer maintained, birds, among other animals, may experience stress.

How the animal is handled during various procedures can create additional stress. Depopulation of birds from laying facilities has shown that increased handling resulted in increased stress and has been observed to compromise the physical, physiological, and behavioral well-being of the birds (Tinker *et al*., 2004). Likewise, inverting, crating, and other handling procedures have been documented to increase the stress response in birds (Tinker *et al*., 2004). It has also been observed that corticosterone levels are higher immediately post handling (Tinker *et al*., 2004), indicating a measurable biological response to the stressor. Duncan (1989) found that birds that had been caught, handled, and transported for 40 minutes had higher plasma corticosterone levels compared to birds that we caught and handled alone. Therefore, inducing management practices that involve minimal catching and handling procedures should benefit bird welfare (Tinker *et al*., 2004).

However, in research conducted by Kannon and Mench (1996), handling method in broilers did not change the corticosterone concentration in birds after crating. This was most likely due to crating being a larger stressor than handling method itself. Transport stress does not only affect the circulating corticosterone in birds. Huff *et al.* (2009) found that transport stress increased white blood cell counts and heterophil percentages, which possibly resulted in increased phagocytosis. Heterophils are mobilized from bone marrow and accumulate in the blood, which results in an increased heterophil-lymphocyte ratio (Koutsos and Klasing,

2014). Furthermore, during early responses to corticosterone, both heterophils and lymphocytes increase expression of mRNA for pro-inflammatory cytokines and chemokines (Koutsos and Klasing, 2014). Changes in organ size, cell numbers, gene expression, and functionality indicate a transition of the immune system from a higher state of activation during acute stress to an anti-inflammatory and immunosuppressed state during chronic stress (Koutsos and Klasing, 2014). Chronic stress has been observed to reduce performance levels in birds, decrease immunity, increase mortality, increase heart attacks, and negatively impact other physiological disruptions.

In most cases, the initial reaction that birds display to human beings is fear and it is likely that even uninjured birds will suffer varying degrees of stress (Tinker *et al*., 2004). Individual birds differ in the reactivity and fear response associated with stressful events and fear is linked closely to stress because the physiological stress-related pathways are activated when the animal experiences fear (Erasmus *et al*., 2015). This may also support why some of this stress may be allocated to highly fearful birds that display a freezing response to aversive or novel stimuli during handling and catching (Tinker *et al*., 2004).

Stress has been shown to impact the reproductive status of birds. Many researchers have reported that there is an interrelationship between elevated temperature, cessation of lay, and semen production in birds (Rozenboim *et al.*, 2004). Rozenboim *et al.* (2004) reported that high temperatures accelerated an increase in circulating prolactin levels and increases expression of incubation behavior in laying female turkeys. Additionally, the effects of heat stress suppressed plasma luteinizing hormone and ovarian steroid levels while

shortening the egg-laying period. It was also observed that elevated prolactin levels reduce photo-induced luteinizing hormone release and delay the onset of sexual maturity in their study.

Additionally, there is a large interplay effect between stress and genetics on behavior, physiology, and reproductive function (Huff *et al*., 2013b). As domestication of birds for meat production has occurred, their genetic lines have begun to be selected for fast growth and increased body weight, which has led to changes in both bird behavior and stress response (Huff *et al*., 2013b). Unfortunately, these traits are also associated with poor bird welfare and musculoskeletal deformities. This may even interplay with the reproductive status of birds since some researchers suggest that reproductive failure occurs because of peripheral vasodilation which decreases the blood flow to the reproductive organs (Rozenboim *et al.*, 2004). In addition, the indirect selection for immune response traits can improve genetic resistance to disease (Singh *et al*., 2004); however, this may have an impact on bird performance.

C. Immune System and Response

The main role of the immune system is to distinguish between what is its own and what is foreign to eliminate any threats that may impair homeostasis (Graczyk *et al*., 2006) and the interaction between stress and the immune system can be positive or negative depending on the source and the reaction of the individual's stress (Huff *et al.*, 2009). Changes in the immune system are thought to be orchestrated primarily by hormones; however, direct neural modulation may also be involved (Koutsos and Klasing, 2014).

Psychological stressors experienced during production and transportation of poultry have been determined to impact immunity and a factor in the development of poultry disease (Huff *et al.*, 2009). Multiple genetic and environmental factors affect immune response and disease resistance in birds (Singh *et al*., 2004). The relationship between genetic lines of turkeys selected for increased body weight has led to an increased susceptibility to opportunistic bacterial infection (Huff *et al*., 2013b). Mechanisms of stress-immune interactions in poultry and how these interactions affect the ability to ward off infections is important for food safety (Huff *et al.*, 2009).

The ability to quantify the impact that stress, nutrition, and other welfare related management practices by obtaining data specific to the immune system, offers a practical advantage in experiments where immunity may be of secondary interest (Cotter, 2012). Opportunistic infections, such as *E. coli*, can result in severe respiratory disease in commercial poultry, lesions including osteomyelitis, arthritis, cellulitis, and soft tissue abscesses, which can all enhance the colonization of foodborne pathogens (Huff *et al*., 2013b). Therefore, the immune system plays an important role by aiding against pathogenic colonization within the animal. The immune system is comprised of two major portions, the innate and the adaptive immune systems which both play an extensive role in regular bird function and have the potential to be compromised under stressful conditions.

The innate immune system is nonspecific and protects the host from general infection. The developmental cost of the innate immunity are very low, however, the cost of using it are high which is exacerbated by subsequent exposure to the same pathogen

(Klasing, 2007). The innate immune system is mediated by macrophages, heterophils, and other primitive B1-type lymphocytes that produce natural antibodies (Klasing, 2007). It has been shown that microbial and environmental factors influence the functional competence of the monocyte-macrophage axis within the host (Heggen *et al*., 1998). Leukocytes can move freely throughout the host and are the first line of defense against pathogenic agents. These include natural killer cells, mast cells, eosinophils, basophils and other phagocytic cells. Leukocytes are responsible for the identification and elimination of foreign pathogens in the host.

The production of natural antibodies is important for a wide variety of defense mechanisms, enhances acquired immunity, interacts with complement, and is critical for defense against pathogens (Chen *et al.*, 2014). In humans, some of the mechanisms of the innate system are based on the cross-reactivity of natural antibodies (Cotter *et al*., 2005) and chickens are naturally rich in antibodies that cross react with rabbit erythrocytes (Swamy *et al.*, 2002). Immunoglobulin G, analogous to IgY in poultry, is the most abundant immunoglobulin in serum and is regarded as an indicator of general humoral immune response (Singh *et al*., 2004). In birds, thyroid hormone positively affects thymus growth, affects humoral immunity, and the number of circulating lymphocytes (Koutsos and Klasing, 2014).

Complement system activation can provide important information about innate immunity (Cotter, 2012). The complement system is a portion of the immune system that enhances the ability of antibodies and other immune cells to clear pathogens from the host.

There are 3 major pathways that complement system works through: 1) classical pathway activated by antibody—antigen complexes (adaptive immunity), whereas 2) alternative and 3) lectin pathways are part of the innate immune system and are directly activated by microorganisms (Chen *et al.*, 2014). Natural antibodies have long been believed to originate from stimulus provided by the surface of gut microorganisms (Cotter *et al*., 2005). If titers against natural antibodies are present, this reflects intact B-cell function (Swamy *et al.*, 2002), which indicates aid from the adaptive immune system.

The adaptive immune system includes B-cells which are derived from bursa and T lymphocytes in poultry which are specific to antigens. In addition, androgen receptors are located in the bursa of immature chickens and development of the thymus and bursa are influenced by androgen levels (Koutsos and Klasing, 2014). The adaptive immune system is ineffective at the first exposure to pathogens, however, will provide memory and protection against subsequent exposures to the same pathogen. The adaptive immune system is expensive to develop, but is cheap to use (Klasing, 2007). By observing changes in the immune system, we can further quantify the bird's ability to cope with stressors in its surroundings. Natural antibodies may play an important role in the regulation of specific immune responses (Cotter *et al*., 2005).

In a study conducted by Graczyk *et al.* (2006), it was presumed that there was a decrease in production of antibodies due to temporary changes in CD4CD8 populations. The reactivity of T-cells in birds is evaluated using delayed-type hypersensitivity response to the administered T-cell mitogen (Graczyk *et al*., 2006). Likewise, the consumption of grains

naturally contaminated with *Fusarium* mycotoxins could render turkeys susceptible to infectious diseases where $CD8^+$ T-cells play a major role (Chowdhury *et al*., 2005). T-cells are necessary for thymus dependent antibody responses and there may be a correlation between growth selection and changing T-cell subpopulations, which may affect antibody production (Li *et al*., 2000).

The sheep red blood cell (SRBC) antigen is classified as a thymus-dependent antigen that requires T lymphocytes to produce antibodies and is non-pathogenic to the host (Singh *et al*., 2004; Li *et al*., 2000; Cheng *et al.*, 1991; Nelson *et al.*, 1995). There has been speculation that ellipsoid-associated cells were more effective in presenting SRBC antigen to immunocompetent cells than the antigen-presenting cells of the tissue of the peritoneal cavity (Li *et al*., 2000). Almost all cells of the immune system have receptors for one or more of the stress hormones which implies that hormones have a direct effect on the immune system and are responsible for many of the changes in immunocompetence that occur as a result of development and growth, stress, and dietary changes (Koutsos and Klasing, 2014).

There are several different types of immune responses that can be conducted to determine a bird's immune function. Using antigens to monitor poultry immune responsiveness has been completed and are effective (Li *et al*., 2000). The use of vaccines containing attenuated pathogenic agents has been used to prevent infectious diseases in animals (Graczyk *et al*., 2006). However, others have reported an immunosuppressive effect when birds have been vaccinated with several antigens at once (Graczyk *et al*., 2006). By focusing solely on primary antibody production instead of specific immunoglobulin

production allows a qualitative understanding in the use of a foreign antigen in the form of SRBCs, can be effective at determining a bird's immune status.

The use of SRBC as a source of an immune response challenge has been documented since the 1940's (Friedwald *et al*., 1947). Using SRBC as an immunological challenge induces a cellular response to the non-proliferating erythrocytes. Hemagglutination depends on several different variables, including the amount and type of antibody present; the size, number and location of available antigen sites; and the pH, temperature, and ionic strength of the test medium (Cotter *et al*., 2005). Some of the genes that control the SRBC response are located on the sex chromosome, which may account for differences observed between genders, with females having higher titers on average (Cotter *et al*., 2005; Singh *et al*., 2004; Li *et al*., 2000).

In a study conducted by Chowdhury *et al.* (2005), turkeys, fed contaminated feed with *Fusarium* mycotoxins and given yeast-derived supplementation, were subjected to an intrajugular injection of 1 mL of 5% SRBC twice during the experiment for subsequent IgM and IgG concentrations for both primary and secondary antibody response. In this study, the researchers observed no differences between birds given the supplement and the controls in the response to the SRBC challenge. However, they did note that there was a decrease in hematocrit and hemoglobin concentrations, although, their values were within the normal ranges for turkey. When this is conducted in addition to stress, as reported by Huff *et al*. (2009), it would be interesting to see the interplay between immunity, transport stress, and

phagocytosis rates under these conditions since transporting has been shown to increase serological stress indicators in birds.

D. Alternatives to Antibiotics

Antibiotics have been added to poultry diets to maintain health and production (Yang *et al*., 2009). However, the increased costs of medications necessary for proper bird production and the possibility of drug residues present in the meat, provides the necessity for alternatives to antibiotics in turkey production to accommodate consumer desires (Singh *et al*., 2004). There are several different modes of actions that can be adopted to decrease the need for antibiotics in animal production. For example, commercial yeast extract products having immunomodulatory properties, which have potential as non-antibiotic alternatives to decrease pathogenic bacteria in turkey production (Huff *et al.*, 2010; Swiatkiewicz *et al*., 2014). Additionally, it has been reported that pathogenic bacteria, which display mannose-specific fimbriae, can be removed from the gastrointestinal tract via dietary MOS ingestion and by creating an unfavorable environment for the bacteria to attach to the intestinal lumen (Fairchild *et al.*, 2001). By decreasing unfavorable bacteria in the gastrointestinal tract, there is potential for increased bird performance due to better nutrient absorption in the gut (Fairchild *et al.*, 2001).

Chronic infections of the joints, bones, and soft tissues with opportunistic bacterial pathogens are a problem in commercial turkey production (Huff *et al.*, 2006). In a study conducted by Huff *et al*. (2006), fast-growing male turkeys appeared to be adversely affected by opportunistic bacterial infection combined with management and environmental stressors.

Although their results focused on the decreases in genetic ability to cope with bacterial diseases, some effects may have been observed due to antibiotic resistant bacteria. The shift towards decreasing the use of antibiotics in animal agriculture, and increased restrictions on antibiotic use, may increase the risk of pathogen contamination of poultry products and decrease poultry welfare (Huff *et al.*, 2009). As an additional example, mycotoxins can act as immunosuppressive agents affecting cell-mediated and humoral immune compartments (Chowdhury *et al*., 2005). Since the gut microflora has significant effects on host nutrition, health, and growth performance by interacting with nutrient utilization and the development of the gut system of the host, when pathogens attach to the gut mucosa, the integrity, function, and immune system are severely affected (Yang *et al*., 2009). The focus of alternative strategies has been to prevent proliferation of pathogenic bacteria and modulation of indigenous bacteria so that the health, performance, and immune status can be altered (Yang *et al*., 2009), while still providing a good quality of life for the animal.

