FOOD SAFETY AND HEAT STRESS RESPONSE OF BROILER CHICKENS FED A SACCHAROMYCES CEREVISIAE FERMENTATION PRODUCT

A Thesis

by

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ABSTRACT

A study was conducted to 1) evaluate the effects of feeding S. Cerevisiae fermentation product on *Salmonella* prevalence and numbers in ceca, carcass, and parts of broilers reared in heat stress as well as to 2) evaluate the welfare of broilers fed S. Cerevisiae fermentation product and reared under heat stress temperatures. A total of 480 Ross broilers were fed control diet (**CON**) or S. Cerevisiae fermentation product (**Original XPC^{TM}** Diamond V Mills, Inc Cedar Rapids, IA). Dietary treatments included control (**CON**), S. Cerevisiae fermentation product fed at 1.25 kg/MT (**XPC**), and S. Cerevisiae fermentation product fed at 2.0 kg/MT (**XPC 2**). Half the birds were subjected to either no heat or heat stress from 31 to 42 days. On D 3, *Salmonella* free chicks were gavaged with 0.25 ml of 10^7 CFU/ml of Novobiocin and Naladixic acid resistant *Salmonella* Typhimurium. At the end of the heat stress period, blood was analyzed from 40 birds per treatment for corticosterone (**CORT**), heat shock protein (**HSP70**), and heterophil/lymphocyte ratios (**HL**). On D 42, bilateral metatarsal traits were measured in 40 birds/T to assess physical asymmetry. Hocks and footpads were scored as a measure of bird welfare. Birds fed XPC or XPC 2 had significantly lower CORT levels than CON ($P < 0.001$). Physical asymmetry scores were higher ($P < 0.001$) in CON compared to XPC and XPC 2. HL ratios were greater in CON than XPC and XPC 2 birds ($P < 0.01$). No differences were observed between CON broilers and those fed XPC in HSP70. However, heat stress did increase ($P < 0.001$) HSP70. Feeding XPC did not significantly influence footpad or hock scores.

On D 42 & D56, 8 birds per pen were processed following industry practices. Individual ceca (**C**), wing (**W**), right breast filet (**BF**), and ground left breast filet (**GBF**) were collected for *Salmonella* prevalence and enumeration. No significant two-way interactions between dietary treatment and environment were observed, so data was pooled for analysis. *Salmonella* prevalence was significantly lower in GBF for XPC fed birds ($P<.046$), indicating the potential for XPC to reduce *Salmonella* levels in ground meat.

DEDICATION

This work and all efforts leading into it are dedicated to God and the glory of the gift of Jesus Christ. Without complete and constant commitment and dedication to His will, there would be no direction or purpose to this. This work is also dedicated to my beautiful and amazing children and wife. They are the joy in my life that makes all work fulfilling and manageable. Their gift in my life will never be equaled. Dedication goes to my parents Tom and Betsy, who have given me more than any child has ever deserved and created an environment that allowed me to dream of and pursue the highest levels of success. Thank you for showing me what confidence can do for a person's direction and purpose and for always trying to instill that confidence. Thank you to my "new" family, my in-laws Mandy and Alan, for being so incredibly supportive of these efforts. I am especially grateful for your making them possible by being there at every moment when needed at home.

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Part 1. Faculty committee recognition

This work was supervised by a thesis committee consisting of Greg Archer, Ph.D., (Committee chair; Dept. of Poultry Science), Craig Coufal, Ph.D., (committee member, Dept of Poultry Science), and Christine Alvarado Ph.D., (committee member Dept. of Poultry Science/Dept. of Food Science and Nutrition).

The data for both experiments was analyzed by Dr. Archer & Dr. Alvarado, and physical asymmetry measurements in Chapter IV were taken and analyzed by Dr. Archer. All other work was performed independently by the student or with assistance from other student workers & company coworkers.

Allen Byrd DVM, Ph.D. of the USDA-ARS provided facilities for the live animal experiments as well as BSL-2 laboratory facilities.

Part 2. Student collaborater contributions

Samples prepared in Chapter III were done in conjunction with the students from the Dept of Food Science and Nutrition under Dr. Alvarado. Their data is under review for publication currently.

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NOMENCLATURE

CORT	Corticosterone
H/L	Heterophil/Lymphocyte
HSP70	Heat Shock Protein 70
C	Ceca
BF	Breast Filet
GBF	Ground Breast Filet
CDC	Centers for Disease Control
USDA	United States Department of Agriculture
FSIS	Food Safety Inspection Services
FDA	Food and Drug Administration
FSMA	Food Safety Modernization Act
TAMU	Texas A&M University College Station
NCC	National Chicken Council
NARMS	National Antimicrobial Resistance Monitoring System
HACCP	Hazard Analysis and Critical Control Points
SSOP	Sanitary Standard Operating Procedure
PAA	Peracetic Acid
CPC	Cetylpyridinium Chloride
PAHP	Peracetic Acid and Hydrogen Peroxide Mixture
TMP/SU	Trimethoprim-Sulfonamides

CFU	Colony Forming Units
MPN	Most Probable Number
MSRV	Rappaport-Vassiliades
RTC	Ready to Cook
WBC	White Blood Cells
BC	Blood Cells
ACTH	Adrenocorticotropic Hormone
AS	Antisymmetry
DA	Directional Asymmetry
L-R	Left Minus Right
AGP	Alpha1 Acid Glycoprotein
TI	Tonic Immobility
GP	Globus Pallidus
POM	Medial Preoptic Area
Tn	Nucleus Taenia of the Amygdala
PVN	Paraventricular Nucleus of the Hypothalamus
GC	Germinal Centre
PCR	Polymerase Chain Reaction
NCDV	Newcastle Disease Virus
ST	*Salmonella* Typhimurium
SH	*Salmonella* Heidelberg
SE	*Salmonella* Enteritidis

MOS	Mannan-Oligosaccharide
IgG	Immunoglobulin G
IAMM	Intestinal Activity Modifier Model

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CHAPTER I

INTRODUCTION

USDA's Food Safety Inspection Services (FSIS) maintains sampling data for foodborne pathogens of concern in poultry. The 2011 signing of the Food Safety Modernization Act has led to FSIS setting the first ever standard for maximum allowable *Salmonella* positives in chicken and in ground chicken, as well as lowering the maximum allowable *Salmonella* positives for whole carcasses to 7.5 percent (Federal registrar 2016). The impetus for this change can be traced directly to the CDC's annual report card for food borne illness, which showed *Salmonella* to be the most common foodborne illness causing pathogen (CDC Foodnet 2014). Of greater concern was that Salmonellosis infection rates remained unchanged from the previous year despite a national prevalence rate of less than 4 percent positives in whole birds. According to the National Chicken Council the American consumption of chicken has changed to reflect that over 40 percent of all chicken is now sold in cut-up parts. FSIS has not previously mandated sampling of *Salmonella* levels in parts, though that has now been addressed by implementing the first ever standards for parts effective July 1, 2016.

Most pathogen control interventions in the U.S. poultry industry are focused on the processing plant, specifically using a combination of pH, temperature, and time to control levels of *Salmonella*. Wideman (2015) conducted a study on different control points using antimicrobials using a survey among six broiler plants and found that peracetic acid (PAA) was the most effective antimicrobial currently in use in the U.S. They also demonstrated that the use of a post-chill antimicrobial immersion tank and/or

use of a cetylpyridinium chloride (CPC) spray cabinet resulted in further reduction in microbial levels when the primary chiller was not sufficient (Wideman, 2015). The use of PAA and its results in this study are of interest, as the U.S.broiler industry relies heavily upon this antimicrobial. Bauermeister (2008) studied Peracetic acid mixture (PAHP), a mixture of PAA & Hydrogen peroxide that has been approved as an antimicrobial for use in poultry chillers. PAHP at 85 ppm reduced *Salmonella* on positive carcasses exiting the chiller by 92 percent, whereas treatment with 30 ppm of chlorine reduced the loads by 57 percent.

These technologies employed as interventions at the processing plant are accepted widely across the industry. However, the forecasted prevalence of *Salmonella* in parts samples indicates a higher than desired level of pathogen still entering the processing plant and surviving these interventions. To confirm this concern of pathogen connection back to the farm, Berghaus (2013) conducted a correlation study in which 55 commercial broiler flocks were sampled both at the farm and the processing plant. This study showed *Salmonella* was recovered in 90.9 percent of farm samples and 94.5 percent of processing plant samples, indicating strong correlation between the two steps (Berghaus et al, 2013). Higgins and Malo (1982) measured *Salmonella* from the hatchery to 6 weeks into the flock showing another strong connection between positive chicks and positive flocks. Arsenault (2007) also looked at risk factors for prevalence of *Salmonella* in commercial broiler flocks. This study indicated the value of biosecurity on the farm level as odds of *Salmonella* colonization were 2.6 times greater for chicken flocks in which the grower failed to lock the chicken house permanently.

In recent years, there has been more focus on control of *Salmonella* in live birds at the farm through vaccination programs. *Salmonella* vaccines have shown to be effective against select serotypes in broilers when breeders are vaccinated (Young, 2007). Berghaus (2011) also studied the prevalence of progeny broilers from *Salmonella* vaccinated hens. Broiler flocks that were the progeny of vaccinated breeders had significantly lower *Salmonella* prevalences and loads than those from unvaccinated breeders (Berghaus, 2011).

Van Immerseel (2005) showed butyric acid in feed decreased cecal colonization shortly after infection, decreases fecal shedding, and as a consequence, decreases environmental contamination *by S. Enteritidis*-infected broilers. However, complete elimination can probably only be achieved with a combined approach using both hygienic measures and different protection measures, as the broilers still carried *S. Enteritidis* bacteria in the ceca at slaughter age, although at enrichment level. (Van Immerseel, 2005).

To investigate the use of probiotics, Park (2014) found that the administration of a *Bacillus subtilis* strain resulted in a quadratic reduction of the intestinal *Salmonella* load as well as a linear reduction (Park, 2014). De Oliviera also looked at in ovo probiotic inoculation on *S.* Enteritidis challenged chicks. A significant reduction in the number of *S.*Enteritidis positive chicks was observed when chicks were in ovo inoculated with *E.* Faecium and continued receiving it in the diet (de Oliveira, 2014).

The fermentation metabolite product Original XPC^{TM} has been shown to have strong preharvest *Salmonella* reduction effects. In 2012 Nsereko utilized the Intestinal

Activity modifier (IAMM) model to show a 91 percent reduction in CFU/g of *S.*Enteritidis ex vivo in the digestate of birds fed Original XPC™ or control. In 2013 Nsereko showed in the same IAMM model a 46 percent reduction in *S. Heidelberg* CFU/g of laying hen digestate. In 2012, a study by Nsereko in which live birds were challenged with *S.*Typhimurium, a 61 percent reduction in *Salmonella* population recovered from the cecum was observed. The Iowa State Center for Veterinary Medicine challenged birds with *S.*Enteritidis and showed a 37 percent reduction in *S.* Enteritidis prevalence recovered from the cecum, as well as a 90 percent reduction in the CFU/g counts of the same *S.* Enteritidis. Hofacre showed a 54 percent reduction in *Salmonella* positives from XPC fed bird birds in a large commercial live bird pen challenge with S. Heidelberg by measuring cecal colonization (Hofacre, 2015).

Heat stress is also a persistent industry concern for both the health and welfare of commercial broilers. This is, in part, due to geographic location of many commercial farms in hot climates in the U.S. The continuous selection for fast growth seems to be associated with increased susceptibility of broiler chicken to heat stress (Berrong and Washburn, 1998; Tan et al., 2010; Soleimani et al., 2011). Today's chickens seem to be particularly susceptible to high environmental temperatures and suffer from multiple patho-physiological alterations, such as immune dysregulation, gut barrier dysfunction and cellular oxidative stress after heat exposure, resulting in decreased productivity and increased susceptibility to infectious diseases and higher mortality (Syafwan, 2011, Varasteh, 2015). *Salmonella* colonization in broilers has been shown to increase as a result of gut barrier dysfunction, as higher rates of prevalence have been found in flocks

also experiencing dysbacteriosis (Soleimani, 2011). Periods of high heat stress have been shown to affect broiler immune regulation and particularly gut barrier function, potentially indicating heat stress as concern for *Salmonella* colonization increase (Cooper, 1998; Arjona, 1988). As a measure of the impact on carcass quality, Zeferino studied heat stressed broilers and found heat stress reduced slaughter and carcass weights, average daily gain and feed intake, and increased feed conversion (Zeferino, 1998). Atilio (2016) conducted experiments where indications were that Heat stress decreased the duodenal concentrations of secretory Immunoglobulin A (sIgA), a trend toward similar findings of sIgA concentrations was observed in the chickens' jejunum. Changes in spleen and Bursa of Fabricius relative weights as well as in spleen morphometry were also noted in heat stressed animals, infected or not. Based on this, it was surmised that heat stress changes germinal centre (GC) formation in chickens, which may lead to failures in vaccination protocols as well as in the host resistance to infectious diseases during periods of exposure to heat stress (Atilio, 2016).

Rimoldi (2015) also found corticosterone levels increased significantly in the blood of heat stressed broilers, due to the activation of the hypothalamic-pituitary-adrenocortical axis (Rimoldi, 2015). If this occurs and too much corticosterone is present, it can retard the longitudinal growth of bones by depressing the proliferation and differentiation of chondrocytes in broilers. According to Zhang's work it depressed the longitudinal growth of the long bones by inhibiting the proliferation and differentiation of chondrocytes in growth plate in the birds (Zhang, 2012).

With an interest in immune benefits to alleviate heat stress in mind, Sohail looked at the effect of mannan-oligosaccharide product (MOS) and saw that MOS only partially alleviated heat stress effects. Among supplemented groups, the heat stress-MOS had higher body weight gain and lower FCR compared with the heat stress-control group (Sohail, 2012). Sohail in 2010 also showed that MOS and probiotic together or in combination reduced serum cortisol and cholesterol concentrations, and increased thyroxine concentrations vs a control heat stressed group (Sohail, 2010).

Gao (2009) noted the measured effects on the broiler immune system with a fermentation product XP, which was shown to increase sIgA content, IgA plasmacyte count and intraepithelial lymphocyte count in the duodenum and cecal tonsil, as well as serum IgM and lysozyme content (Gao, 2009). Al-Mansour (2011) showed that yeast culture treatment resulted in lower white blood cell counts, significant decrease in thrombocyte counts, decreased H/L ratio (Al-Mansour, 2011). Field studies with an experimental version of this yeast culture in liquid phase fermentation have been put into commercial experimental use in recent years. It was observed in 2015 that addition to the diet during a temporary heat stress significantly improved egg production and reduced mortality in caged layer flocks (W. Michael, Diamond V Mills Inc, Cedar Rapids, IA personal communication).This effect was repeated in 2015 in the Midwest, and then the product was added to commercial turkey diets during heat stress. Mortality of toms was reduced in the yeast culture treated house by 40 percent (from 6 percent to 2.5 percent) giving further credence to the effect of yeast culture on stress amelioration (D. Kenyon, Diamond V Mills Inc, Cedar Rapids, IA personal communication).

CHAPTER II

LITERATURE REVIEW

Foodborne Illness

Salmonella in chicken products is of major concern in human health, as foodborne salmonellosis causes numerous illnesses and deaths across the globe. In 2013, *Salmonella* was the cause of over 1,000,000 illnesses, 378 deaths, and 19,000 hospitalizations, all of which totaled a cost of 3.7 billion dollars (foodsafety.gov). Over 19 percent of all foodborne illnesses are attributed to poultry meat (CDC.gov). *Salmonella* infection onset is 12-72 hrs after infection, and includes diarrhea, cramps, and fever. Severe diarrhea and dehydration are the main cause for hospitalizations, according to the CDC. In these cases, *Salmonella* infection may spread from the intestines to the blood stream, and then to the other body sites. It is then that *Salmonella* can cause death if not properly treated with antibiotics. Many patients can experience a latent joint pain effect months or years later known as reactive arthritis (Carter & Hudson, 2009).