E. Fermentation Derived Products Added to Animal Feed

Nutritional immunomodulation may be defined as diet supplementation with specific nutrients or feed additives to affect aspects of immune function in order to achieve an intended goal (Swiatkiewicz *et al*., 2014). The components derived from whole yeast products or yeast cell walls have been used to improve growth and affect the physiology, morphology, and microbiology of the intestinal tract in turkeys and broiler chickens (Huff *et al.*, 2010). For example, brewer's yeast, *S. cerevisiae*, has been added to animal feed since it has nutritional value including good quality proteins and B vitamins (Huff *et al*., 2010;

Swiatkiewicz *et al*., 2014). *S. cerevisiae* can be broken down into several different types of feed additives depending on which stage of the fermentation process products are harvested and is considered a fungal probiotic. Although the content of the active substance in different yeast products are highly variable, the containment of compounds with immunomodulatory properties such as β glucans, mannan oligosaccharides, nucleotides, inositol, and glutamine have been extracted (Swiatkiewicz *et al*., 2014).

In a study conducted by Chowdhury *et al.* (2005), the use of a glucomannan mycotoxin adsorbent (GMA) was used in turkey feed to counteract mycotoxin contamination. Fungal or yeast cell wall components can be added to diets to modulate the immune system, presumably via direct stimulation (Koutsos and Klasing, 2014). However, mycotoxins are fungal secondary metabolites toxic to vertebrates (Chowdhury *et al*., 2005), and therefore, feeding to animals may have a detrimental effect on performance and livability. In their study, the GMA was derived from the cell wall of *S. cerevisiae.* There are two main sections of *S. cerevisiae* in which the outer cell wall of yeast composed of a complex mixture of carbohydrates that contain mannose and a mixture of mannose-proteins, and, the inner surface, which contains glucans and other complex carbohydrates. In cell walls that contain polysaccharides, proteins, and lipids, they may exhibit numerous adsorption mechanisms, such as hydrogen binding and ionic or hydrophobic interactions (Chowdhury *et al*., 2005).

Beta-glucans are long-chain polysaccharides, comprised of numerous D-glucose monomers linked by β-glycosidic bonds that represent ~85% of cell wall β-glucan (Swiatkiewicz *et al*., 2014). The difference in bonding structure of yeast β-glucan (1.3/1.6),

from cereal β-glucan (1.3/1.4), is responsible for its greater biological activity (Swiatkiewicz *et al*., 2014). Yeast-derived β-glucans can bind macrophage specific CR3 receptors founds on the epithelial surface, which activates macrophage phagocytosis and leads to the production of cytokines and eicosanoids: IL-1, TNF-α, and PGE2 (Swiatkiewicz *et al*., 2014). This ultimately leads to the proliferation of $CD4^+$ and $CD8^+$ lymphocytes, meaning an increase in both the innate and adaptive immune response (Swiatkiewicz *et al*., 2014).

Turkey diets that have been supplemented with MOS has been shown to have an immunological stimulatory effect by increasing plasma IgG and IgM levels and decreasing T lymphocyte percentage in the peripheral blood (Swiatkiewicz *et al*., 2014). MOS have been shown to have immunomodulating effects and have the ability to agglutinate and eliminate pathogenic bacteria with type I fimbriae (mannose binding lectins) and to improve gut morphology and health (Huff *et al*., 2010; Swiatkiewicz *et al*., 2014). Pathogens can adsorb to the MOS instead of attaching to the intestinal epithelial cells and, therefore, move throughout the intestine without colonization (Swiatkiewicz *et al*., 2014). In addition, MOS can act as a nonpathogenic antigen, which may have adjuvant-like activity and beneficially modulate the host immune function because of its wide variety of surface defense cells involved in antigen recognition (Swiatkiewicz *et al*., 2014).

In monogastric animal diets, exogenous enzymes are used to improve digestibility including phytate, fiber, and protein (Yang *et al*., 2009). The effects of enzymes on gut microflora occur in two phases: the ileal phase and cecal phase (Yang *et al*., 2009). In the ileal phase, enzymes reduce the number of bacteria by increasing the rate of digestion and limiting the amounts of substrates available to the microflora. In the cecal phase, enzymes

produce soluble, poorly absorbed sugars which feed the beneficial bacteria which produce VFAs that can aid in controlling populations of unwanted bacteria and, perhaps, providing an energy source to the bird (Yang *et al*., 2009). In a study conducted by Solis de los Santos *et al.*, (2007) gastrointestinal maturation was accelerated in poults fed a brewers yeast extract containing β-glucans and MOS. Early in a poults life, the intestines are rapidly growing and are associated with a high metabolic rate during the establishment of the microflora in the intestinal tract.

F. Summary

In this thesis research project, the objectives were to evaluate the effects of increasing management related stress in turkey hens and the ability of two *S. cerevisiae* derived products to alter performance, aid poults in coping with stress responses, and immune system development and function in birds. Additionally, since these two supplements were provided via different routes of administration, the rate of supplementation and mode of action may vary in their ability to aid birds during stress. Therefore, each product was evaluated by itself and in tandem with each other. Subsequent and increasing environmental stress was delivered in different, but similar manners, during each trail to determine if there was a higher limit to the product's ability to aid in bird performance. Although both nutritional supplements were derived during the yeast fermentation process, the rate, inclusion level, and possible modes of action may differ depending on the product. The first product was a liquid fraction (AviCare^{TM} liquid, Diamond V Mills, Inc., Cedar Rapids, IA, USA), a new product that is harvested during the first stage of fermentation by proprietary processes in

fermentation derivation which results in a different metabolite profile and accumulation of yeast cell wall fragments. The second product was a dry fraction (Original XPC^{TM} dry, Diamond V Mills, Inc., Cedar Rapids, IA, USA), considered a yeast culture harvested during the second stage of the fermentation derivation process. Our interest with these two products, although similar, were to observe if there is an advantage of using a liquid or dry product depending on the age, induced stress level, inclusion rate, and duration of feeding on bird performance, ability to modulate a stress response, and immune system function and development.

Nutritional supplementation of products derived during yeast fermentation processes of *S. cerevisiae* may be a good alternative to antibiotic growth promoters for bird performance, immune system development and maintenance, and coping with the stress response. However, there is limited research conducting in turkeys regarding the ability and degree of immune and stress protection that these products may have on commercially raised turkey hens.

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MANUSCRIPT I. The Effects of Management Related Fasting Stress in Turkey Hens Supplemented with *Saccharomyces cerevisiae* Derived Fermentation Products.

ABSTRACT: A study was conducted to determine the effects of a mild 12 hour fasting management induced stress at 35 days in turkey hens supplemented with two yeast derived fermentation by-products (Original XPC™ dry and AviCare™ liquid, Diamond V Mills, Inc., Cedar Rapids, IA, USA) brooded until 5 weeks of age. Poults were randomly assigned to 48 pens, with 20 birds per pen, and assigned to 1 of 4 different treatment groups consisting of two controls and two supplementation treatments: negative control (unsupplemented, non-stressed), positive control (unsupplemented, stressed), liquid product in water (180ml/100L), and liquid product in water combined with a dry product in the feed (180mL/100L, 1.25 kg/MT), respectively. All birds were housed in a curtain-sided facility; with clay subflooring covered with pine shavings as litter and poults were provided with feed and water *ad libitum* except at 35 days, which was the day of mild stress. Immune system stimulation was conducted at 0 days in which birds received a live coccidiosis spray-on vaccine (Immucox® T, CEVA Animal Health, USA) and shed oocysts in fecal matter were quantified. Fecal samples were collected from the floor of each pen and analyzed by pen at 19, 36, 49, and 62 days for oocyte per gram determination of shed coccidiosis in fecal matter. Fecal samples were shipped to the University of Georgia for classification and enumeration of coccidian oocysts. Body weight and feed rations were determined by pen on 0, 7, 28, 35, 36 (8:00am and 8:00 pm), 42, and 63 days and feed/gain (FCR) was calculated.

Two different feed formulations were used during this study; industry standard starter 1 and starter 2 feeds and were provided by the NCSU feed mill that was unsupplemented or

supplemented with Original XPC^{TM} dry (1.25 kg/MT) fed continuously in the feed. AviCare^{TM} liquid was either supplemented in tandem with Original XPC^{TM} dry, or by itself intermittently, from 0 to 10 days and 28 to 42 days in water. Blood samples were collected from 3, not previously bled, individual birds via the brachial vein for serum corticosterone concentrations on 35, 36 (8:00am and 8:00pm), 42, and 63 days. Serum corticosterone was determined using a corticosterone EIA assay kit (Cayman Chemical, Ann Arbor, MI, USA).

There was no difference observed between treatments for livability or water consumption, except day of stress, during this study. Birds that were supplemented with either combination of products performed intermediately with the control groups with regards to stress responses measure by serum corticosterone levels. Body weights were significantly reduced in the positive control, and the two supplementation groups were intermediate following the fasting stress at 35 days ($P<0.05$) compared to the negative control. Feed conversion was improved at 35 and 36 days in supplemented treatment groups since they were similar to the negative control ($P<0.05$). Body weight gain was greatest for hens provided both supplement treatments at 63 days compared to birds in all other treatments. Supplementing hen poults during brooding with a liquid or a combination of dry and liquid fermentation derived products was able to alleviate some of the stress response observed during the fasting stress as seen by intermediate responses in performance and serum corticosterone levels.

(Keywords: turkey, *Saccharomyces cerevisiae,* stress)

Description of Problem

Movement of turkeys between different facilities and locations is a necessary management practice in today's commercial turkey production. Since birds have different nutritional and environmental requirements during different stages of production, this shift towards specialized locations for each stage of production has become industry standard. After birds are brooded under ideal conditions for the first 5 weeks of their life, they are taken off of feed and water, handled, cooped, transported, and placed at a new location for rearing to market age (grow-out production). During transport, birds can be exposed to several different types of stressors resonating from the environment. Environmental stressors include extreme ambient temperatures (hot or cold), high humidity, various types of precipitation, and wind. Birds may also experience social stress due to mixing of the population and moving to a new location. In addition, the birds are placed into coops which may elicit a stress response. Any and or all of these may have a negative effect on bird performance, such as decreased body weight, unfavorable FCR, impaired immune function, and increased mortality rates.

Due to stressful situations and their growth promoting abilities, antibiotic supplementation has been used in commercial poultry production to modulate intestinal microflora and to compensate for environmental, immunological, and social stressors (Huff *et al*. 2013a). However, antibiotic alternatives sourced as nutritional supplementation are available in commercial production to aid birds during management events which include prebiotics, probiotics, organic acids, essential oils, and other various plant extracts. Given the widespread development of antibiotic resistance in human and animal pathogens, it is

important to identify antibiotic alternatives to protect the health and wellbeing of commercial poultry (Huff *et al*., 2013a). Forms of yeast used in poultry production include whole yeast, fermented yeast products, yeast cell wall derivatives, yeast extracts, and yeast by-product metabolites. Products prepared from the yeast *Saccharomyces cerevisiae*, one variety of yeast common in animal feed supplementation, has been shown to elicit positive responses in animals during several different stress related events (Huff *et al*., 2013a).

S. cerevisiae can be prepared in a variety of ways to be added into animal feed. Under different fermentation conditions, different yeast cell fractions and metabolites can be harvested. During the fermentation process, yeast cells are under distress, which causes them to generate metabolite by-products or bioactive compounds (Francois and Parrou, 2001). After the fermentation process is complete, the product can be dried in a way that will preserve the bioactive compounds so that they can be added into animal feeds (Yalcin *et al.*, 2008). Inclusion of yeast cultures in poultry diets has led to marked improvements in bird performance. Positive results have been seen by Haldar *et al*. (2011); Gao *et al*. (2008); Gao *et al*. (2009); Yang *et al*. (2009); Zhang *et al*. (2005) in broilers, and Merska *et al*. (2015); Swiatkiewicz *et al.* (2014); Huff *et al*. (2013a) in turkeys.

Since these products have been shown to have positive modulating effects in poultry, it is possible that these products may have beneficial effects on birds during known times of induced stress, as seen during routine management practices. By improving a bird's response to the stressful event, improved growth and performance may also be observed after an acute stress (that which may last less than 24 hours), such as movement between brooding and grow-out facilities. The study reported herein was designed to deliver a short (less than 24

hours) immune stress challenge to young poults and to subject those birds to an additional fasting stress at the age in which they would be placed under similar conditions during movement between brooding and grow-out facilities. This stress was not designed to be a chronic or severe stress, and instead, to determine how small changes in management can affect bird health and performance. Induced stress has the potential to negatively affect growth and performance after the stress period for an extending period of time, although it is highly dependent on the type of stress applied (Lara and Rostagno, 2013). In this study, two products, derived and harvested during the yeast fermentation process of *S. cerevisiae* were used. These products are produced by fermenting various liquids and raw cereal grain ingredients in *S. cerevisiae*, brewers yeast, and then further processing the product's metabolites without damaging the products viability (www.diamondv.com). The objectives of this study were to determine the effects of management related induced stress events and the subsequent affect of supplementation with two different *S.cerevisiae* derived fermentation products ability to reduce stress effects on commercial turkey hen performance parameters.

Materials and Methods

Birds and Housing

A total of 960, day of hatch, Large White Nicholas commercial turkey hens (Aviagen, Lewisburg, WV) were hatched at Butterball Hatchery (Goldsboro, NC) and were transported to a curtain-sided facility consisting of 48 pens, approximately 2.44m by 3.6m, arranged with 12 pens allocated to each quarter of the building, considered as 4 separate blocks at North Carolina State University's Turkey Education Unit. Poults were randomly allocated into 48

pens with 20 hen poults per pen. Pen assignment within the facility was generated using a random numbers table. Poults did not receive any vaccinations prior to placement and were beak and toe conditioned at the hatchery. Only healthy looking poults that were free of notable defects were placed in this trial. Poults were assigned to 1 of 4 different treatment groups consisting of two controls and two supplementation treatments: negative control (unsupplemented, non-stressed) (NC), positive control (unsupplemented, stressed) (PC), a liquid product supplemented in the water (AviCare™ liquid, 180ml/100L, Diamond V Mills, Inc., Cedar Rapids, IA, USA) (AV), and the liquid products in the water and a dry product in the feed (AviCare™ in water and Original XPC™ in feed, (180mL/100L, 1.25 kg/MT, respectively), Diamond V Mills, Inc., Cedar Rapids, IA, USA) (AVX). Inclusion levels were based on pilot study conducted at North Carolina State University (unpublished).