In diagnosing *Salmonella* as the cause of illness, actual confirmation is received through the process of "culture confirming". In this process blood or stool from human clinical subjects must be added in a specific environment such as a petri dish coated with nutrients (CDC, 2015). If *Salmonella* grows, then it has been culture confirmed. In the majority of cases, patients recover in five to seven days. However, in these more invasive cases involving spread of infection in the body, appropriate antibiotic use is of great importance

Antibiotic resistance concern. Resistance to antimicrobial agents is not uncommon in *Salmonella*. Work by Cui (2005) indicated that *Salmonella* isolates from conventionally raised chicken showed more resistance to antimicrobials than *Salmonella* isolates recovered from organic chickens (Cui, 2005). This could indicate some pressure on *Salmonella* to develop resistance when faced with the common presence of antibiotics in conventional birds. Work done in Brazil comparing antimicrobial resistance from S.Enteritidis isolates taken from poultry, humans, and food products showed the highest percentage of antimicrobial resistant cultures to be in poultry samples (de Oliviera, 2005). More recently, U.S. supermarket samples have shown that multi drug resistant strains of E.Coli were lower in samples from poultry raised without antibiotics than that of samples from conventionally raised chicken (Milman, 2013). Data from the National Antimicrobial Resistance Monitoring System (NARMS) show that 5 percent of non-typhoidal *Salmonella* are resistant to five or more antimicrobial agents. The public concern over antibiotic resistance in foodborne pathogens has risen to dramatic heights in recent years, as more highly publicized foodborne illness outbreaks have occurred. The 2014 outbreak of salmonellosis linked to Turlock, CA based Foster Farms chicken brought the issue to the forefront of the U.S. consumer consciousness month after month while authorities & company personnel struggled to control the epidemic. The CDC reports that a total of 634 people in 29 states were infected with multi-drug resistant strains of S..*Heidelberg* between March 2013 & July 2014 (CDC.gov 2015). By the second month of the outbreak, 62 percent of the recovered *Salmonella* isolates from human illness exhibited resistance to one or more antibiotics,

and 38 percent of the isolates exhibited multidrug resistance to combinations of the following antibiotics: ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulfisoxazole and tetracycline CDC says that, although these antibiotics are not typically used to treat *Salmonella* bloodstream infections or other severe *Salmonella* infections, antibiotic resistance can be associated with a higher risk of hospitalization in infected individuals (foodsafetynews.com). For such a large-scale outbreak to occur in a time in which news is transmitted and absorbed by the U.S. population so rapidly, it could not help but raise a massive consumer concern about the safety and reliability of the retail food supply, specifically chicken.

Much of the public concern over antibiotic resistance has led to consumer preference for chicken raised without antibiotics, or labeled as antibiotic free. Sales of antibiotic free chicken were up 34 percent in 2013 alone (consumerreports.com 2013). Studies have indicated that removal of certain antibiotics from feed can lead to increased prevalence of *Salmonella* in broiler intestinal samples and environments (Ansari-Lari, 2014; Cengiz, 2015). With such a large potential decrease in U.S. antibiotic usage on the industry horizon, there is a need to investigate alternative methods of decreasing *Salmonella* colonization rates.

***Surveillance and control of* salmonella.** National Surveillance of *Salmonella* infections began in 1962 in the U.S. In this surveillance approach, *Salmonella* isolates from human subjects are analyzed by clinical diagnostic labs and submitted to state health agencies. The Kaufman White method of serotyping at these clinical diagnostic laboratories is first performed, via Phenotypic characterization of strains based on the

immunologic reactivity of two surface structures: y Lipopolysaccharide (O antigen) y Flagellin protein (H antigen) (aphl.org). Then if the isolate is unrecognized or if it is unable to be typed, this information is then submitted to the CDC where it can be serotyped in the National Reference Laboratory (CDC.gov).

Since 1996, the Foodborne Diseases Active Surveillance Network, or "FoodNet", has been compiling data through foodborne illness reports. This collaborative program of the CDC involves state health departments in ten states across the U.S. Foodnet personnel at these locations can report and share information with diagnostic laboratories (CDC.gov). These reports are added to the CDC's Laboratory-based Enteric Disease Surveillance & PulseNet, which is representative of over 48,000,000 people. The *Salmonella* outbreak rate for 2014 according to PulseNet reporting was 15.45 per 100,000 people, a figure which did not decrease from the previous year. By comparison, E.Coli cultured infection per 100,000 person basis dropped over 32 percent from 2013 to 2014 (CDC.gov).

The National Veterinary Services Laboratories (NVSL) of USDA-FSIS reports on isolates from not just humans but animals, their environment, and their feed. Serotyping is conducted based on samples received from veterinary diagnostic laboratories across the U.S. Samples are both clinical (animals presenting with signs of salmonellosis) and non-clinical (obtained through national routine environmental, feed, and herd/flock monitoring programs) (Atlas of *Salmonella*, 2011).

Federal government foodborne illness control. In 2006, the USDA clarified officially "Cooking poultry to a temperature of 165°F will ensure it is safe to eat'. The

guideline is based on advice from the USDA's National Advisory Committee on Microbiological Criteria for Foods (NACMCF). According to the USDA Under Secretary for Food Safety Dr. Richard Raymond, "The Committee was asked to determine a single minimum temperature for poultry at which consumers can be confident that pathogens and viruses will be destroyed". This temperature recommendation is the basis for the consumer food safety message that is still prevalent throughout industry and health guidelines today. The continued reporting of large scale outbreaks of salmonellosis from raw and undercooked chicken products however serves as a strong indicator of the need for food safety knowledge at the individual consumer level. Recommendations for safe preparation of poultry meat have not proven effective enough in controlling consumer consumption and cooking practices. While there has been a concerted effort to curb infection by way of mass dissemination of public health messages, it is more likely that the USDA FSIS will continue to pressure poultry processors to produce products that are free of *Salmonella*.

Healthy People 2020 and food safety control. Due to the launch of the federal Healthy People 2020 initiative, the office of Disease Prevention and Health promotion has undertaken a broad ranging set of public health goals. Part of this affects the food supply system, specifically the poultry production industry. In an effort to control the incidence of foodborne salmonellosis, the USDA-FSIS must follow the goal of reducing human illnesses from *Salmonella* by 25 percent and campylobacter by 33 percent by the year 2020 (FSIS Draft compliance guide). The most immediate area of importance in which these efforts impacts the commercial poultry supply chain would be in the

development of new FSIS performance standards for chicken parts and comminuted chicken. Since issuance of the most recent version of the compliance guidance in 2010, there have been several outbreaks associated with consumption of raw poultry products, including chicken parts and comminuted turkey products. In 2011, there were two *Salmonella* outbreaks associated with ground turkey products (specifically, turkey burgers and ground turkey) that resulted in a total of 148 illnesses and 40 hospitalizations.

FSMA and new FSIS parts and ground pathogen standards. With the 2011 signing into action of the Food Safety Modernization Act by President Barack Obama, FSIS was forced to make definitive regulation changes that decrease the threshold maximum acceptable standards for chicken parts and comminuted chicken. As of the final issuance of the new standards published in the Federal Register, for a rolling number of 52 samples, the maximum acceptable positive *Salmonella* samples for chicken parts is 8 of 52, or 15 percent. The maximum acceptable positive *Salmonella* samples for comminuted chicken is 13 of 52 samples, or 25 percent. The whole bird carcass tolerance is listed at 9.8 percent maximum acceptable positive (fsis.usda.gov 2016). This new limit is a strong commitment to monitoring the entire chicken product supply, in contrast with previous controls that were limited to whole carcass rinses. FSIS implemented performance standards for whole chickens in 1996 but has since learned that *Salmonella* levels increase as chicken is further processed into parts (Chen, 2014; Cui, 2005; Milman, 2013). Poultry parts like breasts, wings and others represent 80 percent of the chicken available for Americans to purchase (Consumerreports.com

2016). By creating a standard for chicken parts, and by performing regulatory testing at a point closer to the final product, FSIS can greatly reduce consumer exposure (USDA.gov).

FSIS implemented new sampling procedures in January 2015 when the new standards were proposed. Routine sampling throughout the year, instead of infrequent sampling on consecutive days, was intended to more thoroughly assess whether the facility's processes effectively addressed *Salmonella* and *Campylobacter*. Once USDA-FSIS has completed a 52 set samples under the new standards, results will be posted online with a description of pass, fail or meets standrds for each facility (Acheson group). Table 1 shows the industry averages collected over the course of FSIS's sampling leading up to finalization of the new rule, as well as those thresholds that will be part of the new rule for *Salmonella*.

Table 1. New FSIS prevalence threshold levels (National Chicken Council 2015).

PRODUCT	CURRENT INDUSTRY AVGS	MAX. ACCEPTABLE percent POSITIVE
Broiler Carcass	7.5 percent	9.8 percent
Comminuted Chicken	49 percent	25 percent
Chicken Parts	28 percent	15.4 percent

This food safety regulation change is an important area of focus in achieving the goals of human illness reduction determined by HP2020, as it practically focuses on areas of current concern. About 33 percent of all food related salmonellosis cases are

associated with products regulated by FSIS. Of these FSIS-associated illnesses, poultry represents about 58 percent of the cases with 85 percent being associated with chicken and 15 percent being associated with turkey. Of the illnesses from consuming chicken, FSIS estimates that 81 percent were associated with parts, 13 percent were associated with whole carcasses, and 6 percent were associated with comminuted product. (Federal Register vol80 no16).

In an unprecedented attempt keep consumers and the industry more aware and motivated toward safer food supply, FSIS will begin printing the names and addresses of Federally inspected establishments that do not meet the new standards. Plants will be classified into one of 3 categories based on performance as shown in Table 2:

Table 2. FSIS food safety category levels (Federal Register FSIS.USDA.GOV).

Category 1	establishments continuously achieving 50 percent or less of the pathogen reduction performance standard, i.e., meeting or surpassing the standard
Category 2	establishments that have not continuously achieved 50 percent or less of the pathogen reduction performance standard, nor have they exceeded the standard
Category 3	establishments that have exceeded the pathogen reduction performance standard, i.e., not meeting the standard

Currently in commercial chicken plants, the methods of controlling *Salmonella* and other foodborne pathogens are largely HACCP based. This is a system of prevention that is based on a written document of prerequisite individual Standard Sanitary

Operating Procedures (SSOPs). Conducting analysis of hazards reasonably likely to occur and implementing written plans and procedures to limit the occurrence of these hazards is a large part of an overall food safety plan in poultry processing facilities.

Antimicrobial interventions in processing. Time, temperature, pH of processing environments and physical design of processing facilities can all be effective pieces of a cumulative pathogen control program in broiler processing (Khan, 1972; Felhaber, 1998; Angelotti, 1961). One important area of focus in plants to achieve reliable control is the application of antimicrobials. Wideman (2016) conducted a study on different control points using antimicrobials using a survey among 6 broiler plants and found that peracetic acid (PAA) was the most effective antimicrobial currently in use in the U.S. They also demonstrated that the use of a post-chill antimicrobial immersion tank and/or use of a cetylpyridinium chloride (CPC) cabinet also resulted in a further reduction in microbial levels when the primary chiller was not sufficient (Wideman, 2016). The use of PAA and its results in this study are of great interest, as they coincide with heavy commercial use in the U.S. broiler industry. Bauermeister (2008) studied Peracetic acid mixture (PAHP), a mixture of PAA & Hydrogen peroxide that has been approved as an antimicrobial for use in poultry chillers. PAHP at 85 ppm reduced *Salmonella*-positive carcasses by 92 percent exiting the chiller, whereas treatment with 30 ppm of chlorine reduced *Salmonella* by 57 percent (Bauermeister and Bowers, 2008).

Antimicrobials and carcass quality. With every advantage comes trade offs, and in the case of antimicrobials such as PAA, the question has arisen of what affects this chemical may have on meat quality or product stability. Bauermeister (2008) examined

PAA for quality of product and shelf stability. PAA concentrations as low as 0.0025 percent were observed to be effective in decreasing *Salmonella* spp in comparison with the chlorine treatment. 500 carcasses were also treated with water, chlorine, or varying levels of PAA. The PAA-treated carcasses had an extended shelf-life compared with those treated with water or chlorine. Specifically, on d 15, the only treatments that could be served to sensory panelists were the carcasses treated with higher levels of PAA. These results indicate PAA's ability to effectively reduce microbial levels in chillers, as well as add shelf stability and product life. (Bauermeister & Bowers, 2008). In a further study of PAA, Nagel (2013) found Treatment with PAA had the strongest effect in reducing *Salmonella* and *Campylobacter* when compared to chlorine, lysozyme and water treatments. In this study treatment with the various antimicrobials was not found to harm sensory attributes, suggesting that application of PAA in a post-chill immersion tank effectively reduces *Salmonella* and *Campylobacter* and does not negatively impact product quality (Nagel, 2013).

Antimicrobials and ground meat. Comparison of antimicrobial effects has also been conducted on ground chicken, as its average *Salmonella* prevalence levels continue to exceed whole carcass and parts in national sampling results. Chen & Bauermeister (2014) looked at ground meat & antimicrobials, starting by rinsing parts with either chlorine, PAA, or CPC in a decontamination tank or dip tank. They ground the parts and then plated for *S*. Typhimurium and *C*. *Jejuni*. PAA treatment exhibited the greatest reduction in *Salmonella* and *Campylobacter* and greatest extension of shelf life, while Chlorine was the least effective treatment (Chen, 2014). Sharma (2013) studied the

effects of lauric arginate on *Salmonella* levels in ground chicken for potential as a processing intervention. *Salmonella* was reduced by lauric arginate in peptone suspension, but no suppression of pathogen was observed in ground chicken (Sharma, 2013). Thymol and sodium lactate have been combined in an effort to study antimicrobials in conjuction with reduced usage of essential oils. This was observed to be effective in reducing *S.*Typhimurium in ground fish (Ilhak, 2014).

Processing plant environmental sampling. Multiple step interventions in processing plants is key, and most are under continuous evaluation for efficacy in food safety programs. The key in keeping *Salmonella* under control is measuring its levels throughout the flow of product to the back of the plant, also known as biomapping. Morris and Wells (1970) studied the contamination levels in a poultry plant for over two years and found a rate of 14.2 percent cultures positive for *Salmonella*. Results showed that post carcass wash, cross-contamination or recontamination occurred in more than one other location in the plant such as evisceration and chill tanks (Morris & Wells, 1970). Equipment is an importat potential reservoir for cross contamination as Tadesse (1994) showed in the spread of *Salmonella* in processing. The highest contamination with *Salmonella* was found in the rinse of broiler carcasses, followed by swabs of equipment, and tissues of organs (Tadesse, 1994). Magwood (1967) conducted early work in two Canadian processing plants, and found a rate of 14 percent positive cultures for *Salmonella*. Each of these two plants was subjected to detailed bacteriological examinations over four separate occasions. Samples or specimens for culture were taken from vent areas of carcasses, operators' hands, equipment surfaces and from water in

tanks of iced birds. Only one culture of these was not *S*. Oranienburg or *S*. Infantis. This evidence indicated that *Salmonella* contamination could become widespread in the plant during processing, and despite reductions from interventions, opportunities exist for recontamination during subsequent handling (Magwood & Rigby, 1967).

Farm and plant correlation. The number of parts & comminuted positives a poultry plant may experience in testing can often be correlated with the amount of *Salmonella* being brought into the plant on or in the birds themselves. Bahatia (1979) made observations from farm samples all the way to processing plant. It was found that fresh litter was contaminated with *Salmonella* and could cause infection, thereby making six week litter culture a potentially strong indicator of *Salmonella* levels at processing plants. This study also showed that feed was not positive for *Salmonella* (Bahatia, 1979). Today, formaldehyde is commonly utilized in feed to ensure extra protection against pathogen contamination. In a second study, Bahatia (1980) again examined litter and feed but also hatchery fluff and meconium. It was found that when fluff and meconium were positive for *Salmonella*, litter and carcasses at the processing plant showed a high correlation of positives with the same serotypes. Also it was again found that feed samples were not positive for *Salmonella*, and thus feed was not considered to be an important source of infection (Bahatia, 1980).

Farm and plant sampling. Berghaus et al (2013) conducted a correlation study in which 55 commercial broiler flocks were sampled both at the farm and the processing plant. For environmental sampling, boot socks, drag swabs, litter and fecal samples were obtained. In the processing plant, whole-carcass rinses were performed on 30 birds from

each flock: 12 birds outside the plant with feathers still attached; 6 birds at rehang, which is after defeathering but before evisceration; 6 birds after evisceration and inside-outside bird wash but immediately before entering the chill tank; and 6 birds immediately after exiting the chlorinated immersion chill tank. This study showed Salmonella recovered in 90.9 percent of farm samples and 94.5 percent of processing plant samples, indicating strong correlation between the two (Berghaus et al, 2013). Higgins and Malo (1982) looked at *Salmonella* from the hatchery to the respective six week old flock, showing another strong connection between positive chicks and positive flocks. They looked at *Salmonella* serotypes in dust, litter, feces, water, and feed. Contamination of dust with *Salmonella* was observed even after six of the nine houses were disinfected. This shows that improper cleaning and disinfection of air inlets and fans play a strong role in the recontamination of the house. Fresh intact fecal swabs showed the level of Salmonella shed for the flock. Chickens were often contaminated at the hatchery and showed a significant increase of over 80 percent in two houses by six weeks of age. (Higgins & Malo, 1982).

Salmonella *and biosecurity.* According to White and Baker (1997) in an overview of control in the poultry production chain, prevention of foodborne disease involves an understanding of the multiple entry points of foodborne pathogens in the food chain. A variety of barriers must be established to reduce the potential for *Salmonella* introduction into the food chain. At the farm, these barriers can include sanitation, vaccination, nutritional & biological control, and drug administration. In the processing plant interventions can include counter-flow technology in the scalder &

chiller, routine replacement of used scald water, antimicrobial applications, temperature control, and pH. (White & Baker, 1997).

Many different factors can determine how often flocks are positive for *Salmonella* in the prior to processing. A study was conducted in Iran to examine what role antibiotics administered in feed may play in *Salmonella* prevalence (Ansari-Lari, 2014). The most frequently used antibiotics by flock owners during the production period (75 percent) were fluoroquinolones, combination of trimethoprim-sulfonamides (TMP/SU) and tetracycline. Nearly 60 percent of the flocks which had used TMP/SU were positive for *Salmonella* spp. compared with 10 percent of the flocks which did not use this antibiotic. Increasing flock age was associated with a decreased chance of *Salmonella* spp. detection. In flocks which received infectious bronchitis vaccine, 36 percent were positive for *Salmonella* spp. whereas this was 15 percent for flocks which did not receive this vaccine (Ansari-Lari, 2014).

Arsenault (2007) also looked at risk factors for prevalence of *Salmonella* in commercial broiler flocks. This study indicated the value of biosecurity on the farm level, as odds of *Salmonella* colonization were 2.6 times greater for chicken flocks in which the grower failed to lock the chicken house regularly. Odds of colonization were 4.1 times higher for chicken flocks raised on farms with professional rodent control and 5.2 times higher for flocks with manure heap <200m from the poultry house, and also increased with the number of birds raised per year on the farm and with the age at slaughter (Arsenault, 2007). This corresponds with work showing some indication that

larger farms that hold larger flocks had higher prevalence rates for *Salmonella* (LeBouquin, 2010).

Salmonella *vaccination.* In recent years, there has been more focus on control of *Salmonella* in live birds at the farm level by way of vaccination. *Salmonella* vaccines have shown to be effective against select serotypes in broilers when breeders are vaccinated (Young 2007). Berghaus (2011) also studied the prevalence of progeny broilers from *Salmonella* vaccinated hens. Broiler flocks that were the progeny of vaccinated breeders had significantly lower *Salmonella* prevalence and loads than those from unvaccinated breeders. The difference in prevalence of *Salmonella* was observed to be 62 percent fewer positives in progeny from vaccinated breeder flocks (Berghaus, 2011).

Farm sampling and collection methods. With so many areas of the production and processing chain in which *Salmonella* is found, it is important to note the methods and variations of analysis used on isolating and recovering *Salmonella*. Kingston (1981) conducted an experiment pertaining to evaluate farm sampling/environmental sampling techniques. The sensitivity of both 5 g litter samples and drag swabs was compared in this study in breeder environments, and the methods were observed to be equally sensitive in 32 houses. Both identified the same sheds as being contaminated, and the identical serotypes were recovered. In broiler flocks, however, contamination of seven of thirteen sheds was detected with drag swabs, whereas only five were detected by litter culture. In a repeat experiment in broiler sheds, three sheds were detected as positive by the culture of litter, nine by drag-swab culture. When cost was factored in as well, it was

concluded that drag swabs were a reliably sensitive and cost effective method of detecting *Salmonella* (Kingston, 1981). In 1997, Byrd's work set out to compare the use of skim milk (wet) or no transport media (dry) drag swabs for detecting *Salmonella*. Positives were isolated in 47 percent of wet drag-swab samples, compared with 17 percent for dry drag-swab samples. It was concluded that while skim milk drag swabs are more labor intensive than dry drag swabs, wet swabs are more efficient for detecting *Salmonella* (Byrd, 1997).

Buhr et al (2007) found drag swabs that were stepped on by foot for picking up sample material had comparable *Salmonella* detection level to that for socks. Stepping on the material while in contact with the litter seems to detect a greater incidence of (Buhr, 2007).

***Plant sampling and collection methods*.** The currently accepted method of *Salmonella* sampling in USDA establishments for carcasses and parts is described in step by step format by FSIS (FSIS.usda.gov). This method consists of rinsing a carcass sample or parts sample in a bag of 400 ml buffered peptone water (BPW) for one minute, and then pouring 100mL of the BPW liquid back into a sterile sampling container. Galameu (2015) evaluated several methods of carcass rinse: split-carcass method (rinses of 2 halves of one carcass), repeat rinse method (rinse and rerinse of same carcass), and adjacent pair method (rinses of 2 adjacent carcasses) in the South East in operating commercial processing facilities. The purpose of the work was to determine which method resulted in the most consistency of paired broiler carcass rinses. The adjacent pair method showed moderate agreement over three trials. In one trial, the

repeat rinse method showed a significant difference in prevalence rates between repeated rinses. Even though the split carcass method showed moderate consistency in each of 2 trials, the disadvantages of the split carcass method were that it was more labor and time intensive and the product was damaged when compared to the other 2 methods. Overall prevalence estimates were fairly consistent between pairs by each method (Galameau, 2015). This is of note as the most commonly used methods for industry sampling involve whole bird carcass.

Salmonella load and infectious dose. The importance of testing and recovery of pathogen is motivated by the need to understand the levels of *Salmonella* population in poultry products, as this will be of the most immediate human concern in health control. The level at which *Salmonella* causes infection in humans is still somewhat misunderstood. According to Infectious Diseases by Sherwood Gorbach, In one review of 12 outbreaks of typhoid fever, the estimated number ingested ranged from 17 organisms to 100 billion organisms, and in more than 50 percent of the outbreaks, the estimated number was fewer than 1,000 organisms. In addition to the dose, the serotype is relevant. For example, *S. pullorum*, which is adapted to chickens, produces infection in humans rarely, and only when a large number of organisms (more than 1 billion) are ingested. In contrast, *S.* Typhimurium and *S.* Enteritidis may cause infection in humans with a relatively small number of organisms (Gorbach, 2003).

Crum Cianflone (2008) studied the severity of salmonellosis and found the percentage of infected humans after consumption of food are associated with the level of contamination along with risk factors. It is a dose-response relationship, and several

other factors are at play in levels of susceptible hosts. These include immunosuppression, antibiotic use, age, and gastric conditions (Crum Cianflone, 2008).

Salmonella invasion activity. The method by which *Salmonella* invades has been studied by James & Richardson (1981). Their work on invasion of human HeLa cells by *S.Typhimurium* consisted of three phases. Initially, the motility of the bacteria enabled HeLa cells to attach in a reversible manner (i.e. the bacteria could be removed readily by washing the HeLa cell monolayers with Hanks' Balanced Salt solution). In solutions of low ionic strength, irreversible attachment was prevented. This was likely due to the forces of the primary minimum generated between the HeLa cell and a bacterial adhesion which was capable only acting over short distances between the reversibly attached bacterium and the HeLa cell (i.e. probably less than 15 nm). HeLa cells only internalized bacteria which were irreversibly attached (Jones & Richardson, 1981)

Basics of *Salmonella*

Understanding the need for *Salmonella* control from both a human health and from a regulatory perspective is key in understanding it's role in poultry production. Perhaps even more important with *Salmonella* population control is to understand the organism itself. Its growth, virulence, and susceptibility to antimicrobials are all areas of importance in planning a safe food system with proper controls in place.

Serotypes. *Salmonella* is a Gram-negative rod-shaped bacterium in the Enterobacteriaceae family, but it is not included in the group of bacteria referred to as coliforms. *Salmonella* is one of the principal causes of foodborne illness worldwide and

is also an important pathogen of livestock, causing infections that can be transmitted from animals to humans (zoonotic infections).

Although a great many different strains of *Salmonella* have been identified, there are actually only two recognized species. These are *S. enterica* (which includes 6 subspecies) and *S. bongori*. *S. enterica* subspecies is of the most importance in illnesses of foodborne origin. There are over 2500 subtypes, or serotypes in which *Salmonellae* are classified. Most Salmonellae belong to the species enterica including nearly 1,500 contained in the subspecies enterica. Phage typing, a classification based on infection of bacterial cells by specific viruses (bacteriophages), is another method of determining each serotype. A particular phage type can be denoted using the term PT. For example, *S.* Enteritidis PT4 is an organism commonly associated with eggs and human illness.

Growth parameters. *Salmonella* grows best in the temperature range 7 – 48 ºC, though some slow growth has been reported at below 4^{o}C. Despite low growth, it's survivability in frozen and chilled foods is documented (Dominguez, 2009). Pasteurization is an accepted method of reduction, as D values are usually less than one minute at 70^{o}C. High water activity has been correlated with more heat resistance from few specific serotypes. Certain food attributes such as high fat and oil content have been shown to reduce heat treatment effectiveness (Ilhak, 2014).

pH. A few *Salmonella* serotypes can grow over a range of pH values from 3.7-9.5 under otherwise ideal conditions, but the optimum is 6.5 – 7.5. Although *Salmonella* cannot grow under very acid conditions, the cells are able to survive for some time in acid environments.

Water activity. Salmonellae are not able to grow well in dry environments and require water activity values of at least 0.94 to multiply in foods. The cells will lose viability at lower water activity values, but inactivation can be extremely slow in some products (measured in years), particularly those with very low moisture and high fat content, such as chocolate. *Salmonella* may also survive for some time on dry food production surfaces.

Atmosphere. All Salmonellae can grow with or without oxygen (facultative anaerobes) and in atmospheres containing high levels of carbon dioxide (possibly up to 80 percent in some conditions).

Chemicals. *Salmonella* is not especially resistant to sanitizers used in the food industry, but is able to form protective biofilms if cleaning is inadequate (Wang, 2013).

Environment. O'Connor (2015) found that *Salmonella* was present not only in soil samples of positive farms, but also in that of neighboring almond orchards that surround the production area across the central valley ranches where large numbers of California broilers are produced (R. O'Connor Foster Farms Turlock, CA, personal communication). Hofacre (2007) concluded that *Salmonella* spp is part of the normal intestinal flora of chickens. There is also interaction with other microorganisms that affect prevalence. Research has also indicated increased intestinal disease due to *E. tenella* can increase *Salmonella* levels, though more necrotic enteritis outbreak does not necessarily mean more *Salmonella*. When *E. tenella* is controlled through cocci health programs such as proper vaccination and house cleaning, *S.* Heidelberg colonization is significantly less (Hofacre, 2007).

Temperature and prevalence of pathogens. Conditions of the environment will have an effect on pathogen populations as well. Heat stress is a particular area of concern as Rychlick (2005) indicated during heat stress periods that *Salmonella* & *Campylobacter* populations can increase in environmental samples and in fecal sheds. Sohail (2015) also examined the effect of microbiome in the ceca and trachea in during heat stress and found Lactobacillus was the most abundant genus in trachea and caeca and was more abundant in caeca and trachea of Heat stress groups compared with the thermoneutral control group (Sohail, 2015). Lan (2004) found that in heat stressed broilers the major components of the cecal microbiota were clostridia and lactobacilli. The Clostridium subcluster XIVa was the most predominant group in chicken cecum. (Lan, 2004). In a study of broilers challenged with heat stress and *Salmonella* infection, higher *Salmonella* counts were observed in the spleen of heat stressed broilers. A higher incidence of enteritis was observed in *Salmonella* infected broilers and heat stressed broilers compared with control, indicating the role that stress plays in disruption of the intestinal barrier (Quinterio-Filho, 2012).

Antibiotic Alternatives

Feed additives and water additives are a very popular area of attention in the wake of the Food Safety Modernization Act. These additives in use and in review are most often products that were originally intended to convey an animal health effect in feeding or drinking applications. This is important as the commercial poultry industry continues to receive consumer pressure to remove the use of growth promoting antibiotics and antibiotics of human health importance from poultry production

programs. Probiotics, prebiotics, essential oils, organic acids, short chain fatty acid products, enzymes, phages, and fermentation products are some of the products that can convey in some manner a method of animal health AND food safety potentially. Dr. Randy Singer of University of Minnesota writes *"The health status of food animals that are destined to enter the human food supply chain may be an important, although often overlooked, factor in predicting the risk of human foodborne infections. The health status of food animals can potentially influence foodborne pathogen levels in three ways. First, diseased animals may shed higher levels of foodborne pathogens. Second, animals that require further handling in the processing plant to remove affected parts may lead to increased microbial contamination and cross-contamination. Finally, certain animal illnesses may lead to a higher probability of mistakes in the processing plant, such as gastrointestinal ruptures, which would lead to increased microbial contamination and cross-contamination. Consequently, interventions that reduce the incidence of food animal illnesses might also help reduce bacterial contamination on meat, thereby reducing human illness. Some of these interventions, however, might also present a risk to human health. For example, the use of antibiotics in food animals can reduce rates of animal illness but can also select for antibiotic-resistant bacteria which can threaten human treatment options."* (Singer, 2007). Non antibiotic additives are the subject of a great number of scientific studies today.

Butyrate fatty acid. Van Immerseel (2005) reviewed the potential for success with one such additive in the form of a coated butyric acid product, as butyric acid is known to have a destructive effect on various enteric pathogens by decreasing pH

level(Rasschaert, 2016). In Van Immerseel's work, the group of broilers receiving coated butyric acid had a significantly lower number of broilers shedding *Salmonella* bacteria, but cecal colonization at slaughter age was equal for both groups. In conclusion, butyric acid decreased cecal colonization shortly after infection, decreases fecal shedding, and as a consequence, decreases environmental contamination by *S.* Enteritidis-infected broilers. However, complete elimination can probably only be achieved with a combined approach using both hygienic measures and different protection measures, as the broilers still carried *S.* Enteritidis bacteria in the ceca at slaughter age, although at enrichment level.(Van Immerseel, 2005).

Chlorate. Totten & Farrar (2012) looked at a chlorate product for cecal reduction of *Salmonella*. For broilers fed experimental chlorate product, the concentration of *Salmonella* in the ceca was decreased by 0.61 log(10)cfu/g in the treated group compared to the control group.Intracellular enzymes in the respiratory process of *Salmonella* convert chlorate to chlorite, which builds up in the cell and causes cell death (Byrd, 2007). .

Organic acids. Organic acids as a preharvest intervention have been implemented across U.S. broiler farms to some degree of success for years. Andreopoulou (2014) looked at the acids propionic acid, formic acid, and citric acid for potential to affect the gut environment of host chickens. The work is summarized in stating diet supplementation of organic acids has trophic effects on the intestinal mucosa, modifying the morphologic characteristics of intestinal villi and crypts and maintaining epithelial integrity. Organic acids have anti-inflammatory and immunostimulating

properties, and diet acidification increases gastric proteolysis and the utilization of proteins and amino acids, affects pancreatic secretions and mineral absorption. (Andreopoulou, 2014).

Parker (2011) administered an organic acid blend or control diet only to broilers inoculated with a marked strain of S. *Heidelberg*, and measured prevalence in both inoculated and non inoculated birds housed together. A reduction in prevalence was observed in the birds that were not inoculated but fed the organic acid blend vs in birds that were not inoculated and fed control only. This indicated the ability of organic acids to reduce horizontal transmission, however no effect was seen on birds that were inoculated with *Salmonella* and given organic acid blend (Parker, 2011).