At placement, all birds, excluding the negative control, were sprayed with a 1X dose live coccidiosis vaccine (Immucox® T, CEVA Animal Health, USA) and allowed to consume the gel and dry for at least 2 hours prior to placement. Litter that had previously been used was mixed with fresh bedding to act as an additional stress challenge, excluding NC pens which received fresh bedding to reduce the possibility of stress associated with fecal contaminated used bedding. Liquid supplementation was given during times of induced management stress from 0 to 10 days and from 28 to 42 days. The dry product was supplemented, in addition to the liquid product, continuously in the feed at 1.25 kg/MT in the AVX treatment. At 35 days, birds were subjected to a 12 hour fast in which both water and feed were pulled overnight from the pens, except for negative control birds.

Measurements

Birds were weighed by pen on 0, 7, 28, 35, 36 (2Xs: 8:00am and 8:00 pm), 42, and 63 days. Body weight, feed and water intake were measured while weight gain and feed conversion were calculated. Body weights and feed intake were determined by measurement in order to calculate body weight gain and FCR.

Blood Collection

Approximately 2-3mL of blood was collected from 3, not previously bled, individual birds via the brachial vein for serum corticosterone concentrations using a 23 gauge, 1" needle on 35, 36 (8:00am and 8:00pm), 42, and 63 days. All 3 birds were bled simultaneously by trained individuals to decrease trauma to the blood vessel and all birds were restrained similarly for each bleed using a modified procedure by Benton *et al.* (1977). For the present study, each blood sample was placed into its own individual disposable 15 X 85mm borosilicate glass culture tube (Thermo Fisher Scientific, Waltham, MA, USA) by removal of the needle from the end of the syringe and gently pushing the plunger down to trickle blood along one side of the inside of the tube within 1 minute after collection, but before coagulation occurred in the syringe. Once all blood samples were collected, blood was covered using Parafilm^{TM} and stored undisturbed for at least 2 hours to ensure proper coagulation of blood before centrifugation. After refrigeration, samples were rimmed using a micro spatula to gently dislodge the blood sample from sticking to the inside wall of the glass tubes and to ensure good separation of serum from blood clot after centrifugation. Samples

were placed into the centrifuge at 500g for 1 hour, to ensure proper serum separation. Samples were re-spun at 500g for 30 minutes if there appeared to be less than 2mL worth of serum to be used for laboratory testing. Serum was taken off using a sterile micropipette and samples were placed into -20°C until they were fully frozen, then stored at -80°C until corticosterone analysis could be completed using a corticosterone EIA assay kit (Cayman Chemical, Ann Arbor, MI, USA).

Fecal Sampling

Fecal samples were collected from the floor of each pen and analyzed by pen at 19, 36, 49, and 62 days for oocyte per gram (OPG) determination of shed coccidiosis in fecal matter. The importance of fecal testing for coccidian oocysts is to determine the disease status of the flock. The flock disease dynamic will vary based on the stocking density, environmental humidity, and the influence of the previous flock (i.e. re-used litter). In the present study, OPG analysis was not intended to determine the disease dynamic of the flock or possible interactions of the supplements with a coccidiosis exposure, but instead to ensure that coccidiosis was delivered to the birds through a live vaccine. Upon collection, fecal samples were shipped to the University of Georgia for classification and enumeration of coccidian oocysts.

Diet Formulation

Two different feed formulations were used during this study; industry standard starter 1 and starter 2 feeds were provided by the NCSU feed mill (Table 1).

Statistical Analysis

This study was analyzed using a complete randomized block design using the GLM procedure of JMP 11.2 (SAS Institute, Cary, NC, 2015) for ANOVA. Means were separated using the LSMeans procedure within JMP and significance was recognized at $P \leq 0.05$ for all performance and corticosterone data.

Animal Ethics

This study was conducted under the supervision guidelines set forth by the Institution Animal Care and Use Committee at North Carolina State University. All husbandry, bird well-being, and euthanasia procedures were performed with full consideration of animal welfare.

Results

Immediately following the fast at 35 days, the PC group had significantly higher serum corticosterone versus NC with AV and AVX groups not different from either control (Table 2). Body weights were significantly reduced in PC, AV, and AVX treatments compared to NC at 35 days after the fasting stress (Table 3). However, at 63 days AVX birds were as heavy as NC birds compared to AV and PC (Table 3). Feed to gain from 34 to 42 days and cumulative 42 days was improved in AVX and NC compared to PC and birds in the AV treatment performed intermediately (Table 3). Oocysts per gram of fecal matter were highly variable and sporadic throughout the trial and there were no treatment interactions with the type or amount of coccidiosis shed (Figure 1). No differences were observed

between treatments for livability or water consumption during this study and there was no stress induced mortality throughout the study (data not shown).

Discussion

The objective of this study was to determine the effect of a coccidiosis exposure and a short, 12 hour induced fast on the stress response and subsequent performance in turkey hens brooded to 9 weeks of age that were supplemented with two yeast derived fermentation products. The corticosterone data observed at 35 days, immediately following the stress, indicate that there was a biological, however, not statistically significant, effect seen by the supplemented birds since they had an intermediate response. Since supplemented birds were placed under the same environmental conditions as the positive control and were exposed to the same amount of induced stress, it would have been expected that they would have similar results as the positive control; however, they were also not different from the negative control birds that received no supplementation and were non-stressed in the present study. In a study conducted by Huff *et al*. (2011), corticosterone levels in turkeys were decreased in groups that were supplemented with a yeast extract.

Since corticosterone is known to have high individual variability between birds (Huff *et al.*, 2013b), which may have accounted for some of the overlap between treatment groups in the present study. Additionally, the results obtained in the present study may be due to a low sample size relative to the amount of total birds in this study which may also account for a small separation between treatment groups since there is the possibility for such large differences between individuals. Given that stress can be a combination of both specific and non-specific effects, this intermediate response may be indicative of a partial physiological

response in coping with the stressors in this study. Since stress can be physical, chemical, or physiological in nature (Szabo *et al*., 2012), it is difficult to determine the exact causes of this intermediate response.

Although each bird was handled and bled as similar as possible, variation between each handler and personal bleed technique may have also increased the overlap between treatment groups in the corticosterone data. In the present study, if differences were strictly due to different handling techniques between treatments or days, the corticosterone data should have been significant throughout the entire study over time. It has been documented that after 15 minutes of handling in Japanese quail significant increases in serum corticosterone in high stress strains verses low stress strains was observed, however, there was still a wide variation within treatment groups (Cockrem *et al*., 2010). Although the birds in the present study were minimally handled, the corticosterone data appeared to be highly sensitive in response to the fasting stress.

Since the stress response and the accumulation of corticosterone in the bird are regulated by the hypothalamic-posterior-adrenal (HPA) axis through negative feedback mechanisms, this feedback loop may account for the differences in responses. Since glucocorticoids inhibit activation of the HPA axis through delayed feedback mechanisms and there is additional no genomic feedback system that is sensitive to the rate of glucocorticoid secretion (Smith and Vale, 2006), the rate of clearance in supplemented birds may have been impaired by the fasting stress. However, they may have been recovering from the stress at a faster rate than birds that were stressed but unsupplemented; however, this was not apparent in the results at 36 days following the fasting stress. In the present study, sequential bird

bleeds showed decreases in serum corticosterone levels, which may have been indicative of birds adjusting to being handled throughout the study for other measurements used for feed intake, body weight, and FCR calculations. Additionally, as subsequent bleeds occurred, techniques for handling and bleeding also became enhanced, resulting in fast and more accurate bleeding by the trained individuals, which may also account for the numerical decrease in serum corticosterone.

It has been observed that there is a connection between the stress response, indicated by an increase in corticosterone and a decrease in immune system function. However, in a study conducted by Huff *et al*. (2011), the addition of a yeast extract significantly decreased corticosterone and observed stimulation of the immune system. Throughout the study, shed coccidiosis OPG was highly variable between and within treatment groups and over time. It was not a main objective to determine the disease dynamic of the flock in the present study; however, fecal samples were analyzed for the presence of coccidian oocysts as a housekeeping measure of our vaccination practices. Although negative control birds were not vaccinated, they were still shedding coccidian in excreta in this study. It was presumed that this may have occurred in two ways. First, these pens were not excluded from daily foot traffic where booties or coveralls were not changed between pens. Additionally, this may have also been a natural coccidiosis challenge due to other birds in surrounding pens shedding throughout this trial. However, for the basis of this study, the objective was to evaluate how a coccidiosis exposure given at placement would affect bird performance. In previous studies in broilers challenged with *Eimeria tenella*, as a coccidiosis challenge, birds had lowered average daily gain, average daily feed intake, and FCR compared to controls

(Gao *et al*., 2009). With the addition of a yeast fermentation product, there were no significant effects on growth performance from 1 to 21, and birds fed 0.5% of the product had lower average daily gain compared to the control group.

In a study conducted by Huff *et al.* (2011), turkeys supplemented with a yeast extract provided stimulation of the innate immune system via an increase in oxidative burst activity (OBA) of heterophils and decrease OBA related to transport and bacterial stressors. However, in the present study, decreased body weights were not observed, and all treatments performed similarly at the 35 day stress, even with the negative control birds shedding at high levels at 36 days. Additionally, birds fed a combination of products had significantly better FCR from 34 to 42 days, which contradicts the results observed in Gao *et al.* (2009) study, although this difference may be due to a low coccidiosis exposure in the present study compared to the other researchers study.

At 35 days, the negative control birds had significantly higher body weight gain, which was expected since these birds had continued access to feed and water throughout the fasting stress. At 63 days, birds in the negative control group had significantly greater body weight gain compared to the positive control. Cumulatively, at 63 days, birds that were supplemented with a combination of products (AVX) gained similarly to birds that were non-stressed and unsupplemented (NC). Since yeast cell cultures contain yeast cells as well as peptides, organic acids, oligosaccharides, amino acids, aroma flavors, and unidentified growth factors, which have been proposed to produce beneficial performance results (Gao *et al.*, 2008). Based on the results of this study, through an unknown mechanism, birds that received a combination of yeast derived products had a beneficial body weight gain

compared to birds that received one product or no products under stress conditions. This difference between the two supplemental products ability to alleviate stress may be due to the individual animal response to the type of metabolites in each product. These same affects were seen in improved performance in turkey and chicken fed dietary supplementation of MOS and β-glucan by Zdunczyk *et al.* (2005) and Mikulski *et al.* (2008). Prior studies in broiler chicks (Zhang *et al.*, 2005) showed beneficial results from the addition of yeast on their performance parameters and Ognik and Krauze (2012) had significant higher body weights in turkey hens fed a prebiotic mannanoligosaccharides (MOS) additive at 16 weeks of age compared to the controls. However, there were no other significant differences between treatments prior to 16 weeks. This was similar to the current study in which significant gains were not apparent for the two weeks following the stress, but manifested by the termination of this trial.

Conclusion and Applications

1. Stress was observed in birds by reduced performance and increased serum corticosterone levels present after the 12 hour fast at 35 days.
2. Nutritional supplementation with liquid and dry fermentation derived products had an intermediate response in corticosterone levels following a fasting stress in turkey hens; however, it was not statistically different from either control group.
3. Feed conversion was improved, intermediately, with nutritional supplementation in this study.

4. Body weight gain at the conclusion of the trial was greatest for control hens and hens provided with liquid and dry fermentation derived products compared to birds in the other treatments, indicative of compensatory gain in birds given a combination of supplements.
5. Based on the results of this study, turkeys may be susceptible to stress created by and during management related practices which may be partially alleviated through nutritional supplementation of yeast derived fermentation products.

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Table 1. Composition and nutrient content of diets fed to turkey hens from placement until 9 weeks of age

	Starter 1 (kg)	Starter 2 (kg)
Corn	183.25	224.07
Soybean Meal	170.10	127.01
Poultry Meal	45.36	45.36
Fat	27.22	36.29
Limestone	8.39	6.40
MonoCal	10.89	8.39
Salt	1.13	1.13
Mineral Mix	0.45	0.45
Vitamin Mix	0.45	0.45
Se Mix	0.23	0.23
Choline Cl	0.91	0.91
Lysine	2.04	1.70
Methionine	1.93	1.13
Sodium Bicarbonate	0.68	0.68
Threonine	0.45	--
Total	453.48	453.59

Composition	**Starter 1 (kg)**	**Starter 2 (kg)**
CP	13.38	11.39
ME kcal/kg	639.57	685.38
Crude Fat	4.02	4.08
Lysine	0.85	0.70
Methionine	0.39	0.29
M + C	0.59	0.47
Trypt	0.15	0.12
Thre	0.54	0.42
Arg	0.88	0.74
Val	0.69	0.60
Calcium	0.68	0.56
Av P	0.34	0.28
Sodium	0.09	0.09
Chloride	0.10	0.10
Age Fed:	**0 – 28d**	**28 – 42d**
Form:	***Crumble***	***Pellet***

Table 2. Serum corticosterone levels following a 12 hour fasting stress on turkey hens grown to 9 weeks of age and supplemented with liquid or liquid combined with a dry fermentation derived supplement.