Probiotics. JE de Oliveira (2014) examined an area of earlier pathogen control in the production chain, and examined the effects of probiotic inoculation of chick embryos combined with and without the feed additive effect of probiotic. The feasibility of establishing probiotic bacteria in the intestine of broiler chickens by in ovo inoculation was investigated, followed by verifying possible subsequent protection against *S.* Enteriditis infection. In a first study, 7 commercially available probiotics were screened for compatibility with in ovo inoculation. Two of these probiotics, one being a *Enterococcus faecium* and the other a Bacillus subtilis, were selected for colonizing the chick gut without compromising hatchability. In a second study, these 2 products were administered in ovo and in the feed to chicks reared until 18 d in comparison with noninoculated chicks and with chicks fed an antibiotic. Results showed reduced performance of *S.*Enteritidis challenged chicks fed no additives compared with

challenged chicks fed antibiotic, but no significant differences in mortality was observed. Probiotics offered in ovo or through the diet could only partially recover performance compared with antibiotic-fed chicks. A significant reduction in the number of *S.* Enteritidis positive chicks was observed when chicks were in ovo inoculated with *E.* Faecium and continued receiving it in the diet (de Oliveira, 2014).

Bacteriophages. Bacteriophages have also been examined for their destructive effect on *Salmonella* and other gram negative bacteria. Lysogenic phages are capable of invasion and replication within host bacteriae. A study by Kim (2013), where in the result of a 14 d record after *S.* Enteritidis challenge of 160 birds from 4 previous treatments, indicated mortality was linearly decreased with increasing anti-SE bacteriophage level ($p<0.05$), and *S.* Enteritidis concentration in the cecum was decreased with increasing levels of anti-SE bacteriophage (Kim, 2013). Park & Kim (2014) also found that the administration of a bacillus subtilis strain reduced the intestinal *Salmonella* load both linearly and quadratic (Kim & Park, 2014).

FSIS preharvest interventions guidance. The FSIS released document *Controlling Salmonella and Campylobacter for poultry* summarizes the governmental view of pre-harvest pathogen control technology as of 2015: FSIS is not aware of a single pre-harvest intervention that eliminates *Salmonella* and *Campylobacter* as a pre-harvest hazard. Instead, FSIS recommends that as many interventions are employed as practical — a "multi-hurdle" approach. A multi-hurdle pathogen reduction approach means that multiple sequential pathogen interventions can have an additive effect to reduce pathogens. Implementing multiple interventions and controls beginning at pre-

harvest extends the multi-hurdle approach to *Salmonella* and *Campylobacter* prevention and control across each bird's life. Using interventions with differing modes of action can further improve the extent of pathogen reduction when using a multi-hurdle (FSIS controlling *Salmonella* and *Campylobacter*).

Overall, the FSIS view on several preharvest interventions is described as follows:

Vaccines. Only specific serotype *Salmonella* vaccines are currently available. Approved live-attenuated vaccines are available for use in breeder flocks and in young chickens and young turkeys, and are administered orally or by injection. Other vaccine types, such as inactivated vaccines, may require multiple doses or take longer to produce the immune benefits, limiting but not excluding their usefulness in chicks due to the short growout period. Special approvals from APHIS are required for long-term use of autogenous vaccines or for use with multiple flocks.

Competitive exclusion and probiotics. Some products can be used on the day of hatch to establish healthy gut flora in chicks. Other products can be added to water and feed for both breeders and young chickens and used to boost competition against pathogens throughout the bird's lifetime or when otherwise indicated (e.g., stress). Antimicrobial use should be limited to avoid killing the beneficial bacterial species.

Prebiotics. Prebiotics are nondigestible feed ingredients that promote the growth of beneficial microorganisms in the intestine. They can be added to feed for broilers and turkeys, and the most common supplements include yeast extracts such as beta-glucans and mannan oligosaccharides.

***Organic acids*.** Can be added to both feed and water for breeders and young chickens. Adding to water during feed withdrawal is particularly important. After feed is withdrawn, birds may be more likely to peck at litter and may ingest pathogens. Organic acids in the water will lower the pH in the crop and reduce pathogen colonization and growth.

With each of these broad categories being recognized as conferring some benefit on preharvest intervention for *Salmonella*, it is important to also review them objectively. A national survey of broiler production management personnel, company nutritionists, veterinarians, and food safety managers has revealed the following:

***Competitive exclusion and probiotics*.** The majority of these products must survive pelleting temperatures that can reach over 200 degrees F in commercial feed mills. It is difficult to ensure that the correct level of live microorganism can reach the bird after exposure to these extremes. It is also evident in commercial settings that when birds experience health challenges that affect the intestinal lining of the gut as is common in crowded housing conditions, live probiotic products are unable to perform with a lack of health intestinal binding site for them. The heavy and necessary use of sanitizing antimicrobials in cleaning houses between flocks will effectively reduce populations of preferred bacteria as well.

***Prebiotics*.** These products can include mannan oligosaccharaides such as yeast cell wall extract and beta glucans, but the field results of MOS products alone does not show any consistent effect on keeping *Salmonella* reduced in broiler flocks. In conjunction with other products, MOS may have a stronger *Salmonella* control effect.

***Organic acids*.** Mansoub (2011) showed that organic acids while weak in activity, can still provide some early chick health. They work to inhibit the growth of certain pathogens while allowing the growth of other beneficial microflora (Mansoub, 2011). Russell & Diez Gonzalez (1998) also added organic acids to growout early and late, and found that this inhibited *Salmonella* and *Campylobacter* growth and horizontal contamination (Russell & Diez Gonzalez, 1998). However, Luangtongkum (2006) found that there is some risk of resistance. They found 46 percent of recovered *Campylobacter* isolates from farms where organic acids were used were resistant to antimicrobials vs 2 percent resistance on farms where organic production practices prohibited the use of organic acids as antimicrobials (Luangtongkum, 2006). As *Campylobacter* is responsible for more human illness than *Salmonella*. this in effect could be causing a larger problem than it solved to risk administering organic acids.

XPC biological effects. When investigating the preferred method of preharvest *Salmonella* control by ingestion product for birds, the level of documented repeated results is paramount for understanding a product's ability. Manufactured by Diamond V Mills Inc of Cedar Rapids, IA, the fermentation product XPC has 31 scientific studies to date that show reduction in *Salmonella* prevalence and enumeration as of February 2016. Produced via 2 stage anaerobic fermentation, the product contains over 150 different metabolites and nutritional compounds such as peptides, polyphenols, nucleotides, yeast factors, vitamins, antioxidants, organic acids, and minerals. In combination, these nutritional metabolites have been shown to improve gut morphology as Zhou showed in the significant increase in microvilli length and crypt depth ratio in tilapia, as well as

change microbial populations of the gut as Roto and Ricke showed in cecal maturity via microbiome analysis (Zhou, 2009). The results from the current study indicate the effect of maturity on the cecal microbiome in the reduction of ST and S. Cerevisiae fermentation product may accelerate the time it takes to reach mature levels. The XPC F control suggests S. Cerevisiae fermentation product works in concert with the cecal microbiome in the reduction of ST (Roto, 2016).

XPC immune response & viral challenge. The XPC effects in poultry are well documented in the gut as well as the immune system. In 2007 Al-Homidan and Fahmy measured growth performance, chemical analysis, immunity, ileum villus heights and bacterial counts in broilers fed XPC and found that Intestinal villi volume density at day 42 of age, carcass protein as a percentage of dry matter, and antibody titers to Newcastle Disease were significantly improved. In 2008, Gao published work showing birds fed S. Cerevisiae fermentation product even at a reduced rate (1.25lbs/T) below the current company recommended 2.5lbs/T exhibited significantly higher antibody titers to Newcastle Disease Virus (NCDV), as well as significantly increased secretory Immunoglobulin-A (sIgA) antibody in the duodenum. In an indication of immune balance effect, Gao also showed that XPC fed bird had a marked increase in serum immunoglobulin-G (IgG) (Gao, 2008). Fathi et all in 2011 showed significantly increased antibody titers to NCDV as well (Fathi, 2011). In following this path of consistent effect on response to viral challenge, Park undertook perhaps the most in depth review of XPC's effects on the immune system to date. They vaccinated cobb broilers via the nasal route with B1 and Lasota strains on day 1 & 21 respectively.

Antibody titer response was significantly higher and earlier in XPC fed birds, and it was learned that T-cell marker CD3 which makes killer Tcells, T helper cell CD4 which makes B-Cell antibodies, and Cytotoxic T-cell CD8 which also helps make killer T cells were significantly increased. Spleen and thymus organ weights and sizes were also significantly increased. In this same study, an 84 gene array was monitored from the spleen and PCR assay identified a 2 fold change in 24 immunomodulatory genes that were induced, while 7 were repressed (Park, 2014).

XPC IBV challenge. Toro in 2014 found that when challenged with infectious bronchitis virus, XPC fed birds exhibited a significantly reduced level of lymphocyte infiltration in the trachea, increased IgA and lymphocytes in the spleen, along with CD4 & CD8 in the spleen. Clinical signs of IBV were significantly lower in XPC birds 5 days post challenge.

There has been shown to be a repeated benefit in the morphology of the intestine and health of the gut lining during pathogenic challenge. McIntyre (2013) showed a reduction in intestinal lesion scores from *E. Maxima, E. Tenella, and E. Acervulina*, and also showed a significant reduction in mortality and intestinal lesions during Necrotic Enteritis challenge of XPC fed broilers (McIntyre, 2013). Lensing showed in improvement in intestinal lesions from E. maxima in 2008, and Nsereko showed that XPC reduced the proportion of total bacterial DNA accounted for by C. septicum to less than a tenth of that measured in the Control treatment. This analysis also showed that XPC Reduced pH & Increased acetate, propionate and total volatile fatty acids (VFA) concentrations by 15, 13 and 15 percent, respectively.

Displaying the effect of reduction on enteric pathogens, Zhou & Zhen in 2005 showed that S. Cerevisiae fermentation product fed broilers had reduced E.Coli intestinal populations of a full Log10 lower than those fed trisulmix antibiotic growth promoter (Zhou & Zhen, 2005).

XPC load reduction in live birds. A discovery was brought to the attention of company veterinarian from the Midwest in 2011, and it was learned that in 3 field trials of egg type broiler breeder houses sampled monthly by NPIP protocol, 0 positives for *Salmonella* were found in XPC houses vs continuous Group B & C *Salmonella* in Control houses. In 2012 Nsereko utilized the IAMM model to show a 91 percent reduction in CFU/g of *S.Enteritidis* ex vivo in the digestate of birds fed XPC or control. In 2013 Nsereko showed in the same IAMM model a 46 percent reduction in *S.Heidelberg* CFU/g of laying hen digestate. 2012 Nsereko experimentation with live bird challenge of *S.Typhimurium* showed a 61 percent reduction in *Salmonella* population recovered from the cecum. The Iowa State Center for Veterinary Medicine challenged birds with *S.Enteritidis* and showed a 37 percent reduction in *S. Enteritidis* prevalence recovered from the cecum, yet also a 90 percent reduction in the CFU/g counts of the same *S. Enteritidis*. Hofacre showed a 54 percent reduction in positives in a large commercial live bird pen challenge with *S. Heidelberg* by measuring cecal colonization (Hofacre, 2015). Colorado Quality Research's Steve Davis showed 16 percent reduction in prevalence of *S. Typhimurium* in fecal colonization, but an 87 percent reduction in CFU/g populations of those same positives. This translated to a 79 percent reduction in CFU/g in cecal colonization.

Salmonella virulence; genetic effects of XPC. In 2015 Iowa State University Professor of Pharmacology Steve Carlson DVM PhD conducted a series of 3 repeated studies on intestinal colonization, fecal shedding, invasiveness, and antibiotic resistance of *S.Typhimurium* in XPC and control fed broilers. At the 42 day measurement point for fecal shedding of *Salmonella* in all 3 studies, XPC reduced the CFU/g of *Salmonella* shed by over 80 percent. Intestinal colonization was reduced by 70-75 percent as well. In this study, unlike other *Salmonella* isolate analysis studies, Carlson included an assay for invasiveness of the isolates. In this process a tissue culture assay was performed using mammalian host cells and *Salmonella* isolates from control and XPC were given an hour to invade the cells. XPC reduced the invasive ability of the *Salmonella* recovered by over 80 percent. It has been found that the level of invasiveness in *Salmonella* directly corresponds with the expression of the genetic mechanism Hyper invasive locus-A (hila) (Lee et al, 1992), and in this study Carlson also measured the expression of hilA in recovered isolates. As hilA is the main transcriptional regulator of the SPI-1 island, Transcriptional regulators are responsible for regulating the conversion of DNA to RNA (transcription), thereby controlling gene activity. hilA activates the expression of invasion genes by a direct action at their promoters (Betts & Finlay,1992). *Salmonella* recovered was also exposed to 3 antibiotics to measure resistance levels. Ceftiofur, Florfenicol, and Enrofloxacin were all used in this study and it was shown that *Salmonella* from XPC fed birds was restored to susceptibility to antibiotics. The decrease in resistance was almost 50 percent between control to XPC (Feye, 2015).

Such a consistent history of reduction on cecal *Salmonella* by XPC coupled with a strong relationship between pre harvest *Salmonella* populations and RTC meat levels led to a study being undertaken in conjunction with USDA ARS in Athens GA. This challenge model studied carcass rinses after live bird *Salmonella* challenge and also ground meat from breast of the carcasses in the study. It was found that despite efforts to keep control and XPC carcasses both 100 percent positive, XPC reduced the prevalence of the whole bird carcass rinse by 30 percent. In ground meat comminuted meat sample testing, the XPC treatment reduced *Salmonella* prevalence by over 70 percent.

Further study of comminuted meat samples is suggested, and the need for similar testing of parts is needed. There appears to be a consistent effect in place for XPC to reduce cecal prevalence, load CFU/g, fecal shedding, and even virulence/antibiotic resistance of *Salmonella*. However this effect needs to be traced further into parts.

Heat Stress and Broiler Production

The majority of the U.S. poultry industry's broiler production is centered in the Southern geography of the United States, with the top 5 broiler producing states in order being Georgia, Arkansas, Alabama, North Carolina, and Mississippi (USDA ERS). These states regularly experience temperature ranges in the spring and summer months of 60F-100F+ (Nat.Weather Service). Despite state of the art technology in the form of climate controlled computers and high speed tunnel ventilation being available, only about 75 percent of housing is estimated to have this installed (McDonald USDA ERS). With this percentage of production situated in an area of such historically high risk to

high temperatures, heat stress in broilers is an area of definite interest to production companies.

Heat stress exposure and rapid growth. Morita's (2016) work showed an interesting way of looking at heat stress in predicting its effects based on egg incubation temperatures. They found that exposure to high temperature during late embryonic development has long-lasting effects on the thermoregulatory system of broiler chickens by affecting the heat tolerance of these chickens. Moreover, the preferred ambient temperature of the chickens from heat-treated eggs correspond to those recommended for the strain under study, whereas for the cold-treated and control-chickens it was 1°C below, indicating that incubation temperature might have consequences on the ambient temperature chickens require during the rearing phase (Morita, 2016). More work is needed but this could certainly serve as a base for understanding how to better help broiler flocks cope with expected high summer temperatures.

Some work has been done to show that the resistance to heat stress of strains selected for rapid growth is significantly lower than that of slow-growing strains. Further, the continuous selection for fast growth seems to be associated with increased susceptibility of broiler chicken to heat stress (Berrong and Washburn, 1998; Tan et al., 2010; Soleimani et al., 2011).

Genetic factors for predisposition to stress. Today's chickens seem to be particularly susceptible to high environmental temperatures and suffer from multiple patho-physiological alterations, such as immune dysregulation, gut barrier dysfunction and cellular oxidative stress after heat exposure, resulting in decreased productivity and

increased susceptibility to infectious diseases and higher mortality (Syafwan 2011, Varasteh, 2015).

There are concerns with physiological effects of heat stress, as it can damage to the performance and the yield of parts of chickens. Geraert found endocrinological changes could stimulate lipid accumulation through increased de novo lipogenesis, reduced lipolysis and enhanced amino acid catabolism under chronic heat exposure (Geraert, 1996). Willemsen showed that heat stress can induce mechanisms similar to those of oxidative stress such as increased lipid peroxidation (Willemsen, 2011).

At the cellular level, it has ben shown to reduce uncoupling proteins, which are present in mitochondria and relevant to heat production (Ledesma, 2000). Abe (2000) showed that this uncoupling mechanism in ATP assists in reducing production of reactive oxygen species (Abe, 2000). It can be inferred that reduction in uncoupling proteins could lead to oxidative stress (Del Vesco, 2015).