		Age (Days)			
		35d	36d	42d	63d
Treatment	**Dietary Supplement**	**---------Serum Corticosterone (pg/dL)-----**			
NC	None	254[b]	190	130	33
PC	None	791[a]	238	172	21
AV	Liquid[2]	513[ab]	238	291	59
AVX	Dry[1] and Liquid[2]	583[ab]	155	327	35
Source of Variation		**----------(P-Value)----------**			
P-Value		***0.007***	0.700	0.500	0.100

[1]AviCare (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the water at 160mL/100L in AV and AVX treatment groups.
[2]Original XPC (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the feed at 1.25 kg/MT continuously in AVX treatment group.
[a b] Superscripts within same column are significantly different ($P < 0.05$).

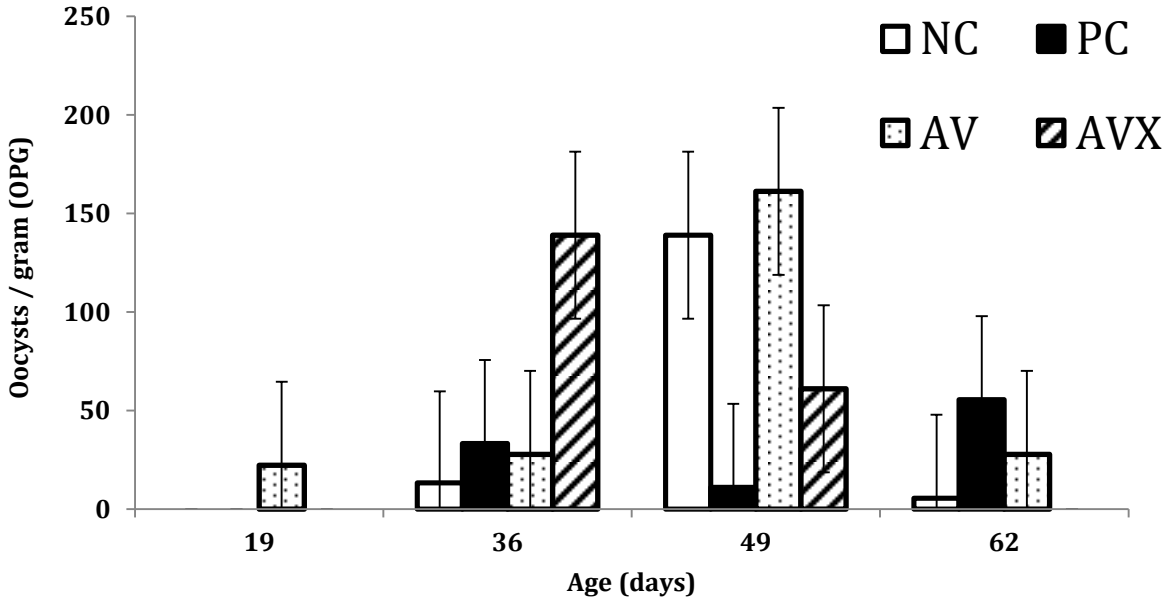
Table 3: Growth performance of turkey hens subjected to a 12 hour fasting stress and grown to 9 weeks of age and supplemented with liquid or liquid combined with a dry fermentation derived supplement.

		Age (Days)						
		0d	34d	35d	36d	42d	49d	63d
Treatment	**Dietary Supplement**	**----------(kg Body Weight Gain/pen)----------**						
NC	None	0.0596	1.39	1.39[a]	1.49	2.05	2.81	4.75[a]
PC	None	0.0582	1.38	1.27[b]	1.48	2.02	2.80	4.59[b]
AV	Liquid[2]	0.0587	1.37	1.26[b]	1.47	2.01	2.77	4.60[b]
AVX	Dry[1] and Liquid[2]	0.0588	1.39	1.28[b]	1.48	2.05	2.83	4.78[a]
Source of Variation		**----------(P-Value)----------**						
SEM		0.0330	0.009	0.008	0.009	0.017	0.02	0.05
P-Value		0.07	0.08	***0.0001***	0.17	0.19	0.38	***0.01***

	34d	36d	42d	49d	34 – 42d	42 – 49d	49 – 63d	63d
	----------(kg Feed: kg Gain) ----------							
NC	1.518	1.552	1.640[b]	1.630	1.902[b]	1.608	1.706	1.660
PC	1.527	1.551	1.680[a]	1.636	2.064[a]	1.542	1.694	1.667
AV	1.532	1.554	1.660[ab]	1.629	1.943[ab]	1.552	1.720	1.661
AVX	1.508	1.531	1.637[b]	1.622	1.904[b]	1.564	1.683	1.646
Source of Variation	**----------(P-Value)----------**							
SEM	0.010	0.010	0.010	0.008	0.044	0.032	0.021	0.008
P-Value	0.80	0.95	***0.02***	0.80	***0.05***	0.53	0.74	0.61

[1]AviCare (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the water at 160mL/100L in AV and AVX treatment groups.
[2]Original XPC (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the feed at 1.25 kg/MT continuously in AVX treatment group.
[a b] Superscripts within same column are significantly different ($P < 0.05$).

Figure 1. Oocysts per gram (OPG) of feces by treatment and age of turkey hens grown to 9 weeks of age and subjected to a 12 hour fasting stress supplemented with liquid or liquid combined with a dry fermentation derived supplement.



AV: AviCare (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the water at 160mL/100L in AV and AVX treatment groups.
AVX: Original XPC (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the feed at 1.25 kg/MT continuously in treatment group.

MANUSCRIPT II. The Effects of Management Induced Cold Stress on Performance and Immune System Function in Turkey Hens Supplemented with *Saccharomyces cerevisiae* Derived Fermentation Products.

ABSTRACT: A study was conducted to determine the effects of an 18 hour fasting and cold management induced stress at 34 days in turkey hens supplemented with two yeast derived fermentation by-products (Original XPC™ dry and AviCare™ liquid, Diamond V Mills, Inc., Cedar Rapids, IA, USA) in turkey hens brooded until 9 weeks of age. The cold stress was applied via the removal of heat lamps from pens; temperatures were reduced from 21°C to 10°C in all pens, excluding the negative control group. The negative control pens were provided with 4 heat lamps and supplemental feed and water were provided *ad libitum* during this time period. All birds were house in a curtain-sided facility; with clay subflooring covered with pine shavings for litter and poults were provided with feed and water *ad libitum* except for day of cold stress. The poults were randomly assigned to 48 pens, with 20 birds per pen, and assigned to 1 of 4 different treatment groups consisting of two controls and two supplementation treatments: negative control (unsupplemented, non-stressed) (NC), positive control (unsupplemented, stressed) (PC), a liquid fermentation derived product supplemented in the water (AviCare™ liquid (180mL/100L) Diamond V Mills, Inc., Cedar Rapids, IA, USA) (AV), and a combination of the liquid product and a dry product fed continuously in the feed (AviCare™ liquid and Original XPC™ dry in feed (180mL/100L, 1.25 kg/MT, respectively) (AVX). Poults were fed an industry standard ration that was either supplemented or unsupplemented with Original XPC™ (1.25 kg/MT) fed continuously in feed. The liquid supplement was either administered by itself, intermittently, from 0 to 10 days and 28 to 42 days in water, or in tandem with the dry fermentation derived product for

the same duration. Immune system stimulation was conducted at 0 days (Immucox® T, CEVA Animal Health, USA) in all birds, except negative controls. Additional immune system stimulation was invoked by using a 7% sheep red blood cell suspension in 1X PBS injected intravenously via the branchial vein at 11 days with 0.5mL, and 37 days with 1.0mL, for hemagglutination primary antibody titers. Body weight and feed consumption were determined by pen on 0, 34, 35, 42, and 63 days and FCR was calculated. Two different feed formulations were used during this study; industry standard starter 1 and starter 2 feeds, provided by the NCSU feed mill were either unsupplemented or supplemented with Original XPC™ dry product (1.25 kg/MT) fed continuously in the feed. Blood samples were collected from 3, not previously bled, individual birds via the brachial vein for serum corticosterone concentrations on 35 days immediately following the induced stress. Serum corticosterone was determined using a corticosterone EIA assay kit (Cayman Chemical, Ann Arbor, MI, USA).

Large White commercial turkey hens (Lewisburg, WV) were supplemented with either combination of products performed intermediately with the control groups with regards to stress responses measured by the serum corticosterone immediately following the cold and fasting stress on 34 days. There was no difference observed between treatments for livability or water consumption (except day of stress) during this study. Body weights were significantly reduced in the positive control, and the two supplementation groups were intermediate following the fasting stress at 35 days. Feed conversion was improved at 35 days and 36 days in supplemented treatment groups since they were similar to the negative

control. Body weight gain was greatest for hens provided both supplemented treatments at 63 days compared to birds in the other treatments.

(Keywords: turkey, *Saccharomyces cerevisiae*, cold stress, hemagglutination)

Description of Problem

The effects of stressors in a turkey production environment are varied and often unpredictable (Huff *et al.*, 2011) and significant economic losses are incurred by the US livestock industries since animals are raised in locations and seasons where effective temperature conditions venture outside their zone of thermal comfort (St-Pierre *et al*., 2003). Placing birds in conditions outside of their thermal zone of comfort exposed those birds to environmental stress. Exposing birds to extreme temperatures can impact immune responses, and the effect of heat or cold stress is generally inhibitory toward lymphocyte-mediated responses (Koutsos and Klasing, 2014). The concept of placing animals in commercial production settings that are unfavorable for them is common in industry. As seen in previous portions of this study, stress is capable of changing the performance level of birds when they are placed under mild stress conditions and was seen as reduced body weight gain. However, supplementation with *Saccharomyces cerevisiae* derived fermentation products have been shown to increase FCR (Huff *et al*., 2013a). Likewise, increased handling and movement to new locations all pose as stressors to animals in production (Huff *et al*., 2013a).

Early life experiences may influence interactions with the environment, health, welfare, and productivity of birds (Dixon *et al.*, 2016). At a young age, poults may be placed under stressful conditions when they are taken out of brooding pens and relocated to a new

facility for the remainder of their production lifetime. Several environmental and nutritional changes occur at this time, which may alter the bird's ability to cope with these stressors. Management practices usually entail birds to endure an extending feed and water withdrawal, in addition, to handling and cooping stress. During transport, there is an array of environmental stress related factors that may arise (Huff *et al*., 2013a). Extreme ambient temperature (hot and cold), humidity, wind, and increased precipitation can all induce stress in poultry and can be seen as negative effects in bird performance.

During brooding, poults are placed under ideal environmental conditions for optimal growth and development. The temperature within the facility during this time is monitored and tightly regulated. In addition, extensive care is given to the poults to ensure they are eating and drinking effectively. Once poults go through the feed and water withdrawal associated with movement between brooding and grow-out locations at 5 weeks of age, suppressed feed intake is common. In addition, the environment during transport between locations, and at the final destination may no longer be tightly regulated and ideal for the best for the birds which can be seen as negative effects in bird growth performance and elevated stress levels. In order to combat these negative effects, nutritional supplementation before and after transport may be beneficial.

Several types of nutritional supplementation are available in commercial production to aid birds during stressful events which include prebiotics, probiotics, organic acids, essential oils, and other various plant extracts. Dietary bioactive food components that interact with the immune system have the potential to reduce susceptibility to infectious diseases (Kogurt, 2009). Of these supplements, yeast cultures have become of increasing

popularity since they can be manufactures in a variety of ways and from several different varieties of yeast. During the yeast fermentation process, mannanoligosaccharides (MOS), among other metabolites are produced and have been shown to have immunomodulatory effects, which have also translated into better growth performance of supplemented animals and may serve as alternatives to antibiotics (Yang *et al*., 2009). Huff *et al*. (2013a) documented a decrease in turkey performance after transport and that yeast products may vary in their ability to alleviate the stress response in birds as seen by differences in feed efficiency.

Under mild heat stress conditions, there may be a redistribution of lymphocytes and immune system changes, although these changes may have a minimal impact on resistance to some disease challenges (Koutsos and Klasing, 2014) and there are several types of products available on a commercial basis that may aid poultry in their development and function of the immune system. The administration of antimicrobials has been used to improve health and performance of birds by decreasing or altering the bacterial populations present in the gastrointestinal tract (Fairchild *et al*., 2001). This change in the intestinal flora during the grow-out period may also protect the animal from pathogenic organisms, to increase weight gain, and to improve meat quality (Fairchild *et al*., 2001). However, the fear of antibiotic resistant bacteria in poultry production has resulted in alternative means for protection needed in the industry.

The nutritional supplementation of products derived from yeast have been shown to have a positive effect on both the immune function and gastrointestinal health in poultry. Most studies indicate that yeast derivatives may have a beneficial influence on the immune

system (Swiatkiewicz *et al*., 2014). Gao *et al*. (2008) have shown increases in serum lysozyme IgM activity and increases in secretory IgA concentrations in the duodenum in broilers fed a yeast culture supplement. Solis de los Santos *et al*. (2007) observed an acceleration in the gastrointestinal maturation in turkeys supplemented with a yeast extract. Likewise, Haldar *et al*. (2011); Gao *et al*. (2008); Solis de los Santos *et al*. (2007) have observed increases in villas height and gut functionality when fed MOS products in broilers. Commercially available products, derived during *S. cerevisiae* fermentation, have been shown to have a direct impact on the health and wellbeing of bird by impacting the innate and adaptive immune systems and may be able to aid birds during times of stress when feed consumption may be suppressed (Ozpinar *et al*., 2012). The objectives of this study were to determine effects of an 18 hour fast and a management-related induced cold stress on turkey hens that were previously subjected to a coccidiosis exposure and vaccinated with a 7% SRBC solution on the potential of two *S.cerevisiae* derived products ability to alleviate the stress response.

Materials and Methods

Birds and Housing

A total of 960, day of hatch, Large White Nicholas commercial turkey hens (Aviagen, Lewisburg, WV) were hatched at Butterball Hatchery (Goldsboro, NC) and were transported to a curtain-sided facility consisting of 48 pens, approximately 2.44 m by 3.66 m, arranged with 12 pens allocated to each quarter of the building, considered as 4 separate blocks at North Carolina State University's Turkey Education Unit. Birds received no prior

vaccinations at the hatchery and were randomly allocated into 48 pens with 20 hen poults per pen. Poults were assigned to 1 of 4 different treatment groups consisting of two controls and two supplementation treatments: negative control (unsupplemented, non-stressed) (NC), positive control (unsupplemented, stressed) (PC), a liquid product supplemented in the water (AviCare™ liquid, 180mL/100L, Diamond V Mills, Inc., Cedar Rapids, IA, USA) (AV), and the liquid products in the water and a dry product in the feed (AviCare™ in water and Original XPC™ in feed, 180mL/100L, 1.25 kg/MT, respectively), Diamond V Mills, Inc., Cedar Rapids, IA, USA) (AVX). At placement, all birds, excluding the negative control, were sprayed with a 1X dose live coccidiosis vaccine (Immucox® T, CEVA Animal Health, USA) and allowed to consume the gel and dry for at least 2 hours prior to placement in pens to act as a primary immune system activator. Additional immune system stimulation was conducted by using a fresh 7% SRBC suspension in 1X PBS injected intravenously via the branchial vein at 11 days with 0.5mL and 37 days with 1.0mL for hemagglutination primary antibody titers. Serum was collected from these vaccinated birds at 37, 42, 52, and 63 days for primary antibody $\log_2$ analysis. Previously used litter from Part I was caked out and top dressed with ½" of fresh pine shavings in all pens to act as an additional stress for the birds, except negative control pens, which received fresh bedding for this trial. At 34 days, birds were subjected to an 18 hour fast in which both water and feed were pulled from the pens, except the negative control (NC).

Additionally, at 34 days, a cold stress was applied via removal of heat lamps from pens; temperatures were reduced in pens from 21°C to 10°C in all pens except NC. To accommodate and not apply the cold stress to the NC pens, these pens were provided with 4

heat lamps, cardboard brooding rings were placed around the perimeter of the pen, and feed and water were supplemented *ad libitum* during the stress period at 34 days. Two different supplementation products were giving during this experiment consisting of a liquid and a dry fraction obtained during the yeast fermentation process. Two (AV and AVX) of the four treatment groups (NC, PC, AV, AVX) were supplemented with the liquid product during times of induced management stress from 0 to 10 days and from 29 to 42 days. The dry product was fed continuously in the feed in only one treatment group (AVX).

Measurements

Birds were weighed by pen at 0, 34, 35, 42, and 63 days of age. Feed and water intake, weight gain, and feed conversion were calculated. Body weights and feed intake were determined by measurement in order to calculate body weight gain and FCR.

Blood Collection

Blood collection occurred in the same manner as outlined previously in this thesis with an approximate blood draw of 2-3mL collected from 3, not previously bled, individual birds via the brachial vein for serum corticosterone concentrations using a 23 gauge, 1" needle at 35 days immediately following the cold stress. Each blood sample was placed into its own individual disposable 15 X 85mm borosilicate glass culture tube (Thermo Fisher Scientific, Waltham, MA, USA) and once all blood samples were collected, blood was covered using Parafilm^{TM} and stored for at least 2 hours to ensure the coagulation of blood before centrifugation. After refrigeration, samples were rimmed using a micro spatula to

gently dislodge the blood sample from sticking to the inside wall of the glass tube and to ensure good separation of serum from blood clot after centrifugation. Samples were spun at 500G using the same procedure as Part I of this study to obtain at least 2mL worth of serum to be using for laboratory testing. Serum was taken off using a sterile micropipette and samples were placed into -20°C until they were fully frozen, then stored at -80°C until corticosterone analysis could be completed using a corticosterone EIA assay kit (Cayman Chemical, Ann Arbor, MI, USA).

A fresh 7% SRBC suspension was prepared in 1X PBS to be injected intravenously into the branchial vein of 3 birds in each pen. Birds were identified using paint markings on their backs so that they could be identified for subsequent vaccinations and blood collection. Birds were injected using a 23 gauge, 1" needle with 0.5mL at 11 days of age, and at 37 days of age with 1.0mL of the solution. Blood collection for hemagglutination was collected in the same manner using a 23 gauge, 1" needle at 37, 42, 52, and 63 days of age from the same birds throughout the trial. Each blood sample was placed into its own individual disposable 15 X 85mm borosilicate glass culture tube (Thermo Fisher Scientific, Waltham, MA, USA) using similar techniques from the corticosterone bleeds by removal of the needle from the hub of the syringe and gently pushing the plunger down to trickle blood along one side of the inside of the tube within 1 minute after collection, but before coagulation occurred. This gently transfer was introduced to decrease the chances of lysing of the turkey RBCs. Once all blood samples were collected, blood was covered using Parafilm^{TM} and stored for at least 3 hours to ensure the coagulation of blood before centrifugation under undisturbed refrigeration. After refrigeration, samples were rimmed using a micro spatula to gently

dislodge the blood sample from sticking to the inside wall of the glass tube and to ensure good separation of serum from blood clot after centrifugation. Samples were spun at approximately 500g for 1 hour and checked for serum separation. Samples were re-spun at 500g for 30 minutes if there appeared to be less than 2mL worth of serum to be using for laboratory testing. Serum was taken off using a sterile micropipette was used to transfer samples into 2mL microcentrifuge tubes. Samples were vortexed and split for heat inactivation, with one bulk, untreated sample, and one to be heat inactivated. Heat inactivation was completed by placing samples into a 56°C water bath for 30 minutes to inactivate complement. Samples were then removed from the water bath and placed into storage at -20°C until assays were completed.

Specific form of agglutination was used for blood typing and quantifications of titers from birds vaccinated with a fresh 7% SBRC suspension in 1X PBS in blood serum. For this study, the overall total primary antibody response or non-response was evaluated following immunization with the solution. Previously heat inactivated samples were removed from the -20°C storage and allowed to slowly unthaw while on ice. Hemagglutination assays were performed in 96 well conical-bottom polystyrene plates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) and serial dilutions were performed, in duplicate for each sample.

Dilutions were conducted using 50µL of sterile 1X PBS added into the first row of the 96 well plate. A total of 50µL of vortexed sample were mixed into each well. The plate was covered using a clear adhesive microplate film cover (USA Scientific, Ocala, FL, USA) and plates were placed into a 37°C incubator for 30 minutes. Plates were removed from the incubator and 50µL of PBS were added to remaining wells so that the serial dilutions could

be performed. A multichannel pipette was used to transfer 50µL of gently remixed sample/1X PBS mixture from the first row of wells to the second. A total of 50µL worth of mixed sample from the second row of wells was placed into the third row. This procedure continued until the eighth row was reached, and 50µL of the sample mixture was taken out of the last well to be discarded. Tips were not changed during this procedure, unless they became contaminated with other surfaces during the dilution process. This was done to ensure proper dilutions and to decrease unnecessary use of laboratory materials. A total of 50µL of fresh 2% SRBC suspension was added into each well and the plate was recovered using the same adhesive cover from the first incubation. Plates were placed back into the incubator at 37°C for 30 minutes before they were read.

Plates were removed after the second incubation and placed on a 45° tilt for 20 seconds before the reading took place. Each plate was carefully read from the bottom of the plate to avoid misreading due to bubbles that may have formed during the dilution and SBRC pipetting procedures (Figure 2). For reading, the plate was held above the observer at the 45° angle so that the solution would remain in the wells but a clear reading could be obtained. A button would form in positive well and a teardrop would form in negative wells. Total agglutination was marked by a button and was recorded as the well of full agglutination before a full teardrop was observed. In the case of any ambiguous well agglutination formations, such as a partial button, if the sample was at least 2/3 teardrop, the well above was considered the full agglutination and marked as such.

Statistical Analysis

This study was analyzed as a completely random block design using the GLM procedure of JMP 11.2 (SAS Institute, Cary, NC, 2015) for ANOVA. Means were separated using the LSMeans procedure within JMP and significance was recognized at $P \leq 0.05$ for all performance data and $P \leq 0.10$ for corticosterone data. Antibody titers were transformed to reciprocal $\log_2$ units of greatest dilution showing 100% agglutination prior to analysis and was analyzed using the GLM procedure of JMP 11.2 for ANOVA with mean separation using LSMeans procedure within JMP. A difference with a probability of $P \leq 0.05$ was considered significant.

Animal Ethics

This study was conducted under the supervision guidelines set forth by the Institution Animal Care and Use Committee at North Carolina State University. All husbandry, bird well-being, and euthanasia procedures were performed with full consideration of animal welfare.

Results

Significantly higher serum corticosterone was observed in PC birds compared to NC birds immediately following the cold and fasting stress on 35 days, with birds supplemented with the liquid or dry fermentation derived products being intermediate in their response (Table 4). Feed intake was significantly higher from 0 to 34 days, 0 to 42 days, 34 to 63 days, and 0 to 63 days feed intake was significantly higher in the AVX birds compared to AV, with

PC and NC being intermediate (Table 5). From 34 to 42 days feed intake was significantly higher in the AVX treatment compared to NC and AV, with PC being intermediate in feed intake (Table 5).

Body weight gain from 0 to 34 days was significantly higher in AVX birds compared to NC, PC, and AV hens (Table 5). Body weight gain from 0 to 42 days was significantly higher in NC and AVX birds compared to PC and AV birds (Table 5). However, there was no difference in FCR from 0 to 34 days or 0 to 42 days (Table 5). Additionally, AVX birds had a significantly lower $\log_2$ primary antibody titer response as 42 days compared to all other treatments (Table 6). There were no differences in $\log_2$ titers between treatments at any other time when measurements were taken in this study (Table 6). FCR was significantly improved from 34 to 42 days in NC birds compared to AV and AVX birds, with PC birds being intermediate in response following the fasting and cold stress (Table 5). Cumulatively, AVX birds had significantly higher body weight gain compared to AV, with PC and NC intermediate in response (Table 5). Furthermore, AVX birds were trending at $P<0.10$ being significantly heavier than all other treatments at 63 days (Table 5). No differences were observed between treatments for livability or water consumption during this study and there was no stress induced mortality (data not shown).

Discussion

The objectives of this study were to determine the effect of a coccidiosis exposure, an 18 hour fast, and a cold stress at 34 days in turkey hens that were either vaccinated with a 7% SRBC suspension on immune system functionality, the stress response, and bird

performance. Birds that were subjected to the stressor had an increased corticosterone response compared to those that were non-stress and unsupplemented birds. Those that received either combination of supplements had an intermediate response, as measured by plasma corticosterone. The effect of corticosterone on the immune system of birds is highly dependent on the level and duration of increase and on the components of the immune system measured (Koutsos and Klasing, 2014). Exposure to adverse climate increases glucocorticoid secretion, which are antagonistic to immunity and impairs expression of humoral immune response (Haldar *et al.*, 2011). Elevated corticosterone levels in chickens have been seen within 30 min post treatment of exposure to cold stress and may persist for several hours following a stimulus (Davis and Siopes, 1987). In a study conducted by Davis and Siopes (1987), turkeys were subjected to different routes of administration of ACTH. The observed that plasma corticosterone could be altered by exogenous ACTH or cold exposure. Likewise, in the present study, those given dietary supplementation performed similar to both control groups. Given that the half-life of corticosterone in Japanese quail has been documented to be approximately 10 to 15 min (Cockrem *et al.*, 2010), this may correlate with birds in the present study having an intermediate response between treatments based on when birds were bled after the cold stress was completed. Moreover, pens were bled in sequential order which allowed for differences of time between first pen and last pen bled to be controlled in the statistical model. Results obtained by Gao *et al.* (2009) suggested that a *S. cerevisiae* fermentation product may increase the ability for chickens to cope with stress and in the present study, the partial alleviation of stress seen by an intermediate

response to the cold stress in regards to the corticosterone levels post stress, indicate that this may also be the case in turkey hens.

The primary antibody titers in the group given the combination of products was significantly lower when measured at 42 days. This may have been a result of this interaction between glucocorticoid secretion and the inability of those birds to have an increased immune response after the second vaccination. However, β-1,3/1,6-glucan from the cell wall of *S. cerevisiae*, is recognized as a foreign agent by the immune system of mammals, fish, and birds and has been shown to be protective in a number of disease challenge studies (Huff *et al.*, 2007), which would most likely be associated with a higher titer antibody response in this present study which was not seen. In addition, exposing birds to extreme temperatures can impact immune responses, and the effect of heat or cold stress is generally inhibitory toward lymphocyte-mediated responses (Koutsos and Klasing, 2014). It is well known that upregulation of the innate immune system can have a high energy cost and that immunological stress resulting from environmental bacteria or their products can result in decreased growth (Huff *et al.* 2013a). There has also been research documenting that in selecting for growth in genetic lines is correlated with changes of T cell subpopulations, which would affect the antibody production (Li *et al.*, 2000). Likewise, primary antibody concentration in the secondary response is highly influenced by the number of weeks between the primary and secondary injections (Li *et al.*, 2000). Therefore, this decreased response in primary antibody titers may not have been necessarily associated with a reduced biological immune response in the present study. Moreover, it has been documented that a positive effect of yeast derivatives on the immune system is not always a reflection in an

improvement in production parameters, especially when the experiments are conducted on healthy (non-challenged) birds (Swiatkiewicz *et al*., 2014). Given that this difference in hemagglutination titers was only seen at 42 days, and birds in this supplemented group had the highest body weight gain from 0 to 63 days, this decrease in immunity did not appear to affect the bird's ability to cope with fasting and cold stressors in this trial and may have been beneficial given that they gained similarly to birds that were not subjected to the cold stress. Since immunomodulatory nutrients appear to have a robust dose-response profile (Koutsos and Klasing, 2014) which has been observed in other yeast fermentation products on the immune response (Gao *et al.* 2009), may also account for the difference seen between supplemented treatments in the present study.