Carcass quality. As a measure of the impact on carcass quality, Zeferino studied heat stressed broilers and found heat stress reduced slaughter and carcass weights, average daily gain and feed intake, and increased feed conversion. Additionally, these birds showed increased carcass and abdominal fat percentages, but reduced breast, liver and heart percentages and increased meat pH and negatively affected meat color and cooking loss (Zeferino, 1998). Pale, soft exudative-like changes in meat quality have also been observed in broilers exposed to acute or chronic heat stress pre-slaughter (Northcutt et al., 1994; Sandercock et al., 2001; Lu et al., 2007). Heat-stressed animals often have a blunted lipolytic response to catabolic signals. Either directly because of or

in coordination with this, animals experiencing environmental hyperthermia exhibit a shift toward carbohydrate use (Rhoads, 2013).

Immunosuppression and heat stress. While it is noted that production efficiency will often decrease during a heat stress event, the reasoning why is important. Heat stress has been shown to have some immunocompromising effects as noted in the study on splenic germinal centre formation by Atilio (2016). The indications were that Heat stress decreased the duodenal concentrations of sIgA, which was accompanied by a reduction in germinal centre (GC) number in the duodenal lamina propria; a trend to similar findings of sIgA concentrations was observed in the chickens' jejunum. Changes in spleen and Bursa of Fabricius relative weights as well as in spleen morphometry were also noted in heat stressed animals, infected or not. Together, these data suggest that heat stress change GCs formation in chickens, which that may lead to failures in vaccination protocols as well as in the poultries' host resistance to infectious diseases during periods of exposure to heat stress (Atilio, 2016).

Hosseini-Vashan (2015) found heat stress did not change the relative weights of the lymphoid organs but reduced the total and IgG titers for secondary antibody response to sheep red blood cells and titer against Newcastle disease virus and increased the heterophil/lymphocyte ratio (Hosseini-Vashan, 2015). They also measured tibia ash content on heat stressed broilers and found that Ca, P, and ash were decreased in heat stressed birds.

Calefi (2016) analyzed the effects of heat stress and/or Clostridium perfringens (CP) infection on behavior, intestinal morphology, brain activity, and corticosterone

serum levels in chickens. Their data showed that heat stress and C. perfringens infection produced significant differential responses in the chickens' behavior and in c-fos expression in the paraventricular nucleus of the hypothalamus (PVN), nucleus taenia of the amygdala (Tn), medial preoptic area (POM), and globus pallidus (GP) of the chickens. Heat stress ameliorated some of the intestinal lesions and the neuroendocrine changes induced by C. perfringens in the birds. Results suggest the existence of clear relationships between the degree of intestinal lesions, the chickens' behavioral outcomes, brain activity, and serum levels of corticosterone (Calefi, 2016).

Heterophil: lymphocyte ratios and stress. Heat stress can impact the immune system of broiler flocks not just in production settings, but also in the transport process as shown in Mitchell's (1998) work on transit. It was found that inadequate ventilation results in heterogeneous distributions of temperature and humidity and, thus, thermal loads within the transport vehicle, and, therefore, the existence of a "thermal core" in which the risk of heat stress is increased (Mitchell, 1998). Mitchell (1992) also utilized blood and serum analysis as indications of physiological stress and found that Heterophil:lymphocyte ratios and plasma creatine kinase activities increased and eosinophil counts were decreased during the journey in birds transported in both July and October when the curtain sides of the vehicles were open or closed respectively (Mitchell, 1992).

The measurements of heterophil:lymphocyte ratios (H/L), tonic immobility(TI), basophils, eosinophils have been used widely as indicators of stress levels, and Zulkifili (1999) utilized H/L ratios and tonic immobility both to measure the effects of an

ascorbic acid on stress indicators such as these. It was recorded that the ascorbic acid treatment had reduced underlying fearfulness, as measured by TI reaction (Zulkifili, 1999). These measurements as stress indicators trace back to Davison and Rowell (1983) who described how dietary cortisone could induce lymphocytopenia and granulocytosis and so affect the granulocyte:lymphocyte ratio. They suggested the granulocyte:lymphocyte ratio might provide a convenient measure of adrenal-corticoid hyperactivity (Davison & Rowell, 1983). Gross (1989) indicated that social stress, chilling, and injected Escherichia coli affect the H/L ratio. The H/L ratios of Leghorns showed a marked increase with the addition of corticosterone to the diet (Gross, 1983). Building on this work, significant differences in H/L ratios have also been observed in hens housed in enriched colony cages, aviaries, and conventional cages (Cotter, 2014). Gross (1983) also measured H/L ratio as a response to corticosterone in feed. Lymphocytes in chicken blood samples decreased and the number of heterophils increased in response to stressors and to increasing levels of corticosterone in the chicken feed in this experiment (Gross, 1983).

Density and stress effects on performance. Stocking density is yet another source of potential stress in production poultry, as Purron (1995) and Feddes (2002) observed effects on production parameters associated with stocking densities, and Murphy & Preston (1988) have recorded various behavioral impacts related to stocking density levels. However it was the work of Proudfoot (1979) and Greene (1985) that showed these levels can affect immunity resulting in adverse physiological conditions affecting health (Purron, 1995, Feddes, 2002, Murphy & Preston 1988, Proudfoot, 1979,

Greene, 1985). However, Thaxton & Dozier (2006) did not find that stocking density caused any physiological adaptive changes indicative of stress (Thaxton & Dozier, 2006). Najafi (2015) found both temperature and density had significant effect on alpha1 acid glycoprotein (AGP) and HSP 70. The conclusion was drawn that irrespective of temperature, high stocking density was physiologically stressful to broiler chickens, as indicated by elevated corticosterone, AGP, ceruloplasmin, ovotransferrin, and HSP 70, but not detrimental to growth performance and survivability (Najafi, 2015). Cenzig (2015) studied stocking density as well and did not find serum malondialdehyde, corticosterone, nitric oxide, and plasma heterophil:lymphocyte ratio were affected by stocking density However, feed intake and weight gain were significantly low and feed conversion ratio was poor in broilers at high stocking density (Cenzig, 2015).

Physical asymmetry measures. A measure of stress gaining attention in recent years is that of physical asymmetry, or morphological asymmetry. As challenges can affect growth, birds can over time begin to display this physical response to the stress they encounter. As previously stated, tibia ash, Calcium, and phosphorous have all been shown to be decreased during heat stress conditions (Hosseini-Vashan, 2015). Deviations from perfect symmetry represent asymmetry and may be estimated by the frequency distribution of left minus right (L-R) for a given bilateral trait. There are 3 forms of bilateral asymmetry; fluctuating asymmetry (FA), directional asymmetry (DA) and antisymmetry (AS) (Palmer and Strobeck, 1992). The definition of fluctuating asymmetry is random deviations from perfect growth symmetry that is generally expected in certain body parts when morphological development is successfully

controlled, and it is the result of both genetic factors and environmental conditions. Prieto (2010) found allicin significantly increased fluctuating asymmetry of wing length and tonic immobility duration. The addition of lactic acid or corticosterone resulted in greater fluctuating asymmetry of wing length of heat-stressed chicks, further evidencing the effects of stress hormones on physiological development (Prieto, 2010). Yang (1997) found that degrees of physiological asymmetrical displays can indicate the impact of environmental or genetic stress conditions on individuals, and the ability of those individuals to combat them (Yang, 1997). While Dawkins (2004) and Broom (2006) have served as a popular reference for overall animal physical response to genetic and environmental stress, Moller (1999) was able to show effects both from a genetic stress and an environmental stress (Dawkins, 2004, Broom, 2006). It was found that relative asymmetry was higher in faster growing broiler breeds than slow, and that tarsometatarsus assyemtry was higher for broilers raised in constant lighting program than those with light-dark photoperiods (Moller, 1999).

The effect of most importance on asymmetry from stress is in the leg health and walking ability of commercial birds. Kestin (1999) showed that leg disorders cause changes in gait of birds, and unnatural biomechanical forces on joints resulting leg disorders (Kestin, 1999). Tibial dischondryplasia can lead to femoral head necrosis, both of which are all too common in the commercial poultry industry with an estimated cost between 80 to 120 million annually in losses and condemns (Sullivan UNebraska). According to Riddell (1991), if broiler chickens are kept to roaster weights, lesions due to dyschondroplasia may be much more severe. A high association between leg

deformities and tibial dyschondroplasia has been described in turkeys (Riddell, 1991). Naas (2008) tells us reduced walking or standing ability often leads to breast blisters and hock burns as the bird has to spend a long time crouching on poor quality litter. The welfare concerns from lameness causing behavioral restriction and pain has been reported by Reiter & Kutriz (2001), and Weary et al. (2006). While it has been seen that physical properties of collagen are altered during growth, leading to weak legs and gait problems, with a consequent reduction in feed intake and productivity (Onyango et al., 2003), leg lesions are also a culprit in the causation of limited mobility and the resulting lack of feed intake. Baracho (2011) found there was a positive correlation ($p < 0.05$) between back toe and outer toe asymmetry with the presence of leg lesions (Baracho, 2011).

Corticosterone and stress. In much of the scientific study of poultry welfare and stress, corticosterone is the historical hormone of focus for measurement and indications of stress. This hormone has been added via injection to treatment birds and the results showed that chronic CORT administration resulted in enhanced proteolysis and gluconeogenesis (Lin, 2004). Post (2003) showed that to avoid treatment of animals with physical or physiological stress, addition of the stress-related hormone corticosterone to the drinking water, may serve as a practical alternative to reproducibly investigate hormone-related stress in broiler chickens (Post, 2003). It was observed in this model that uptake of endogenous corticosterone reduced body and spleen growth, increased heterophil counts, and decreased formation of antibodies against sheep red blood cells. Furthermore, corticosterone decreased adrenal gland responsiveness, measured by

corticosterone production, after a challenge with adrenocorticotropic hormone (ACTH) (2003). Rimoldi (2015) also found corticosterone levels increased significantly in the blood of heat stressed broilers, due to the activation of the hypothalamic-pituitary-adrenocortical axis (Rimoldi, 2015). The importance of this axis in stress is evident, as it leads to a rapid release of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) from the cells located in the hypothalamus and pituitary, respectively. ACTH stimulates the synthesis and release of steroids from the adrenal cortex by promoting the uptake of cholesterol and its enzymatic conversion to the glucocorticoid hormone corticosterone (Jones et al., 1988; Fraisse and Cockrem, 2006; Rimoldi, 2015). If this occurs and too much corticosterone is present, it can retard the longitudinal growth of bones by depressing the proliferation and differentiation of chondrocytes in broilers. According to Zhang's (2012) work it depressed the longitudinal growth of the long bones by inhibiting the proliferation and differentiation of chondrocytes in growth plate in the birds (Zhang, 2012). Corticosterone is one of the stress indicator glucocorticoids that has shown to rise in many occasions of stress. Poor feed access or limitation for any number of reasons will raise levels of this hormone (Hangalapura, 2005), as will temperature fluctuations (Mashaly, 2004), social structure pressures (Cheng, 2004), and biological conditions (Shini, 2009; Ghareeb, 2014).

Corticosterone and WBC populations. Post also evaluated automated analysis for measuring blood cell parameters in relation to corticosterone in a recently introduced corticosterone model (2003) because in reviewing different methods of measurement, discrepancies between the methods were encountered especially for monocytes,

eosinophils, and basophils (Sachse et al., 1998; Grimaldi and Scopacasa, 2000). It is important to have the most reliable method of measurement, as evaluating white blood cell counts (WBC) is of great interest for welfare.

Changes in blood leukocyte populations have been noted in bacterial, viral and parasitic infections (Siegel et al., 1987; Latimer et al., 1988; Branton et al., 1997). Interestingly, changes in H/L are related to different forms of stress in avian species (Maxwell, 1993). Maxwell (1992) also delved deeper in the structure itself of certain blood cells (BCs) and their structure, noting Granule counts in basophils after heat treatment showed a significant reduction in their total numbers compared with pre-heat treatment. This depletion of granules corresponded with evidence of degranulation seen at the ultrastructural level. (Maxwell, 1992). In the study after heat stress, there was a significant increase in heterophil lobulation. In several birds there was also evidence of cytoplasmic fragmentation in the form of portions of cells containing granules but no nuclei, lying free in the circulation. Damaged mitochondria, not seen before heat treatment, were frequently observed in the cells. Monocytes also showed significant increases in cytoplasmic lipid droplets after heat stress compared with pre-heat treatment, suggesting the onset of possibly early fatty degeneration (Maxwell, 1992). This is further evidence of the connection between heat stress and immunoresponse. The dimensional and ultrastructural changes identified in the cells after heat stress question their effective functional ability in raising an immune response under such circumstances (Maxwell, 1992).

Heat shock protein. While Heat stress has been discussed as a cause of elevated corticosterone & changed H/L ratios, one of the most obvious changes in physiology during heat stress is the increase in heat shock protein (HSP70). According to Kregel (2002), HSPs are present in both prokaryotic and eukaryotic cells, and their high level of conservation suggests that they play an important role in fundamental cell processes. HSPs were initially discovered in Drosophila melanogaster larvae that were exposed to sudden prolonged heat exposure. They are present in the cytosol, mitochondria, endoplasmic reticulum, and nucleus, although these locations vary depending on the particular protein. (Kregel, 2002)

One relevant feature of HSPs is that overexpression of one or more HSP genes confers protection against subsequent stress (McCormick et al. 2003a; Zhang et al. 2007; Luh et al. 2007).There appears to be a high degree of conservation of these proteins across species, and that coupled with their importance in cell survival in various conditions, suggests that these HSPs are critical for both normal cellular function and survival after a stress. HSPs play an important role in the protection and repair of cells and tissues. Therefore, one of the primary means to gain insight into HSP70 function in both in vitro and in vivo systems has been to assess their cellular responses after a stress-related induction (Kregel, 2002).

Live production interventions for heat stress. In an effort to reduce measurable effects of heat stress in the blood, Ghazi (2015) studied inclusion of essential oils and other compounds and found supplemental oregano essential oil and vitamin C in a combined form decreased the serum concentration of corticosterone, triglycerides,

glucose, and MDA compared with other groups. An increase in the serum concentrations of vitamin C were seen in broiler chicks supplemented with vitamin C (Ghazi, 2015).

Varasteh's (2015) work displayed differences in heat stress response at different points of the small intestine itself, yet found that addition of galacto-oligosaccharides improved the heat stress related changes of the jujenum (Varasteh, 2015). Zhu (2015) experimented with feeding a particular strain of lactobacillus and found egg production, average egg weight, average daily feed intake, feed conversion ratio and percentage of speckled egg, soft shell egg and misshaped egg were significantly improved by the increasing supplementation of the strain in the diet. Shape index, eggshell thickness, strength and weight were increased linearly with increasing lactobacillus supplementation. The level of calcium, phosphorus, glucose, total protein and albumin in serum of the hens fed the strain supplemented diet was significantly higher than that of the hens fed the basal diet, whereas cholesterol level was decreased (Zhu, 2015). Khosravinia experimented with S. khuzistanica essential oils (SkEO) as it was learned they exhibited antioxidant (Abdollahi et al. 2003; Radonic and Milos 2003), antiviral (Yamasaki et al. 1998), antibacterial (Azaz et al, 2002), and antifungal (Skocibusic and Bezic 2004) effects. In Khosravinia's study it was noted that that administration of SkEO at 400 mg/L through drinking water to heat-stressed broiler chickens improves economic efficiency of broiler flocks possibly through promoting the digestion process, creating minute improvement in FCR and lowered mortality rate (Khosravinia, 2015).

Organic acids, mineral immune response and heat stress. In reviewing the effects of a combination of ascorbic acid and Zinc in broiler diets during heat stress,

Chand found that feed intake, body weight and feed conversion ratio, and weight of thymus, spleen, and bursa of Fabricius improved significantly in that combination compared to the other treatments. Antibody titer against Newcastle disease, infectious bursal disease (IBD), and infectious bronchitis (IB) increased significantly in that group. It was also noted that total leucocytes count, lymphocytes, and monocytes increased in all groups treated with zinc, ascorbic acid, or both compare to control (Chand, 2014). Selenium and vitamin E levels have also been researched in heat stressed broilers. Habibian (2014) measured the liver and lymphoid organ weights as well as IgM and IgG, antibody titers for primary and secondary antibody responses to sheep red blood cell & found they were reduced significantly under heat stress. In the experiment, heat stress also resulted in a significant increase in H/L ratio. Dietary vitamin E resulted in improvement of primary and secondary antibody responses both in thermoneutral and heat stress broilers ($P<0.05$). The heat stress birds also showed an improved antibody titer in secondary response with high concentration of Selenium. Vitamin E was shown to reduce H/L ratios in both thermoneutral and heat stresse birds (Habibian, 2014).

Bartlett in 2003 also looked at Zn in broilers during heat stress and saw that Lymphoid organ weights, primary and secondary antibody responses, incidences of macrophages in abdominal exudate cells, phagocytic ability of macrophages, and plasma zinc concentration were all significantly reduced by heat stress (Bartlett, 2003).

Yeast cell wall as a heat stress intervention. With an interest in immune benefits to alleviate heat stress in mind, Sohail (2012) looked at the effect of MOS and saw that MOS only partially alleviated heat stress effects. Among supplemented groups, the heat

stress-MOS had higher body weight gain and lower FCR compared with the heat stress-control group. On d 21 and 42, the heat stress-control group had higher serum corticosterone concentrations compared with the control and supplemented groups. The control group had higher villus height, width, surface area, and crypt depth compared with the heat stress-control group (Sohail, 2012). MOS was also looked at with probiotic combinations in heat stressed broiler intestines & intestinal immunity. In this trial by Ashraf heat stress reduced villus height, crypt depth and surface area in duodenum and ileum, and increased crypt depth in ileum. Villus width decreased in duodenum and jejunum compared to control. However, MOS and probiotic combination treatments reversed all these changes in duodenum, while only increased villus height and surface area in ileum. In jejunum, the villus height and surface area increased with probiotic combination, and crypt depth increased with MOS treatment (Ashraf, 2013). Sohail in 2010 also showed that MOS and probiotic together or in combination reduced serum cortisol and cholesterol concentrations, and increased thyroxine concentrations vs a control heat stressed group (Sohail, 2010).

XPC fermentation and immune response. The effects of yeast culture fermentation are of great interest as they are known to contain some elements of many other previously dietary treatment products. XPC's production process begins with both yeast and lactobacillus in many occasions (P. Manternach Diamond V Mills Inc, Cedar Rapids, IA personal communication) and by the end of the production process it contains a significant number of compounds of which MOS, b-Glucans, antioxidants, peptides, and polyphenols are known to be included. As far back as 1993, Teeter looked at yeast

culture (XP-precursor to XPC) and found that in litter treated groups, XP was able to increase livability significantly (Teeter, 1993).

Gao (2009) noted the measured effects on the broiler immune system with XP, which was shown to increase secretory IgA content, IgA plasmacyte count and intraepithelial lymphocyte count in the duodenum and cecal tonsil, as well as serum IgM and lysozyme content (Gao, 2009). Al-Mansour (2011) showed that yeast culture treatment resulted in lower white blood cell counts, significant decrease in thrombocyte counts, decreased H/L ratio (Al-Mansour, 2011). Field studies with an experimental version of this yeast culture in a liquid phase fermentation have been put into commercial experimental use in recent years. It was observed in 2015 that addition to the diet during a temporary heat stress significantly improved egg production and reduced mortality in caged layer flocks (W. Michael, Diamond V Mills Inc, Cedar Rapids, IA, personal communication 2015). This effect was repeated in 2015 in the Midwest, and then the product was added to commercial turkey diets during heat stress. Mortality of toms was reduced in the yeast culture treated house by 40 percent (from 6 percent to 2.5 percent) giving further credence to the effect of yeast culture on stress amelioration (D. Kenyon, Diamond V Mills Inc, Cedar Rapdis, IA, personal communication 2015).

Heat stress studies of XPC in brown strain commercial layers have also been conducted with a design in which the stressful temperature was obtained by setting the minimum temperature in the heated pens at 23.9°C (75°F) at the start of the trial and raising the temperature by 2.7°C (4.9°F) per week until 29.4°C (85°F) was reached. Each treatment was replicated four times for a total of 16 pens with 20 birds per pen. In

this experiment, it was seen that for the period from June to October, XPC fed birds maintained anywhere from 2-5 percent higher egg production levels (NCSU, 2010).

XPC and stress response. With an understanding of the potential for strengthened immune function to be able to alleviate certain symptoms of physiological, environmental, and genetic stresses, further study on the impact of fermentation product XPC was needed on stress measurements. As a corresponding study to the in processing measurements with and without heat stress, an experiment was conducted to evaluate XPC's effect on stress measures such as HSP70, corticosterone, heterophil:lymphocyte ratios, eosoniphil counts, basophil counts, and physical asssymetry measures.

CHAPTER III

EFFECT OF FEEDING *SACCHAROMYCES CEREVISIAE* FERMENTATION PRODUCT ON *SALMONELLA* ENUMERATION AND PREVALENCE IN CECA, BREAST AND GROUND BREAST MEAT IN HEAT STRESSED AND NON-HEAT STRESSED BROILERS

Introduction

Passage of the Food Safety Modernization Act in 2011 increased the rigorousness with which RTC chicken is monitored by FSIS, specifically chicken parts. After collecting baseline samples from processing plants and retail outlets, a new standard was enacted and written into the federal register (Federal register, 2016). As the CDC reported in 2015, salmonellosis cases had remained unchanged (Pulsenet.com, 2015). This illustrated the need for increased focus on improving the safety of the nation's food supply, and resulted in FDA increasing its involvement in RTC chicken pathogen testing.

FSIS found in their baseline sampling survey that 28 percent of chicken parts were positive for *Salmonella,* and 49 percent of comminuted chicken was positive for *Salmonella*. The maximum acceptable standards for FSIS sampling under the new FSMA rule for parts is 15.4 percent *Salmonella* positive, and the maximum acceptable standard for comminuted chicken is 25 percent *Salmonella* positive. Based on these figures only one in three U.S. broiler processing plants is expected to meet the new standards (National Chicken Council-FSIS). These events have led to a demand for data

and peer reviewed research on interventions used to control pathogens both at the plant and in preharvest.

According to FSIS guidance for controlling *Salmonella* and *Campylobacter* 2008: The feathers, skin, crop, and cloaca of birds brought to slaughter are often highly contaminated with *Salmonella* (Kotula and Pandya, 1995) and *Campylobacter* (Berrang et al, 2000). Cross-contamination of both birds and cages is frequently made worse when the birds are moved to the plants. There can be a 20-40 percent increase in *Salmonella* both inside and outside the birds during movement (Berrang et al., 2003; Slader et al., 2002; Bailey et al., 2001; Corry et al., 2002; Humphrey and Allen, 2002). Moving the birds causes them to pass more fecal material (FSIS). With this being the case, it is imperative to find methods of lowering *Salmonella* in preharvest and studying these effects in RTC product to evaluate the impact.

The FAO CCFH working group report (2007) discussed vaccines for specific serotypes (for example *S*. Enteritidis and *S*. Typhimurium) that are now available. According to the report, the use of vaccination depends on the epidemiological situation. Vaccines have very little chance of eradicating *Salmonella* from an infected flock, but may decrease the infectious burden. The positive effects of vaccines against *S*. Enteritidis and *S*. Typhimurium have been demonstrated by, Hofacre (2002), Feberwee et al. (2000) and Clifton-Hadley et al. (2002).

A variety of combined interventions are used currently in the US poultry industry to curb preharvest levels of *Salmonella*, such as feed withdrawal, feed sanitation, and litter control. Most commercial integrators follow National Chicken Council

recommendations of removing feed from broilers from 8 to 12 hours prior to processing. If kept on feed too long before transport, the gut wall can rupture and cross contaminate other carcasses (NCC, 1992). Additionally, feed is often sanitized. The effects of feed sanitation have been documented as well by Spratt (1985), showing that formaldehyde mold inhibitor products also significantly reduce *Salmonella* survival in broiler feeds. Control of wet litter as a harbor for *Salmonella* growth has also been recommended, specifically by drying litter and adding litter amendments to it (USDA FSIS 2007). Despite these combined methods, processing plant samples remain higher than FSIS would allow, and salmonellosis cases remain unchanged from 2014-2015. Therefore it is pertinent to investigate methods to reduce *Salmonella* growth in the live birds.

One method of preharvest intervention is *S*.Cerevisia fermentation product. Feeding this product has also been correlated with reduced *Salmonella* levels in ground meat by over 75 percent (N.Cox, USDA ARS, Athens, GA unpublished data). The work of Broomhead (2012, 2013), Nsereko (2012, 2013), McIntyre (2013), Feye (2015), Hofacre (2015), Davis (2015), & Mathis (2015) also lend credence to the hypothesis that XPC could reduce *Salmonella* in chicken parts and ground meat in heat stressed and non heat-stressed broilers.

Materials and Methods

A total of 480 Ross male broilers (chicks were placed in one of 24 pens lined with pine shaving litter (0.91 m x 2.74 m). Each pen was equipped with its own bell feeder suspended from the ceiling and nipple drinking system. A 3x2 factorial design was used in this experiment in which birds were assigned at random to one of three

dietary treatments and one of two environmental treatments. The heat stress treatment began at 31 days and temperatures were set at 35C for 18hrs per day. At night temperatures were reduced to 23C for six hours. The facility layout consisted of 2 rooms with 8 pens in each room, and 4 rooms with 2 pens in each room. The 480 birds were divided into 20 birds per pen. Each room was equipped with fluorescent lighting, oscillating fans for additional air flow, and a single pass central ventilation system equipped with HEPA filters per USDA Biosecurity Level 2 protocols (USDA BSL2).

Figure 1. USDA ARS grow out pens

Figure 1 shows the pens with 8 per room, and figure 2 shows the pens with 2 per room and oscillating fan. The pens were .9m by 2.7m and will be .8m high. The pens were constructed of solid black plastic on all but the front side, which will be made of mesh wire. The birds were managed according to the guidelines set forth in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Figure 2. Grow out pen heat stress.

Birds were fed a four-phase diet consisting of a starter (Days 0 to14, crumble Table 3), grower (Days 14 to 28, pellet), finisher 1 (Days 28 to 44 pellet) and finisher 2 (Days 44 to 56, pellet). Birds were allowed unrestricted access to feed and water. A photoperiod of 24L:0D was maintained to 10 days of age and then reduced to 20L:4D

for the remainder of the study. Feed was mixed at Texas A&M Poultry Science Center and Table 3 shows the ingredient inclusions by percentage of total mixed batch.

Table 3. Broiler starter feed fed during 14 day growout

Ingredient name	Percent
CORN	60.34
SOYBEAN MEAL	32.75
DL-METHIONINE	0.28
LYSINE HCL	0.29
FAT, BLENDED	2.43
LIMESTONE	1.57
BIOFOS 16/21P	1.57
SALT	0.47
TRACE MINERALS	0.05
VITAMINS	0.25

Diets fed for this experiment were the feed as mixed in Table 4 and labeled **CON.** Original S. Cerevisiae was obtained from Diamond V Mills Inc of Cedar Rapids, IA and added at the rate of 1.25kg/MT for the dietary treatment **XPC.** Original S. Cerevisiae was added at the rate of 2.00kg/MT for the dietary treatment **XPC2.** These 3 dietary treatments were randomly assigned to pens to be fed to birds therein. Table 4 shows the breakout of dietary treatments as fed along with environmental treatments and their arrangement by pen.

Table 4. Experimental design for commercial broilers challenged with *Salmonella* Typhimurium, fed a diet with and without Diamond V Original S. Cerevisiae and exposed to either a normal or a heat stress environment**.**

	Treatment		
Variable	**CON**	**XPC**	**XPC 2**
Product[1]	N/A	Original S.	Original S.
Product Inclusion Rate	N/A	1.25 kg/MT	2.00 kg/MT
Salmonella Challenge	Yes	Yes	Yes
Heat Stress Pens	4	4	4
Non-Heat Stress Pens	4	4	4
Total Number of Birds	160	160	160

1. Diamond V, Cedar Rapids, IA 52404

Salmonella *typhimurium challenge*. Upon arrival of day-old chicks, the tray pads in each chick box were tested to confirm that all birds were *Salmonella* free. Any birds that were confirmed positive for *Salmonella* were immediately removed from the study and euthanized according to AVMA Guidelines for the Euthanasia of Animals. Prior to inoculation, the *S.*Typhimurium used in this study was revived by washing it three times in Tryptic Soy Broth (TSB) (Becton Dickinson, East Rutherford, NJ) within a 24 h period. Following this 24 h period, the *Salmonella* was washed and diluted to the specified concentration for oral administration to the birds. On Day 3, the chicks were orally challenged with 0.25 to 0.50 mL of 1 x 107 CFU of antibiotic resistant (Nalidixic acid / Novobiocin) marked strains of *S.* Typhimurium. On D 21, 1 bird per pen was euthanized to determine colonization of *Salmonella*. Ceca were aseptically collected from 5 birds from each group in order to verify challenge. A 0.25-g sample of contents from 1 cecum was collected and serially diluted to a final dilution of 1:10, 1:100, and

1:1,000, and 0.1 mL of the 1:10 dilution and 0.1 mL of the 1:100and 1:1,000 dilutions were spread plated on NO and NA BGA plates (Moore et al., 2006). The plates were incubated at 37 C overnight. The second cecum was placed in 15 mL of RV for enrichment and incubated at 42 C overnight, then streaked with 10 ul loop on NO and NA BGA plates. Plates were incubated at 37 C overnight.

On D 50, the remaining birds destined for processing on D 55 were re-challenged using the procedures described above to increase the *Salmonella* counts for enumeration and prevalence. After confirmation of *Salmonella* colonization, birds were inspected daily and feed and water was maintained for ample supply and cleanliness per FASS (2010). Feed was weighed and recorded during daily visits to the growout facility when it was added. Upon change of diets from starter to grower, grower to finisher, finisher to withdrawal, feed from the previous diet left in the feeders was weighed back and recorded (Ohaus Champ CD-11, Pine Brook, NJ).

Processing. At D 41, 8 birds from half of the control (6) pens and 8 birds from half of the heat stressed (6) pens were processed. Birds (8 per pen) from the remaining 6 control and 6 heat stressed pens were processed on D 43. Data from these birds were grouped together and considered the average age of 42 days of age. The 42 day bird processing size was chosen to represent the average of majority commercial birds sold for retail tray pack markets. At 55 days the remaining birds were all non-heat stressed birds, thus making the experiment a 3x1 factorial based only on dietary treatment for this portion of study.

At all processing days, feed was removed from pens 8-12 hours before processing based on industry practices in an effort to reduce contamination at processing (Wabeck, 1972; Bilgili, 1988). The period of feed withdrawal was based on National Chicken Council recommendations, breed guidelines (Cobb-Vantress, Siloam Springs, AR), and industry standard practices (Veerkamp, 1986). Birds were then loaded 8 at a time into broiler plastic transport crates (Kuhl corp; Tek Inc.) and into enclosed TAMU service van for transport 2.7 mi away to TAMU poultry science processing facility.

Upon arrival, the birds were placed in an environmentally controlled holding room until processing. Birds were processed in 12 replicates of 8 birds each (by pen). Each bird in this comparison was considered the experimental unit.

Birds were placed on processing shackles and stunned at 13-15 mA using an electrified knife (Midwest Processing Systems, Minneapolis, MN) and bled following a unilateral neck cut. Following bleeding for 90 sec, in a custom stainless steel water bath bleed tunnel, birds were hard scalded (60C) for one minute or until feathers follicles were loose enough for picking in a Dynagard autocal series scald tank and controller (Dynamation Inc, Ann Arbor, MI) and picked using a rotary drum picker (Pickwick/Knase Co St. Paul, MN). Feet were removed following picking & tagged by individual bird and placed in Ziploc™ (S.C. Johnson, Racine, WI) bags for storage and analysis in stress measure study. Carcasses were manually eviscerated and ceca were collected using clean gloves on each individual bird. Ceca were placed directly into 2oz 3x5" Whirlpak™ bags (Nasco, Ft. Atkinson, WI) and then sent directly back to USDA ARS Southern Plains Agricultural Research Center microbiology laboratory for

Salmonella prevalence and enumeration. Following evisceration, carcasses were immersion chilled for 74 min (4°C) in agitated tap water and ice. After chilling, carcasses were placed on drip racks and allowed to drip for an average of 1 min. Carcass were rinsed with 100 ml of buffered peptone water (ThermoFisher Scientific, Tampa, FL) and rinses were collected and analyzed for *Salmonella* enumeration and prevalence. For each batch of 8 carcasses per pen, the equipment was sanitized (10 percent chlorine and rinsed with water) and new chiller water was used to ensure no cross contamination from batch to batch. Following the carcass rinsate sample collection, carcasses were manually deboned. The right breast filet and the combination of the right and left wings were sampled for *Salmonella* enumeration and prevalence using nalgahide screwtop containers to collect rinsate back from bags and transfer to USDA ARS Souther Plains Agricultural Research Station. In between each deboning, the deboning surfaces were rinsed off with 10 percent chlorine and cleaned with water. Each remaining left breast fillet was individually ground using a 3/16 plate and fed through an electric mixer (Kitchen-Aide tilthead model). A target sample of 25g was weighed and placed in filter bags (Whatman GE Marlborough, MA). This 40ml sample rinsate was sent with wing, carcass, and breast rinsate samples to USDA ARS to be tested for *Salmonella* enumeration and prevalence. Grinding heads were replaced with freshly sanitized (dipped in 10 percent chlorine solution and then rinsed with water) blades between batches from each replicate pen.