Body weight gain was negatively affected in birds that received the liquid product alone compared to the negative control and those that received a combination of products. Huff *et al.* (2007) observed similar results in turkey poults supplemented with a yeast extract feed supplement exposed to a cold stress resulted in lower body weights compared to the control fed birds placed under stress. It was theorized that this decrease in body weight may have been due to the additive effects of cold stress and yeast extract on the immune system which may have caused this to happen. In the present study, birds fed a combination of products had significantly higher body weight compared to those that received the liquid alone going into the stress and persisted for 1 week post stress. However, the lowered hemagglutination titers observed in the group fed the combination may indicate that in this instance, the single product allowed for proper immune function to occur, but suppressed body weight gain, and vice versa in those fed the combination of products. This phenomenon

was not observed during part I of this study, and may be due to the increased environmental stress given in this study, especially since increased but not significant corticosterone levels were observed in these treatments especially since it has been shown that psychological stressors experienced during production and transportation of poultry have been determined to impact immunity and a factor in the development of poultry disease (Huff *et al.*, 2009). Since stress has variable effects on the immune system, in which it can enhance or suppress responses depending on the type, degree of stress, and individual variation in the host response (Siegel, 1995), these differences in responses may be due to different mechanistic modes of action between and within the two products. Since these two products differ in their derivation during the fermentation process, different metabolites harvested during these times may alter the bird's stress response which can be seen as differences in immune response and body weight gain.

Throughout the entire trial, birds that were given the combination of products consistently ate more feed compared to those receiving the liquid product alone and controls were intermediate in their feed intake. Previous trials in male turkey poults fed a MOS supplement or an antibiotic had significantly higher body weight compared to controls (Fairchild *et al.*, 2001). Likewise, turkey hens supplemented with the same MOS product had significantly higher body weight compared to the control in a study conducted by Ognik and Krauze (2012). The present study results indicate that similar increases in body weight were associated with birds fed the combination of products. Not only did these birds have significantly higher body weight going into the cold stress at 34 days, they also maintained a significantly higher body weight until 1 week after the stress was completed, although this

difference diminished for the immediate period following the stress. In the present trial, this was a notable similarity compared to part I, since birds fed the combination had similar gains cumulatively after the stressors were applied. These results indicate that perhaps the addition of the dry fermentation product is able to modulate effects on the birds which allow them to overcome times of the induced management stress and increased environmental stressors.

In the period immediately following the stress until one week post stress, birds that received either form of supplementation had significantly better FCR compared to the controls. Similar results have been reported by Gao *et al.* (2008) in birds supplemented with a yeast culture from 22 to 42 days and cumulatively at 42 days. In the present study, the addition of dietary supplements may have been able to give additional nutritional benefits during this time post stress and may account for the increased efficiency of the animals. Since yeast derived products have metabolites, peptides, organic acids, oligosaccharides and some potential growth factors associated with it during dietary supplementation (Gao *et al.*, 2008), this may have aided birds in the current study during the cold stress. However, birds fed only the liquid product did not have increased gains compared to hens supplemented with the combination of products. Since an immune response was evoked at the time of stress, this may have been a suppression effect on the immune system in which the liquid product only given for a short duration around the time of stress may need to be increased to see long term effects on bird production. At this point in time, the dry product appears to have a longer term effect on bird health, however, it has not been evaluated by itself to determine if it has lasting long term effects on the bird's health status and performance.

Conclusions and Applications

1. Management events of fasting coupled with an environmental challenge, such as cold stress, resulted in a stress response measured by serum corticosterone and the observed stress negatively affected bird performance. Liquid supplementation in water and the water supplement combined with the dry product in the feed reduced corticosterone levels following a fasting and cold stress in turkey hens compared to the positive control.
2. The supplementation of *S. cerevisiae* fermentation derived products partially alleviated the stress response as measured by corticosterone levels and significantly improved performance of turkey hens.
3. Further research using SRBC as an immune system stimulator will need to be conducted to determine if the supplemental products positively affected the immune system in turkey hens, since reduced antibody titers were observed.

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Table 4. Serum corticosterone levels following an 18 hour fasting and cold stress on turkey hens grown to 9 weeks of age and supplemented with liquid or liquid combined with a dry fermentation derived supplement.

Treatment	Dietary Supplement	Age (Days) 35d
NC	None	49[b]
PC	None	273[a]
AV	Liquid[2]	135[ab]
AVX	Dry[1] and Liquid[2]	181[ab]
Source of Variation		**------(P-Value)----**
SEM		59.84
P-Value		***0.10***

[1]AviCare (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the water at 160mL/100L in AV and AVX treatment groups.
[2]Original XPC (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the feed at 1.25 kg/MT continuously in AVX treatment group.
[a b] Superscripts within same column are significantly different ($P < 0.10$).

Table 5. Growth performance of turkey hens subjected to an 18 hour fasting and cold stress supplemented with liquid or liquid combined with a dry fermentation derived supplement.

		Age (Days)				
		34d	42d	34 – 42d	34 – 63d	63d
Treatment	**Dietary Supplement**	**----------(kg Body Weight Gain/bird)----------**				
NC	None	1.58[b]	2.28[a]	0.68	3.74	5.34[ab]
PC	None	1.57[b]	2.20[b]	0.65	3.72	5.28[ab]
AV	Liquid[2]	1.58[b]	2.22[b]	0.65	3.67	5.23[b]
AVX	Dry[1] and Liquid[2]	1.65[a]	2.30[a]	0.67	3.77	5.40[a]*
Source of Variation		**----------(P-Value)----------**				
SEM		0.014	0.015	0.017	0.030	0.080
P-Value		***<0.001***	***<0.001***	0.590	0.270	***0.030***
		----------(kg Feed Intake/bird)----------				
NC	None	2.32[ab]	3.40[ab]	1.06[b]	6.34[ab]	8.68[ab]
PC	None	2.30[ab]	3.38[ab]	1.07[ab]	6.40[ab]	8.73[ab]
AV	Liquid[2]	2.29[b]	3.37[b]	1.06[b]	6.19[b]	8.48[b]
AVX	Dry[1] and Liquid[2]	2.36[a]	3.45[a]	1.09[a]	6.44[a]	8.84[a]
Source of Variation		**----------(P-Value) ----------**				
SEM		0.016	0.019	0.008	0.061	0.080
P-Value		***0.040***	***0.010***	***0.020***	***0.040***	***0.030***
		----------(kg Feed: kg Gain) ----------				
NC	None	1.41	1.45	1.56[b]	1.70	1.61
PC	None	1.41	1.48	1.65[ab]	1.72	1.61
AV	Liquid[2]	1.41	1.48	1.70[a]	1.69	1.60
AVX	Dry[1] and Liquid[2]	1.39	1.46	1.69[a]	1.75	1.62
Source of Variation		**----------(P-Value)----------**				
SEM		0.015	0.001	0.032	0.021	0.001
P-Value		0.480	0.170	***0.020***	0.240	0.520

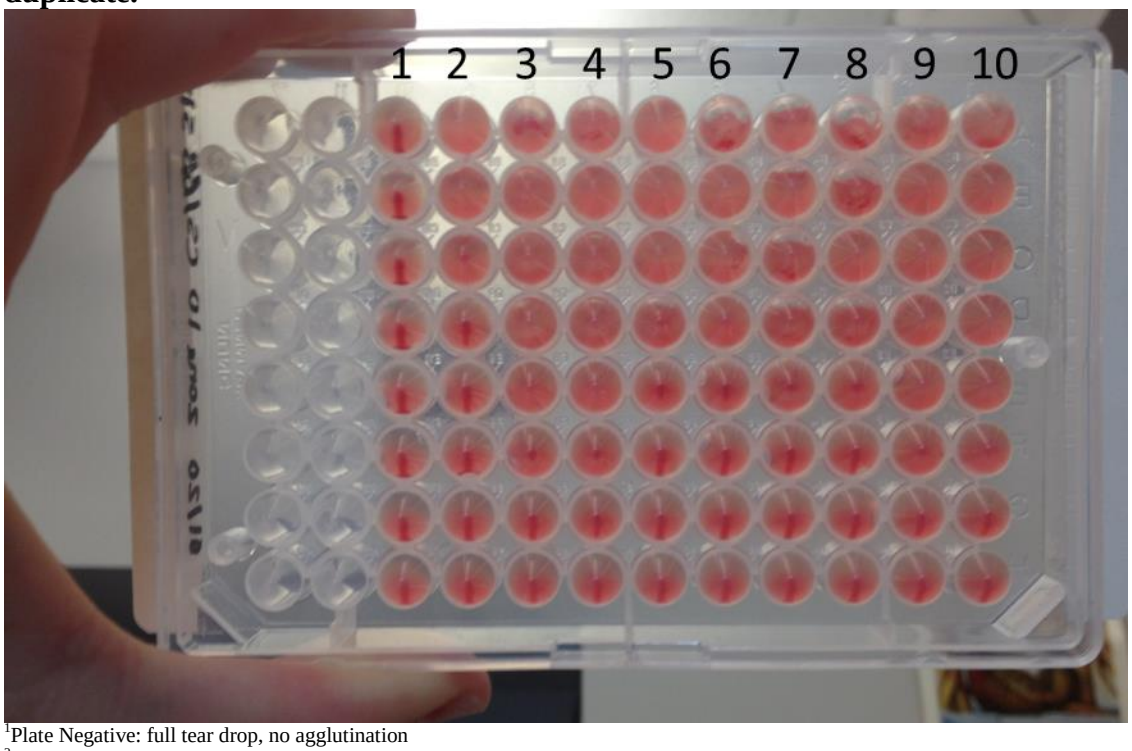
[1]Original XPC (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the feed at 1.25 kg/MT continuously in AVX treatment group.
[2]AviCare (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the water at 160mL/100L in AV and AVX treatment groups.
[a b] Superscripts within same column are significantly different ($P < 0.05$). * indicates trending at ($P < 0.10$).

Table 6. Primary antibody hemagglutination titer response of turkey hens subjected to an 18 hour fasting and cold stress grown to 9 weeks of age and supplemented with a liquid or liquid combined with a dry fermentation derived supplement.

		Age (Days)			
		Day of 2nd Vaccination	Post 2nd Vaccination		
		37d	42d	52d	63d
Treatment	**Dietary Supplement**	**----------(Log$_2$ Primary Antibody Titer)-------**			
NC	None	0.565	5.93[a]	1.88	1.25
PC	None	0.755	5.98[a]	1.66	1.48
AV	Liquid[2]	0.806	5.75[a]	1.92	1.51
AVX	Dry[1] and Liquid[2]	0.631	5.22[b]	1.91	1.53
Source of Variation		**----------(P-Value)----------**			
SEM		0.142	0.116	0.097	0.097
P-Value		0.610	***<0.001***	0.140	0.160

[1]Original XPC (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the feed at 1.25 kg/MT continuously in AVX treatment group.
[2]AviCare (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the water at 160mL/100L in AV and AVX treatment groups.
[a b] Superscripts within same column are significantly different ($P < 0.05$).

Figure 2. **Primary antibody hemagglutination titer dilutions example to illustrate differences between a negative reading, a pooled positive, and example samples run in duplicate.**



[1]Plate Negative: full tear drop, no agglutination
[2]Pooled Positive: 1:8 dilution
[3, 4]Example sample A: read as 1:64 dilution
[5, 6]Example sample B: read as 1:16 dilution
[7, 8]Example sample C: read as 1:32 dilution
[9, 10]Example sample D: read as 1:64 dilution

MANUSCRIPT III. The Effect of Induced Heat and Crowding Stress on Performance and Immune System Function in Turkey Hens Supplemented with *Saccharomyces cerevisiae* Derived Fermentation Products.

ABSTRACT: A study was conducted to evaluate two nutritional supplementation products on bird performance, stress response, and immune system functionality. Four separate management induced stresses were used in the study. The first two were used to invoke an immune response (Immucox® T, 2X concentration of dose, CEVA Animal Health, USA) and vaccination occurred at 14 days of 1.0 mL of a fresh 7% SRBC suspension in 1X PBS, and again, at 38 days with 1.0 mL of the suspension. Subsequent primary antibody titers were obtained via hemagglutination assay from vaccinated birds at 21, 34, 47, 55, and 62 days. At 34 days, an overnight, 18 hour fast was applied. At 35 days, heat and crowding stresses were applied, except for birds in the negative control, to represent cooping and transport between brooding and grow-out locations. This was accomplished by reducing the initial pen size to similar dimensions of transport coops used in production and a supplemental heat lamp was placed over the top of the pen for 6 hours. The two supplementation products consisted of first: a dry fermentation derived product, fed continuously in the feed (Original XPC™ dry, Diamond V Mills, Inc., Cedar Rapids, IA, USA) and a liquid fermentation derived product (AviCare™ liquid, Diamond V Mills, Inc., Cedar Rapids, IA, USA) fed intermediately, from 0 to 10 days, and 29 to 42 days in water, during the experiment within times of known induced stress.

Birds that were supplemented with either combination of products performed intermediately with the control groups with regards to stress responses measured by the

serum corticosterone immediately following the fasting, heat, and crowding stresses applied at 34 days. Body weight gain from 0 to 34 days was similar between the negative control and birds given the combination of supplementation products compared to the other treatment groups. From 0 to 62 days, feed intake was significantly higher in negative control birds compared to the other three treatment groups. The addition of the dry product (XPC + AVX vs. NC + PC) resulted in significantly lower cumulative FCR from 0 to 62 days. There was no difference observed between treatments for livability or water consumption, except day of stress, during this study.