Salmonella bacterial samples (both enumeration and prevalence) were collected during processing as follows: 1) ceca, 2) whole carcasses following 75 min of agitated

immersion ice water bath chilling, 3) boneless skinless breasts, 4) wings and 5) ground breast meat. Enumeration of *Salmonella* included sample pre-enrichment with buffered peptone water (BPW), enrichment into 10 mL of Rappaport Vassiliadis (RV) and incubation at 35˚C for 24 h. Additionally, 0.1 mL of the pre-enriched samples was transferred to 10 mL of RV, and incubated at 42˚C for 24 h. At the end of the enrichment period, a 10uL loopful of the enrichment samples was streaked onto brilliant green agar, and all the plates were incubated at 35˚C for 24 h to measure the prevalence of *Salmonella*.

Data collection and analysis. For each bird, ceca prevalence and enumeration were recorded. Wing rinse prevalence and enumeration was recorded, carcass rinse prevalence and enumeration were recorded, breast rinse prevalence and enumeration were recorded, and for each bird ground sample rinse prevalence and enumeration data was collected. Enumeration counts were based off of a 100 fold dilution. Proceeding with 1 part of the sample into 99 parts of the diluent solution, if there was one colony on the plate only a number of 1 was recorded. Therefore, 1 x 100 = 100, which is a log of 2, making the level of detectability 2. Samples were enriched with RV so if one cell was present then it would grow and be detected on the plate. This count would then be below the value of log 2. If it is below the level of detection in the RV enriched sample then it is assigned the value of "0". If the contents that were negative at the 1:100 dilution on XLT4 plates but positive after RV-XLT4 plates then they were assigned 1.50 log10 *Salmonella* per gram/ml of the sample (Byrd 2015; Corrier-Hinton 1990).

Statistical Analysis

The *Salmonella* prevalence data were analyzed by Chi-square with likelihood ratio P-value calculated. For the *Salmonella* enumeration data GLM was used to test the main effects of diet and heat stress as well as the interaction between diet and heat stress. No significant interactions between diet and heat stress were identified for any of the traits measured so only main effects were further tested. Means were separated for the enumeration data were conducted using Duncan's Multiple Range Test. A probability of less than 0.05 was considered significant for all analyses.

Results and Discussion

Salmonella *enumeration: day 42.* Data for ceca, carcass, wing, breast and ground breast *Salmonella* enumeration at 42 days of age are presented in Table 5. No significant interaction between dietary treatment and heat stress was found for ceca, breast and ground breast. Therefore, pooled means were used to describe the effect of dietary treatment and heat stress on *Salmonella* counts for ceca, breast and ground breast. A significant interaction was found between dietary treatment and heat stress for carcass and wing, so the effect of dietary treatments and heat stress on *Salmonella* counts in both carcass and wing were separated by heat stress and dietary treatments, respectively.

No significant differences in *Salmonella* count were found among the three dietary treatments for ceca, breast and ground breast. The recovery of *Salmonella* from all treatments was considered low and led investigators to re-challenge remaining birds after the 43d sampling. However, these results indicate that the addition of XPC to the

diet at either inclusion rate did not reduce *Salmonella* counts for ceca, breast and ground breast regardless of heat stress environment.

No significant differences in *Salmonella* counts were found between HEAT and NORM broilers for breast, indicating that heat stress had no effect on *Salmonella*. NORM broilers had significantly lower (P = 0.03) *Salmonella* counts in the ceca (0.85 ± 0.06) but significantly higher (P = 0.02) *Salmonella* counts in ground breast (1.45 ± 0.11) compared to HEAT broilers (1.09 ± 0.08 and 1.11 ± 0.09 for ceca and breast, respectively). These results indicate that the effect of heat stress could increase the susceptibility of challenged broilers to *Salmonella* in the ceca, but reduce the susceptibility to *Salmonella* in ground breast which does not correlate with previously reported observations. Heat stress tends to reduce immunological functions and increase shedding of pathogens. However, it is important to note that the lack of *Salmonella* positive samples in the ground breast meat could have affected these results.

As previously mentioned a significant interaction was found between dietary treatment and heat stress for both carcass and wing. For wing in HEAT broilers, *Salmonella* counts were highest for S. Cerevisiae fermentation product 2 (1.89 ± 0.18) and lowest for CON (1.36 ± 0.06) with XPC (1.65 ± 0.17) intermediate but not statistically different from either CON or XPC 2. These results indicate that feeding Original XPC at the higher level of 2.00 kg/MT could possibly increase the susceptibility of HEAT broilers to higher *Salmonella* counts for wings. For NORM broilers, *Salmonella* counts were significantly higher in XPC fed birds (1.96 ± 0.20) compared to CON (1.19 ± 0.09) and S. Cerevisiae fermentation product 2 (1.36 ± 0.09),

which did not differ statistically. The effects of heat stress were only significant for wings in the birds in the XPC 2 treatment group. No differences were observed between HEAT and NORM broilers for *Salmonella* count in wings for CON or XPC fed broilers. For XPC 2, birds that were HEAT had significantly higher *Salmonella* counts than NORM broilers, 1.89 vs. 1.36 log10 CFU/ml, respectively. These data suggest that the addition of Original S. Cerevisiae fermentation product to the diet regardless of inclusion rate, especially in heat stress environments could result in higher *Salmonella* counts in wings compared to CON fed birds.

For NORM broilers, no significant differences were observed for *Salmonella* counts for carcass, but significant differences were observed in HEAT birds between dietary treatments. For HEAT birds, *Salmonella* counts were highest for S. Cerevisiae fermentation product 2 (1.95 ± 0.17) and lowest for CON (1.29 ± 0.12) with XPC (1.62 ± 0.15) intermediate but not statistically different from either CON or XPC 2. These results indicate that feeding Original XPC at the higher level of 2.00 kg/MT could possibly increase the susceptibility of HEAT broilers to higher *Salmonella* counts for the carcass. No significant differences were observed for between HEAT and NORM broilers for birds receiving either the CON or XPC diets. However, a significant effect was observed for XPC 2, with HEAT broilers having higher *Salmonella* counts for the carcass than NORM broilers, 1.95 vs. 1.46 log10 CFU/ml, respectively. These data suggest that in a heat stress situation, broilers fed Original XPC at the higher inclusion rate of 2.00 kg/MT may be more susceptible to having higher *Salmonella* counts for the carcass.

Salmonella *enumeration*: day 55. All birds remaining in the NORM group were re-infected with *Salmonella* at 50d. *Salmonella* counts (log10 CFU/ml) for ceca, carcass, wing, breast and ground breast in birds fed a diet with and without S. Cerevisiae fermentation product are presented in Table 3. No significant difference in *Salmonella* counts were found among three dietary treatments for ceca, carcass, wings, breast and ground breast, indicating that the addition of XPC in diet at either inclusion level did not influence the *Salmonella* counts of big-size challenged broilers within 5d of challenge.

Salmonella *prevalence: day 42*. *Salmonella* prevalence on Day 42 for ceca, carcass, wing, breast and ground breast are presented by either heat stress environment (Table 7) or non-heat stress environment (Table 8). For HEAT broilers, no significant differences in *Salmonella* prevalence were found among three dietary treatments for ceca, carcass, wing, and breast, indicating that addition of Original XPC in diet at either inclusion level did not influence the prevalence of *Salmonella* in these parts. A significant difference ($P = 0.0150$) was found for ground breast with XPC having the lowest *Salmonella* prevalence (5.41 percent) compared to CON (23.53 percent) and XPC 2 (29.41 percent), indicating that feeding Original XPC at the 1.25 kg/MT inclusion rate could reduce the susceptibility of HEAT challenged broilers to *Salmonella* in ground breast (Table 7).

For NORM broilers, no significant differences in *Salmonella* prevalence were found among the three dietary treatments for ceca, carcass, wing, breast, and ground breast, indicating that the addition of Original XPC in diet at either inclusion level did not influence the prevalence of *Salmonella* in these parts. Table 9 shows only one

significant difference by environmental challenge, as ground breast prevalence was lower in birds exposed to heat stress.

Table 5. *Salmonella* counts (log10 CFU/ml) and statistical probabilities for ceca, carcass, wings, breast and ground breast on Day 42 for birds challenged with *S.* Typhimurium, fed a diet with and without Diamond V Original S. Cerevisiae and exposed to either a normal or a heat stress environment.

	Ceca		Carcass		Wing		Breast		Ground Breast	
DIET	**Norm**	**Heat**	**Norm**	**Heat**	**Norm**	**Heat**	**Norm**	**Heat**	**Norm**	**Heat**
CON	0.76 ± 0.10	1.14 ± 0.15	1.4 ± 0.08	1.29 ± 0.12	1.19 ± 0.09	1.36 ± 0.06	1.25 ± 0.08	1.39 ± 0.10	1.60 ± 0.19	1.21 ± 0.18
S. Cerevisiae	0.75 ± 0.10	1.05 ± 0.12	1.8 ± 0.21	1.62 ± 0.15	1.96 ± 0.20	1.65 ± 0.15	1.60 ± 0.15	1.35 ± 0.10	1.35 ± 0.19	0.95 ± 0.14
S. Cerevisiae2	1.05 ± 0.11	1.08 ± 0.17	1.46 ± 0.11	1.95 ± 0.17	1.36 ± 0.09	1.89 ± 0.18	1.36 ± 0.09	1.47 ± 0.17	1.41 ± 0.17	1.17 ± 0.18
Diet Main Effects										
CON	0.96 ± 0.10		1.34 ± 0.07		1.28 ± 0.05		1.32 ± 0.06		1.40 ± 0.13	
S. Cerevisiae	0.91 ± 0.08		1.70 ± 0.12		1.80 ± 0.13		1.47 ± 0.09		1.13 ± 0.12	
S. Cerevisiae2	1.06 ± 0.10		1.71 ± 0.12		1.63 ± 0.11		1.42 ± 0.08		1.29 ± 0.12	
Heat Effect										
Norm	0.85 ± 0.06		1.55 ± 0.08		1.50 ± 0.09		1.41 ± 0.07		1.45 ± 0.11	
Heat	1.09 ± 0.08		1.62 ± 0.09		1.63 ± 0.09		1.40 ± 0.07		1.11 ± 0.09	
Probability (p-value)										
Diet Effect	0.4988		0.0185		0.0015		0.4244		0.3097	
Heat Effect	0.0314		0.6071		0.2929		0.9604		0.0153	
Diet & Heat Effect	0.3928		0.0489		0.0136		0.1816		0.8852	

1. On Day 3, birds were orally challenged with 0.25 to 0.50 mL of 1x107 CFU of antibiotic (Nalidixic acid / Novobiocin) marked strains of *Salmonella* Typhimurium.
2. Means ± SEM.
3. Means across rows within the same variable column with no common superscript differ significantly ($P < 0.05$).
4. Means within the same column with no common superscript differ significantly ($P < 0.05$).
5. Diamond V Original S. Cerevisiae inclusion rate was 1.25 kg/MT for all diets.
6. Diamond V Original S. Cerevisiae inclusion rate was 2.00 kg/MT for all diets.

The effects of environmental stress, HEAT or NORM, on *Salmonella* prevalence are presented in Table 9. No significant difference in *Salmonella* prevalence were found between heat stress and non-heat stress broilers for ceca, carcass, wing, and breast, indicating that heat stress did not affect the susceptibility of heat challenged broilers to *Salmonella* in these parts. However, a significant difference ($P = 0.0135$) was found between HEAT and NORM broilers for ground breast with heat stress broilers lower

Salmonella prevalence than NORM broilers (19.05 vs. 34.38 percent, respectively). This lower *Salmonella* prevalence indicates that heat stress could reduce the susceptibility of heat stress challenged broilers to *Salmonella* in ground breast.

Salmonella *prevalence:* day 55. No significant differences in *Salmonella* prevalence were found among the three dietary treatments for ceca, carcass, wing, breast, and ground breast on Day 55 (Table 10). The lack of significant difference indicates that the addition of *S.* Cerevisiae fermentation product in diet at either inclusion level did not influence the susceptibility of big-size broilers re-infected with *Salmonella* relative to the prevalence of *Salmonella* in ceca, carcass, wing, breast and ground breast. The extremely high *Salmonella* prevalence observed for all parts might be a result of the birds being re-challenged 5 days before the processing which increased the possibility of contamination with *Salmonella*.

Table 6. *Salmonella* counts (log10 CFU/ml) and statistical probabilities for ceca, carcass, wings, breast and ground breast on Day 55 for birds challenged with *S.* Typhimurium, fed a diet with and without Diamond V Original S. Cerevisiae and exposed to no heat stress.

	Treatment			
Variable[2,3]	**CON**	**XPC[4]**	**XPC2[5]**	**P-value**
Ceca	3.92 ± 0.15	3.74 ± 0.13	4.05 ± 0.14	0.277
Carcass	1.95 ± 0.13	2.34 ± 0.18	2.30 ± 0.21	0.238
Wings	2.39 ± 0.22	2.19 ± 0.18	2.42 ± 0.22	0.672
Breast	2.70 ± 0.28	2.19 ± 0.20	2.98 ± 0.23	0.066
Ground Breast	2.29 ± 0.18	2.01 ± 0.15	1.89 ± 0.16	0.210

1. On Days 3 and 50, birds were orally challenged with 0.25 to 0.50 mL of 1x107 CFU of antibiotic (Nalidixic acid / Novobiocin) marked strains of *Salmonella* Typhimurium.
2. Means ± SEM.
3. Means across rows within the same variable column with no common superscript differ significantly ($P < 0.05$).
4. Diamond V Original S. Cerevisiae inclusion rate was 1.25 kg/MT for all diets.
5. Diamond V Original S. Cerevisiae inclusion rate was 2.00 kg/MT for all diets

Table 7. *Salmonella* prevalence (percent) and statistical probabilities for ceca, carcass, wings, breast and ground breast on Day 42 for birds challenged with *S.* Typhimurium, fed a diet with and without Diamond V Original S. Cerevisiae and exposed to a heat stress.

	Treatment			
Variable[2,3]	**CON**	**XPC[4]**	**XPC2[5]**	**P-value**
Ceca	32.35 ± 8.14	37.84 ± 8.08	30.30 ± 8.12	0.7868
Carcass	70.59 ± 7.93	75.68 ± 7.15	91.18 ± 4.94	0.0722
Wings	85.29 ± 6.17	75.68 ± 7.15	88.24 ± 5.61	0.3446
Breast	76.47 ± 7.38	78.38 ± 6.86	73.53 ± 7.68	0.8910
Ground Breast	23.53 ± 7.38[a]	5.41 ± 3.77[b]	29.41 ± 7.93[a]	0.0150

1. On Day 3, birds were orally challenged with a 0.25 to 0.50 mL of 1x107 CFU of antibiotic (Nalidixic acid / Novobiocin) marked strains of *Salmonella* Typhimurium.
2. Means ± SEM.
3. Means across rows within the same variable column with no common superscript differ significantly ($P < 0.05$).
4. Diamond V Original S. Cerevisiae inclusion rate was 1.25 kg/MT for all diets.
5. Diamond V Original S. Cerevisiae inclusion rate was 2.00 kg/MT for all diets.

Table 8. *Salmonella* prevalence (percent) and statistical probabilities for ceca, carcass, wings, breast and ground breast on Day 42 for birds challenged with *S.* Typhimurium, fed a diet with and without Diamond V Original XPC and exposed to no heat stress.