(Keywords: turkey, *Saccharomyces cerevisiae*, heat stress, crowding stress)

Description of Problem

Social and environmental stresses are common in the life of birds which may influence the immune system and their susceptibility to disease (Koutsos and Klasing, 2014). Heat stress is one of the most costly issues in commercial bird production (Lara and Rostagno, 2013) and it has been associated with significant decreases in bird performance, feed intake, and increased mortality. Proper thermoregulation is needed by birds to cope with heat stress and can be observed as changes in behavior. Birds lack the ability to sweat, and must rely on the environment, panting, and other behavioral responses to cope with increased ambient temperature. In order for birds to maintain proper thermoregulation during this time, additional energy is necessary. However, when birds are in the process of being transported, between locations or subjected to a feed withdrawal, the additional energy necessary to maintain thermoregulation must come from the bird's energy stores. Once birds are placed at

their final location, decreased performance is common. Huff *et al*. (2013a) documented a decrease in turkey performance after transport and yeast fermentation additives in feed may vary in their ability to alleviate the stress response in birds as seen by differences in feed efficiency. Likewise, transport may not occur during ideal environmental conditions, subjecting birds to harsh surroundings which may also impact bird performance after placement at the new destination. During transport, birds are subjected to a social stress since they may not be cooped with known conspecifics. Additionally, once unloaded, birds may be mixed with other birds from different locations or pens causing a need for the reestablishment of the social dominance hierarchy and may cause changes in bird behavior, which will require energy to be set forth. With the addition of supplementation of yeast derived products, birds may cope with the stressor better than birds that were not supplemented, since they supply birds with readily digestible by-products and metabolites that can be used during these time periods. *Saccharomyces cerevisiae* supplemental products come in several different forms, which may alter their metabolite properties, which may cause different effects in bird behavior and growth performance. If supplemented birds do not have to use as much of their stored energy to cope with the stress response, they may have an advantage once they are placed in a new location. Furthermore, since these products can be given as a liquid or dry form, the mode of action within the bird's gastrointestinal tract may be affected differently based on which product is given.

Elevated stocking density is negatively correlated with growth of immune organs (Koutsos and Klasing, 2014). Furthermore, psychological stressors experienced during production and transportation of poultry have been determined to impact immunity and to be

a factor in the development of poultry disease (Huff *et al*., 2009). Gao *et al.* (2009) observed that in broilers challenged with a coccidian challenge had improved average daily gain with a *S. cerevisiae* derived fermentation product. Japanese quail that were exposed to a heat stress significantly altered the incidence of bacterial isolation after heat stress with low stress genetic lines having lower levels of colonization compared to the high stress lines (Huff *et al*., 2013a). Gao *et al*. (2009) observed that the supplementation with a yeast extract increased $CD3^+$, $CD4^+$, and $CD8^+$ T-lymphocyte counts, which indicated that the inclusion of their yeast derived product may have improved immune function and growth performance in coccidian infected broiler. Immune function has been observed by some researchers to require a great deal of energy and metabolic resources, which will be partitioned away from growth (Sandberg *et al.*, 2007). Dietary supplementation of yeast and yeast derived products have been validated to have immunomodulatory effects (Swiatkiewicz *et al*., 2014). These effects have been seen as increased antibody titers in broilers (Gao *et al*., 2008), and similar results in turkeys (Li *et al*., 2000).

Commercially available products, derived during yeast fermentation, have been shown to have a direct impact on the health and wellbeing of bird by impacting the innate and adaptive immune system in a positive manner and may be able to aid birds during times of stress when feed consumption may be suppressed. There were two main objectives in this study. The first was to determine effects of management-related induced stress events on turkey performance due to fasting, crowding to invoke a social stress, and heat stress. The second was to determine if fermentation derived products, a dry product (Original XPC™ dry, Diamond V, Cedar Rapids, IA, USA) and a liquid product (AviCare™ liquid, Diamond

V, Cedar Rapids, IA, USA) combined with the dry product, are effective at alleviating negative effects on performance associated with stress.

Materials and Methods

Birds and Housing

A total of 960, day of hatch, Large White Nicholas commercial turkey hens (Aviagen, Lewisburg, WV) were hatched at Butterball Hatchery (Goldsboro, NC) and were transported to a curtain-sided facility consisting of 48 pens, approximately 2.44m by 3.66m, arranged with 12 pens allocated to each quarter of the building, considered as 4 separate blocks at North Carolina State University's Turkey Education Unit. Poults were randomly allocated into 48 pens with 20 hen poults per pen. At 0 days of age, all birds, excluding the negative control (NC), were sprayed with a double dose of live coccidiosis vaccine (Immucox® T, 2X concentration dose, CEVA Animal Health, USA). Additional immune system stimulation was conducted by using a fresh 7% SRBC suspension in 1X PBS injected intravenously via the branchial vein at 13 days with 1.0mL and at 36 days with 1.0mL for hemagglutination $\log_2$ primary antibody titers. Serum was collected from these birds at 21, 34, 47, 55, and 62 days for primary antibody $\log_2$ analysis. Previously used litter from Part II was caked out and top dressed with ½" of fresh pine shavings in all pens to act as an additional stress for the birds, except negative control pens, which received fresh bedding for this trial.

At 34 days, an overnight feed & water withdrawal was applied (except for NC). At 34 days, in the evening, birds were subjected to an 18 hour fast in which both water and feed were pulled from the pens. At 35 days, a crowding and heat stress was applied (except for

NC) to represent cooping and transport between brooding and grow-out locations. This was accomplished by reducing pen size to 0.028 m^2 per bird and a heat lamp placed over the top of the pen for 6 hours. Fans inside the facility were turned off during this procedure. Poults were assigned to 1 of 4 different treatment groups consisting of two controls and two supplementation treatments: negative control (unsupplemented, non-stressed) (NC), positive control (unsupplemented, stressed) (PC), a dry product supplemented in the water (Original XPC^{TM} dry, 1.25 kg/MT, Diamond V Mills, Inc., Cedar Rapids, IA, USA) (XPC), and the liquid products in the water and a dry product in the feed (AviCare^{TM} liquid in water and Original XPC^{TM} dry in feed, 180mL/100L, 1.25 kg/MT, respectively), Diamond V Mills, Inc., Cedar Rapids, IA, USA) (AVX).

Measurements

Birds were weighed by pen at 0, 34, 35, 42, and 63 days of age. Feed and water intake, weight gain, and feed conversion were calculated. Body weights and feed intake were determined by measurement in order to calculate body weight gain and FCR.

Blood Collection

Blood collection occurred in a similar manner to previous sections of this thesis with an approximate blood draw of 2-3mL from 3, not previously bled, individual birds via the brachial vein for plasma corticosterone concentrations using a 23 gauge, 1" needle at 35 days immediately following the cold stress. Blood plasma was used in this study since previous blood collected in parts I and II had high levels of post centrifugation gelling of the serum.

Each blood sample was placed into its own individual disposable 15 X 85mm borosilicate glass culture tube (Thermo Fisher Scientific, Waltham, MA, USA) by removal of the needle from the end of the syringe and gently pushing the plunger down to trickle blood along one side of the inside of the tube within 1 minute after collection (before coagulation occurred). Once all blood samples were collected, blood was covered using Parafilm^{TM} and stored for at least 2 hours before centrifugation. Samples were spun in a similar manner at 500G and checked for plasma separation. Samples were re-spun at 500g for 30 minutes if there appeared to be less than 2mL worth of plasma to be using for laboratory testing. Plasma was taken off using a sterile micropipette and samples were placed into -20°C until they were fully frozen, then stored at -80°C until corticosterone analysis could be completed using a corticosterone EIA assay kit (Cayman Chemical, Ann Arbor, MI, USA).

A fresh 7% SRBC suspension was prepared in 1X PBS to be injected intravenously into the branchial vein of 3 birds in each pen. Birds were identified using paint markings on their backs so that they could be identified for subsequent vaccinations and blood collection. Birds were injected using a 23 gauge, 1" needle with 1.0mL at 11 days of age, and again at 37 days of age with 1.0 mL of the solution. From the previous section, the initial amount of the SRBC suspension was increased to ensure a robust primary antibody response post initial injection. Blood collection for hemagglutination was collected in the same manner as previous using a 23 gauge, 1" needle at 37, 42, 52, and 63 days of age from the same birds throughout the trial. Each blood sample was placed into its own individual disposable 15 X 85mm borosilicate glass culture tube (Thermo Fisher Scientific, Waltham, MA, USA) and using gentle transfer techniques to decrease the chances of lysing of the turkey RBCs. Once

all blood samples were collected, blood was covered using Parafilm^{TM} and stored for at least 3 hours to ensure the coagulation of blood before centrifugation under undisturbed refrigeration. After refrigeration, samples were rimmed using a micro spatula to gently dislodge the blood sample from sticking to the inside wall of the glass tube and to ensure good separation of serum from blood clot after centrifugation. Samples were spun at 500g and checked for serum separation. Samples were re-spun at 500g for 30 minutes if there appeared to be less than 2mL worth of serum to be using for laboratory testing. Serum was taken off using a sterile micropipette was used to transfer samples into 2 mL microcentrifuge tubes. Samples were vortexed and split for heat inactivation, with one bulk, untreated sample, and one to be heat inactivated. Heat inactivation was completed by placing samples into a 56°C water bath for 30 minutes to inactivate complement and occurred in the same manner as Part II of the thesis study. Samples were then removed from the water bath and placed into storage at -20°C until assays were completed.

Specific form of agglutination was used for blood typing and quantifications of titers from birds vaccinated with a fresh 7% SBRC suspension in 1X PBS in blood serum following the same procedures as Part II. Overall total primary antibody response or non-response was evaluated following immunization with the solution by assaying previously heat inactivated samples from the -20°C storage. Assays were performed in 96 well conical - bottom polystyrene plates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) and serial dilutions were performed, in duplicate for each sample following the procedure outlined in Part II. Plates were removed after the second incubation and placed on a 45° tilt for 20 seconds before the reading took place. Each plate was carefully read from the bottom of the

plate to avoid misreading due to bubbles that may have formed during the dilution and SBRC pipetting procedures. Agglutination was assessed using the same procedure outline in Part II with total agglutination marked by a button and was recorded as the well of full agglutination before a full teardrop was observed. In the case of any ambiguous well agglutination formations, such as a partial button, or if the sample was at least 2/3 teardrop, the well above was considered the full agglutination and marked as such.

Statistical Analysis

This study was analyzed as a completely random block design using the GLM procedure of JMP 11.2 (SAS Institute, Cary, NC, 2015) for ANOVA. Means were separated using the LSMeans procedure within JMP and significance was recognized at $P \leq 0.05$ for all performance and corticosterone data. Antibody titers were transformed to reciprocal $\log_2$ units of greatest dilution showing 100% agglutination prior to analysis and were analyzed using the GLM procedure of JMP 11.2 for ANOVA with mean separation using LSMeans procedure within JMP. A difference with a probability of $P \leq 0.05$ was considered significant.

Animal Ethics

This study was conducted under the supervision guidelines set forth by the Institution Animal Care and Use Committee at North Carolina State University. All husbandry, bird well-being, and euthanasia procedures were performed with full consideration of animal welfare.

Results

Significantly higher plasma corticosterone was observed in PC birds versus NC birds immediately following the stress event on 35 days, with XPC and AVX birds being intermediate in their response (Table 7). Feed intake was significantly higher from 0 to 34 days in NC and XPC birds compared to PC birds with AVX birds being intermediate (Table 8). Feed intake was significantly higher from 0 to 45 and 34 to 45 days in NC birds compared to PC birds, with XPC and AVX birds being intermediate in response (Table 8). From 34 to 62 days and cumulative 62 day feed intake was significantly higher in NC birds compared to all other treatment groups (Table 8).

Body weight gain from 0 to 34 days was significantly higher in NC compared to PC and AVX birds, with XPC birds being intermediate in response (Table 8). Body weight gain was significantly greater from 0 to 45 days in NC birds compared to PC, with XPC and AVX being intermediate (Table 8). There was no difference in FCR from 0 to 34 days, 0 to 45 days or 34 to 45 days (Table 8). Feed to gain ratio was significantly improved in XPC and AVX compared to NC from 34 to 62 days with PC birds responding intermediate in their response; however, there was no difference between treatments cumulatively (Table 8). Upon further analysis, a contrast of treatments receiving the dry dietary additive to the feed versus controls showed significantly improved FCR in AVX + XPC treatments compared to PC + NC groups (Figure 3). There were no differences in $\log_2$ primary antibody titer response between treatment groups in this study (Table 9). Likewise, there were no primary antibody level interactions effects over time by treatment seen in this study (Figure 4). No differences were

observed between treatments for livability or water consumption, except day of stress, during this study and there was no stress induced mortality in this study (data not shown).

Discussion

In the present study, corticosterone levels were significantly elevated in birds that received no supplementation and placed under the heat and crowding stress. However, those given dietary supplementation performed similar to both control groups. Due to great individual bird variability, corticosterone levels may be hard to determine since this was a short term induced stress. Similar results were obtained in all 3 parts of this study with the negative control birds having the lowest corticosterone levels immediately following the induced stress compared to the positive control, with supplemented birds performing intermediately. In a study conducted by Davis and Siopes (1987), turkeys were subjected to different routes of administration of ACTH. They observed that plasma corticosterone could be altered by exogenous ACTH or cold exposure. In a study conducted by Huff *et al.* (2013b), they observed that Japanese quail exposed to heat stress and an *E.* coli challenge did not affect the serum corticosterone levels in birds selected for high and low stress lines compared to the control, however, their control genetic lines had increased body weights compared to their low and high stress genetic lines. Similar results were obtained in the present study with supplemented birds performing similar to both controls. Gao *et al.* (2009) has suggested that a *S. cerevisiae* fermentation product may increase the ability for chickens to cope with stress. In the present study, the partial alleviation of stress seen by an intermediate response to the heat and crowding stress in regards to the corticosterone levels

post stress, indicate that this may also be the case in turkey hens, however, significant increases in body weight gain during the stress period was not observed in this study.

The present results show that the total anti-SRBC titers were similar between all treatments in this study. Li *et al.* (2000) observed similar results with differences seen in IgM levels between sexes, but no differences in IgG between treatments. Likewise, Gao *et al.* (2008) observed that broilers given a dietary yeast culture increased IgM, however, there were no differences seen in serum IgA or IgG content. This same discovery was observed in from Gao *et al.* (2009) when broilers were challenged with coccidiosis and birds had significantly higher IgM content but still had no influence IgG content. In the present study, specific antibodies were not assayed, and instead, primary response antibodies were determined via hemagglutination. Therefore, it is difficult to quantify which immunoglobulins were being agglutinated during the hemagglutination of this study. However, the basis of this study was not to determine levels of specific antibodies, but instead, if the stress and subsequent supplementation products were able to modulate differences in immune system responses.

Although the same total amount of antigen was used at each injection in the present study, the age and size of the bird may have altered the agglutination response to the SRBC and therefore, the titer response may have been lower post-secondary compared to post-primary. This same phenomenon has been previously record by Munns and Lamont (1991). In the present study, there were no differences observed in time by treatment interactions in SRBC antibody response. It has been reported that the kinetics of antibody response can be easily altered for chicken genetic lines selected for high or low early antibody response to *E.*

coli (Nelson *et al.*, 1991). However, the present study did not show differences in titer responses based on the environmental factors of this trial.

Body weight gain was significantly higher in the negative control compared to the positive control and those fed a combination of products and birds that received the dry fermentation product alone performed intermediate going into the induced stress at 34 days. Exposure to adverse climate increases glucocorticoid secretion, which are antagonistic to immunity and impairs expression of humoral immune response (Haldar *et al.*, 2011) and it has been well documented that increased glucocorticoid levels may result in decreased performance. This same phenomenon was observed in this study in which birds exposed to the stressor had negative effects on body weight gain. However, FCR for birds given either form of supplementation were significantly better than birds not placed under stress conditions. Similar results were seen in Gao *et al.* (2008), in which broilers were supplemented with a dietary yeast culture at 2.5g/kg had improved average daily gain and FCR from 22 to 42 days, but this was not apparent in the starter phase from 0 to 21 days.

In a comparison of contrasts between treatments supplemented with the dry product versus controls (XPC + AVX vs. NC + PC), birds fed the dry product has significantly improved cumulative FCR. Results from Huff *et al.* (2013a) in which turkeys were subjected to a transport stress, determined that the addition of a yeast culture during these stressful practices was able to partially alleviate the negative effects seen in FCR. They also noted that since nutritional requirements may increase during stress, birds that were subjected to transport and the *E. coli* challenge had significantly decreased production values and adding the yeast culture to the diet may have provided necessary nutrients to aid birds during these

times. Similar results were seen in the present study in which birds that were supplemented had similar body weights after the day of stress until the end of the trial however, they had less feed intake which resulted in better FCR.

Conclusions and Applications

1. Turkeys are susceptible to stress due to environment and management related events, including social stress.
2. The combination of supplements was able to reduced plasma corticosterone levels following a multifaceted stress event in turkey hens; however, it was not statistically different.
3. Addition of dietary the dry supplementation product resulted in improved FCR (XPC + AVX vs. NC + PC).
4. The supplementation of fermentation derived products partially alleviated stress and significantly improved performance of turkey hens.

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Table 7. Plasma corticosterone levels of turkey hens subjected to an 18 hour fast and a 6 hour heat and crowding stress grown to 9 weeks of age and supplemented with a dry or dry combined with a liquid fermentation derived supplement.

Treatment	Dietary Supplement	Age (Days) 35d
NC	None	131[b]
PC	None	1010[a]
XPC	Liquid[2]	574[ab]
AVX	Dry[1] and Liquid[2]	590[ab]
Source of Variation	**----------(P-Value)----------**	
SEM		179
P-Value		***0.009***

[1]Original XPC (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the feed at 1.25 kg/MT continuously in XPC and AVX treatment groups.
[2]AviCare (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the water at 160mL/100L in AVX treatment groups.
[a b] Superscripts within same column are significantly different ($P < 0.05$).

Table 8. Growth performance of turkey hens grown to 9 weeks of age and subjected to an 18 hour fast and a 6 hour heat and crowding stress supplemented with a dry or dry combined with a liquid fermentation derived supplement.

		Age (Days)				
		34d	45d	34 – 45d	34 – 62d	62d
Treatment	**Dietary Supplement**	**----------(kg Body Weight Gain/bird)----------**				
NC	None	1.45[a]	2.22[a]	0.75	3.14	4.64
PC	None	1.38[b]	2.13[b]	0.77	3.15	4.54
XPC	Dry[1]	1.41[ab]	2.19[ab]	0.78	3.18	4.61
AVX	Dry[1] and Liquid[2]	1.40[b]	2.16[ab]	0.76	3.20	4.60
Source of Variation		**----------(P-Value)----------**				
SEM		0.013	0.020	0.016	0.030	0.028
P-Value		***0.002***	***0.026***	0.769	0.544	0.138
		----------(kg Feed Intake/bird)----------				
NC	None	2.30[a]	3.57[a]	1.30[a]	4.88[a]	7.92[a]
PC	None	2.18[b]	3.41[c]	1.15[b]	4.52[b]	7.52[b]
XPC	Dry[1]	2.26[a]	3.51[ab]	1.24[ab]	4.56[b]	7.53[b]
AVX	Dry[1] and Liquid[2]	2.25[ab]	3.47[bc]	1.22[ab]	4.59[b]	7.55[b]
Source of Variation		**----------(P-Value) ----------**				
SEM		0.017	0.026	0.024	0.048	0.059
P-Value		***<0.001***	***0.001***	***<0.001***	***<0.001***	***<0.001***
		----------(kg Feed: kg Gain) ----------				
NC	None	1.52	1.56	1.69	1.48[a]	1.64
PC	None	1.53	1.56	1.58	1.43[ab]	1.64
XPC	Dry[1]	1.53	1.56	1.61	1.42[b]	1.61
AVX	Dry[1] and Liquid[2]	1.53	1.56	1.59	1.43[b]	1.61
Source of Variation		**----------(P-Value)----------**				
SEM		0.016	0.012	0.035	0.012	0.011
P-Value		0.963	0.996	0.127	***0.011***	0.058

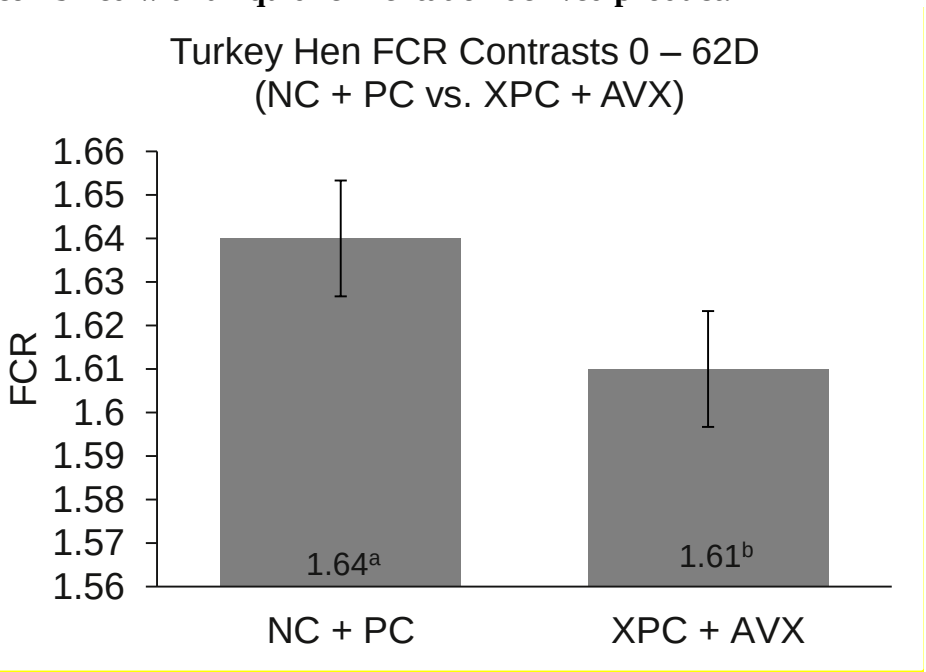
[1]Original XPC (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the feed at 1.25 kg/MT continuously in AVX treatment group.
[2]AviCare (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the water at 160mL/100L in AV and AVX treatment groups.
[a b] Superscripts within same column are significantly different ($P < 0.05$).

Table 9. Primary antibody hemagglutination titer response of turkey hens grown to 9 weeks of age and subjected to an 18 hour fast and a 6 hour heat and crowding stress supplemented with a dry or dry combined with a liquid fermentation derived product.

		Age (Days)				
		21d	34d	47d	55d	62d
Treatment	**Dietary Supplement**	**----------(Log_2 Primary Antibody Titer)----------**				
NC	None	7.19	2.52	5.50	3.05	2.51
PC	None	6.78	2.76	5.17	3.14	2.61
XPC	Dry[1]	6.93	2.57	5.56	3.05	2.35
AVX	Dry[1] and Liquid[2]	6.90	2.24	5.25	2.89	2.51
Source of Variation		**----------(P-Value)----------**				
SEM		0.170	0.162	0.121	0.125	0.153
P-Value		0.365	0.155	0.072	0.593	0.704

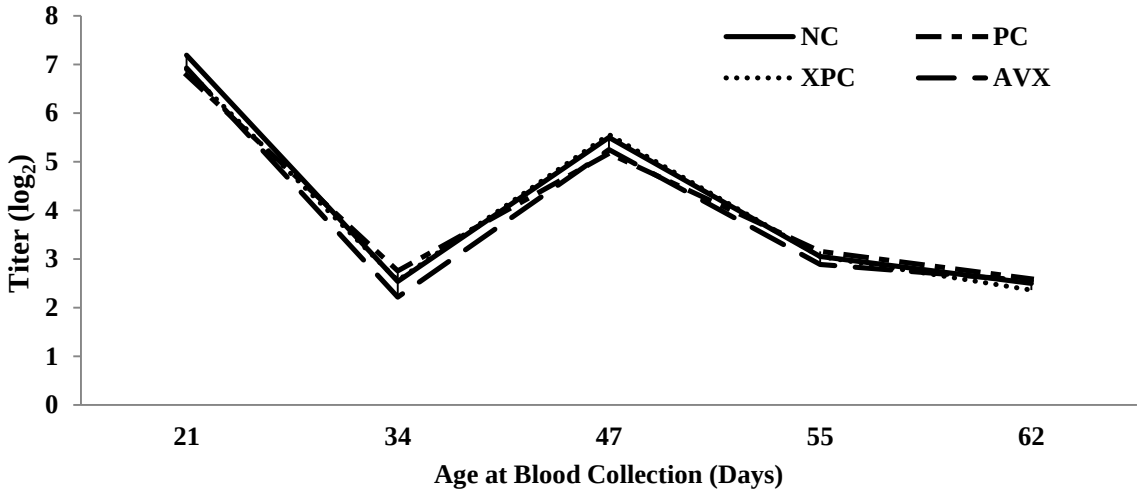
[1]Original XPC (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the feed at 1.25 kg/MT continuously in AVX treatment group.
[2]AviCare (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the water at 160mL/100L in AV and AVX treatment groups.

Figure 3. Contrasts analysis of FCR of turkey hens grown to 9 weeks of age and subjected to an 18 hour fast and a 6 hour heat and crowding stress supplemented with a dry or dry combined with a liquid fermentation derived product.



[1]Original XPC (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the feed at 1.25 kg/MT continuously in AVX treatment group.
[2]AviCare (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the water at 160mL/100L in AV and AVX treatment groups.
[a b] Superscripts within same column are significantly different ($P < 0.05$).

Figure 4. Bleed date by treatment interaction for primary antibody hemagglutination titers of turkey hens grown to 9 weeks of age and subjected to an 18 hour fast and a 6 hour heat and crowding stress supplemented with a dry or dry combined with a liquid fermentation derived product.



[1]Original XPC (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the feed at 1.25 kg/MT continuously in AVX treatment group.
[2]AviCare (Diamond V Mills, Inc., Cedar Rapids, IA, USA) was supplemented in the water at 160mL/100L in AV and AVX treatment groups.

SUMMARY

Environmental conditions and management practices can elicit a stress response in turkeys. This response can be objectively measured by circulating blood corticosterone in both the serum and plasma fractions. This physiological stress response resulted in reduced performance by growing turkeys as observed by reduced body weight and negative changes in FCR. When birds were placed under mild stress conditions, supplementation with fermentation products was able to partially alleviate the stress response and improve the subsequent growth response. Hemagglutination titer results were not conclusive. Therefore, further investigation of hemagglutination titers against SRBCs for primary antibody will require additional research to determine if there was indeed a biological response in supplemented birds as seen in the second and third studies.

However, based on the results obtained in heat and crowding stress study (study 3), at this time, it is possible that the differences observed in the primary antibodies titers in the previous cold stress study may not be associated with a true biological response. It would also be beneficial to determine if these products are viable at partially alleviating the stress response associated with chronic stress. Since this research focused only on a short term (~24 hours) stress response and conditions were immediately reverted back to favorable, it would be beneficial to determine how long these products last in chronic stress situations since they were able to partially alleviate some stress associated with acute stressors.