	Treatment			
Variable[2,3]	**CON**	**XPC[4]**	**XPC2[5]**	**P-value**
Ceca	18.75 ± 7.01	18.75 ± 7.01	40.63 ± 8.82	0.0777
Carcass	84.38 ± 6.52	71.88 ± 8.08	87.50 ± 5.94	0.2491
Wings	68.75 ± 8.32	81.25 ± 7.01	81.25 ± 7.01	0.4009
Breast	75.00 ± 7.78	81.25 ± 7.01	81.25 ± 7.01	0.7809
Ground Breast	37.50 ± 8.70	28.13 ± 8.08	37.50 ± 8.70	0.6551

1. On Day 3, birds were orally challenged with a 0.25 to 0.50 mL of 1x107 CFU of antibiotic (Nalidixic acid / Novobiocin) marked strains of *Salmonella* Typhimurium.
2. Means ± SEM.
3. Means across rows within the same variable column with no common superscript differ significantly ($P < 0.05$).
4. Diamond V Original S. Cerevisiae inclusion rate was 1.25 kg/MT for all diets.
5. Diamond V Original S. Cerevisiae inclusion rate was 2.00 kg/MT for all diets.

Table 9. *Salmonella* prevalence (percent) and statistical probabilities for ceca, carcass, wings, breast and ground breast on Day 42 for birds challenged with *S.* Typhimurium, fed a diet with and without Diamond V Original XPC and exposed to either a normal or a heat stress environment.

	Environment		
Variable[2,3]	**NORM**	**HEAT**	**P-value**
Ceca	26.04 ± 4.50	33.65 ± 4.66	0.2396
Carcass	81.25 ± 4.00	79.05 ± 3.99	0.6958
Wings	77.08 ± 4.31	82.86 ± 3.70	0.3059
Breast	79.17 ± 4.17	76.19 ± 4.18	0.6128
Ground Breast	34.38 ± 4.87[a]	19.05 ± 3.85[b]	0.0135

1. On Day 3, birds were orally challenged with a 0.25 to 0.50 mL of 1x107 CFU of antibiotic (Nalidixic acid / Novobiocin) marked strains of *Salmonella* Typhimurium.
2. Means ± SEM.
3. Means across rows within the same variable column with no common superscript differ significantly ($P < 0.05$).

Table 10. *Salmonella* prevalence (percent) and statistical probabilities for ceca, carcass, wings, breast and ground breast on Day 55 for birds challenged with *S.* Typhimurium, fed a diet with and without Diamond V Original XPC and exposed to no heat stress.

	Treatment			
Variable[2,3]	**CON**	**XPC[4]**	**XPC2[5]**	**P-value**
Ceca	100.00 ± 0.00	96.88 ± 3.13	100.00 ± 0.00	0.3298
Carcass	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	1.0000
Wings	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	1.0000
Breast	96.88 ± 3.13	93.75 ± 4.35	96.88 ± 3.13	0.7816
Ground Breast	78.13 ± 7.42	71.88 ± 8.08	75.00 ± 7.78	0.8462

1. On Days 3 and 50, birds were orally challenged with a 0.25 to 0.50 mL of 1x107 CFU of antibiotic (Nalidixic acid / Novobiocin) marked strains of *Salmonella* Typhimurium.
2. Means ± SEM.
3. Means across rows within the same variable column with no common superscript differ significantly ($P < 0.05$).
4. Diamond V Original S. Cerevisiae inclusion rate was 1.25 kg/MT for all diets.
5. Diamond V Original S. Cerevisiae inclusion rate was 2.00 kg/MT for all diets.

At 42 day processing, birds fed S. Cerevisiae fermentation product at either treatment did not exhibit reduction in CFU/ml of *Salmonella* for ceca, breast or ground breast. Results for *Salmonella* prevalence in birds not exposed to heat stress are listed in Table 8, and are shown to exhibit no significant differences. XPC fed at 2.00kg/MT to heat stressed birds did result in an increase of *Salmonella* CFU/g in wing and carcass samples compared to CON. XPC fed at 1.25kg/MT to non heat stressed birds also showed an increase in *Salmonella* CFU/g in wings rinses. Heat stress effect across treatments exhibited a decrease in *Salmonella* counts in ground breast meat, and an increase in counts in cecal samples, and no effect in breast meat. XPC fed at 2.00kg/MT under heat stress showed increased *Salmonella* counts in wing and carcass rinses vs those from non heat stressed birds.

In all parts sampled at 42 day processing, prevalence and counts were observed to be lower than expected for ceca, carcass, wing, breast, and ground breast. It was believed by USDA ARS staff that the Novobiocin and Nalidixic acid resistant strain of *S.* Typhimurium used to inoculate birds was losing its virulence. It has been found *Salmonella* Pathogenicity Island-1 encoded type III secretion system regulates invasiveness, but its induction is affected by a variety of environmental factors. Changes in these factors can also affect *Salmonella's* replication (Sridhar, 2016). These factors have not been accurately pinpointed and many can be at play. Therefore the decision was made to inoculate the remaining birds a second time with .25-.50 mL of $1x10^9$ of nalidixic acid/novobiocin resistant *S.* Typhimurium.

Day 55 processed broilers consisted of only birds that were non heat stressed, though this processing age group served to represent a larger market bird size. Neither dietary treatment showed any effect on reducing counts on any of the parts sampled. Day 42 processed birds did show a significant increase in cecal prevalence in non heat stress conditions and fed XPC at 2.00kg/MT compared with CON and S. Cerevisiae fermentation product fed at 1.25kg/MT. Day 42 processed birds exposed to heat stress and fed XPC at 1.25kg/MT did show significant reduction in *Salmonella* prevalence of ground breast meat rinse samples. In past work levels of XPC higher than 1.25kg/MT have shown less effect on reduction of *Salmonella* (Berghaus, 2014). It appeared that XPC fed at 1.25kg/MT was effective at reducing *Salmonella* prevalence in ground meat. Despite this significant effect in ground meat reduction, there did not appear to be any correlation with prevalence or CFU/mL in cecal samples. The results of the study have an interesting comparison to previous work in regard to *Salmonella* prevalence, as Hofacre's 2015 study showed prevalence to be higher in XPC fed bird environmental swabs. That work did show a significant reduction in *Salmonella* prevalence for carcass rinses (Hofacre, 2015). There are many factors at play in transfer of pathogens in a processing plant environment, therefore it is difficult to predict correlation levels between cecaal prevalence and RTC product. Al-Zenki (2007) found carcass prevalence rates of double what the prevalence rates were in ceca (Al-Zenki, 2007). This could be due to cross contamination from certain high CFU/mL cecal content spreading to other carcasses, or even release of pathogens from debris. In the majority of industry experimentation with XPC, *Salmonella* prevalence has correlated with reductions in

RTC product rinses directly correlating with reductions in cecal load (D. Smith, USDA ARS Athens, GA, personal communication; S. Carlson, Iowa State University, Ames, IA, personal communication).

Work by Feye (2015) showing S. Cerevisiae fermentation product treatment to affect *Salmonella* virulence and antibiotic levels does add some potential explanation for the discrepancy between cecal prevalence levels of CON vs XPC fed broilers and ground meat prevalence of CON vs XPC fed broilers. XPC has been shown to down regulate the hilA gene encoded on SPI-1, and to expel resistance gene encoded SG1 integron or resistance gene encoded plasmids, thereby reducing invasiveness, virulence, and antibiotic resistance. These genetic effects that combine to weaken *Salmonella* coupled with XPC's known history of having no direct killing or eliminating effect but rather a reduction effect, could potentially be making *Salmonella* more susceptible to antimicrobials or unfavorable environmental conditions. This could explain the observation of high cecal *Salmonella* in XPC compared to CON fed birds, yet low 42 day ground breast prevalence of XPC fed bird vs CON fed birds. Any further study on this should most likely involve virulence assays, hilA gene expression analysis, SG1 analysis, and antibiotic resistance assays for recovered isolates.

Conclusion

Ground breast meat processed at 42 days showed a reduction in *Salmonella* counts (log10) by dietary treatment. Birds fed XPC at 2.00kg/MT and birds fed S. Cerevisiae fermentation product at 1.25kg/MT resulted in ground breast meat *Salmonella* counts that were lower compared to CON fed birds ground breast meat. This

was the case in both heat stressed and non-heat stressed birds fed either treatment. Heat stressed birds exhibited lower *Salmonella* counts in ground breast meat than non-heat stressed birds. Non heat stressed birds processed at Day 55 and fed XPC at 1.25kg/MT showed a reduction in *Salmonella* counts (log10) that was approaching significance for breast meat and for ground breast meat. While there did not appear to be a consistent difference in levels between CON parts sampled and parts sampled from birds fed either treatment of XPC, the reduction of prevalence at 42 days in ground meat and counts at day 55 in ground meat are of great interest as this is normally the most difficult area of control due to cross contamination at plants and spread of as meat is further processed. This study also showed the importance of virulence in marked *Salmonella* strains as the counts and prevalence at 42 days were much lower than expected. Repeated inoculation in future studies could ensure that there is a measurable level of *Salmonella* from which to start in challenged birds. However, feeding S. Cerevisiae fermentation product at 1.25kg/MT still showed potential for reduction of *Salmonella* in ground breast meat.

CHAPTER IV

EFFECTS OF FEEDING ORIGINAL XPC TO BROILERS ON STRESS MEASURES DURING HEAT STRESS AND NON-HEAT STRESS

Introduction

As the broiler industry has progressed in its mission to provide the most efficient and high quality meat product to consumer markets, a number of practices with regard to live production have become commonplace and necessary to achieve maximum profitability. Special attention has been paid to stocking density, with today's national average hovering around .75sqft/bird in commercial broiler barns (NCC 2015). While commercial production environments are designed to provide adequate air flow, humidity, access to feed/water, and constant temperature of around 80F for growout (Aviagen Ross mgmt. guide), conditions may still be debatably crowded and even stressful on birds. The modern consumer has become increasingly more conscious of the perceived plight of food production animals in confinement and a market category has begun to focus on more humane treatment of these animals. In the past 10 years, American Humane Heartland, Humane Farm Animal Care, Global Animal Partnership, and Animal Welfare Alliance (just to name a few) have all formed non-profit third party corporations to ensure the protein industry's production of humanely raised food animals. Multinational Corporations now have created corporate positions such as "Chief Sustainability Officer", "Vice President of Responsible Sourcing and Compliance", and "Corporate Animal Welfare Director" in an effort to position

themselves as aware and focused on improving conditions of food production animals in the protein supply chain. Nestle and General Mills both for example have made public written and recorded commitment to the Five Animal Freedoms (UK Farm Animal Welfare Council 1979) as part of their corporate animal welfare policy in sourcing protein.

While the aforementioned third party animal welfare advocate groups can conduct farm level inspections of environment and treatment, it is important to consider what measures may be available to producers to induce a less stressful experience for animals in production. Access to food and water, clean environments, and safe housing are all considered basic prerequisites for producing comfortable and healthy animals. There is however, room to focus further on food and water ingestion as a key area of controlling stress. As the number one goal of producing food animals is to increase their weight to market demands, ingestion of quality feed is paramount. Selection by genetic companies for fast growing, high-yield broilers has been shown to have detrimental effects on birds ability to cope with heat stress (Berrong and Washburn, 1998; Tan et al., 2010; Soleimani et al., 2011).

Syafwan (2011) showed that heat stress in broilers utilized in the industry today can lead to cellular oxidative stress, which increases susceptibility to infectious diseases (Syafwan, 2011). This oxidative stress was shown to lead to lipid peroxidation in other studies (Willemsen, 2011), indicating an effect on carcass quality via an increase in free radicals. Habibian's (2015) work indicated that this could be due to a fluctuation in the immune system by changing cytokine expression (Habibian, 2015).

The effects of heat stress are numerous and detrimental to performance and quality of commercial broilers, but heat stress has also been shown to affect welfare. Tonic immobility measures in broilers are used as an indication of fear or stress response (Liu, 2016), and they have also been measured in response to heat stress. Altan (2003) showed that heat stress exposure increased duration of tonic immobility in broilers, indicating that heat stress is correlated with more fearful birds. This same study also found an increase in heterophil/lymphocyte ratios in bird exposed to heat stress (Altan, 2003).

Stress measures such as tonic immobility can be correlated with decreased production efficiency, as shown by Wang (2013). In this work birds with longer duration of tonic immobility were also shown to have higher levels of the stress hormone corticosterone & more pododermatitis (Wang, 2013). There is some indication that broilers exposed to heat stress early in life will cope with this stress better later in life, exhibiting better feed conversion and efficiency of weight gain (Arjona, 1988). Morita (2016) showed similar effects in egg incubation temperatures that correlated to increased tolerance of heat stress by broilers (Morita, 2016). Understanding how broilers will perform in heat stress conditions is of great value in commercial production where efficiency is paramount. This was displayed in Cooper's work, which showed a strong correlation between the body temperature of commercial broilers and their efficiency of feed conversion, weight gain, as well as feed consumption (Cooper, 1998).

To assist in mitigating the effects of heat stress, environmental controls are designed based on a house temperature 5^{o}F warmer than the outside temperature (U.KY,

2009). However to further combat the detrimental effects of excessive heat, nutritional and health program strategies are needed. Selenium for example, has been shown to reduce oxidative stress by altering cytokine production. This in turn could improve performance measures in production broilers (Habibian, 2015). Zinc also plays an important role in the immune system and antioxidant defense, and has been improve feed efficiency in heat stress situations (Sahin, 2009). Ryu (2016) showed that broilers under heat stress exhibit changes in serum antibodies, intestinal microflora, volatifle fatty acid levels, and corticosterone levels when fed at different times of night (Ryu, 2016). The addition to diets of heat oxidized soy protein isolate has shown to decrease crude protein and dry matter digestibility, further indicating the importance of antioxidant defense in broiler efficiency during heat stress (Chen, 2015).

Prior studies have shown that Diamond V Original S. Cerevisiae can balance the immune system and stress hormone levels in production poultry (HughesJones 1987; Firman, 2013). Gao's work (2005) showed increased secretory IgA and intestinal IgM in birds fed XPC (Gao, 2005), and Al-Mansour (2011) showed that XPC significantly decreased H:L ratios in broilers (Al-Mansour, 2011). XPC has also shown to have a positive impact on weight gain, feed conversion and mortality in broilers that were challenged with ingestion of used litter during a heat stress period (Teeter, 1993). For this reason, a study was undertaken to measure the effects of stress on broilers fed Original S. Cerevisiae during heat stress and non-heat stress.

Materials and Methods

For the purposes of this experiment 480 Ross Broilers (Aviagen Inc. Huntsville, AL) were transported to College Station TX (Tyson Inc Center, TX). At USDA ARS Southern Plains Agricultural Research Center, Chicks were placed in one of 22 pens lined with pine shaving litter (0.91 m x 2.74 m). Each pen was equipped with its own bell feeder suspended from the ceiling and nipple drinking system (Lubing Inc.). A 3x2 factorial design was used in this experiment in which birds were assigned at random to one of three dietary treatments and one of two environmental treatments. The facility layout consisted of 2 rooms with 8 pens in each room, and 4 rooms with 2 pens in each room. The 480 birds were divided into 20 birds per pen. Each room was equipped with fluorescent lighting, oscillating fans for additional air flow, and a single pass central ventilation system equipped with HEPA filters per USDA Biosecurity Level 2 protocols (USDA BSL2). Two rooms of pens were 8 per room, four rooms of pens were 2 per room with oscillating fan. The pens were 3 ft. by 9 ft. and will be 2.5 ft. high, and were constructed of solid black plastic on all but the front side, which was made of mesh wire. The birds were managed according to the guidelines set forth in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Birds were fed a four-phase diet consisting of a starter (Days 0-14, crumble), grower (Days 14-28, pellet), finisher 1 (Days 28-44 pellet) and finisher 2 (Days 44-56, pellet). Birds were allowed ad libitum access to feed and water. A photoperiod of 24L:0D was maintained to 10 days of age and then reduced to 20L:4D for the remainder

of the study. Feed was mixed at Texas A&M Poultry Science Farm and table 11 shows the ingredient basics

Diets fed for this experiment were the feed as mixed in Table 11 and labeled **CON.** Original S. Cerevisiae was obtained from Diamond V Mills Inc of Cedar Rapids, IA and added at the rate of 1.25kg/MT for the dietary treatment **TRT1.** Original S. Cerevisiae was added at the rate of 2.00kg/MT for the dietary treatment **TRT2.** These 3 dietary treatments were randomly assigned to pens to be fed to birds therein. Table 12 shows the breakout of dietary treatments as fed along with environmental treatments and their arrangement by pen.

Table 11. Broiler starter feed fed during 14 day growout

Ingredient name	percent
Corn	60.34
Soybean meal	32.75
Dl-methionine	0.28
Lysine hcl	0.29
Fat, blended	2.43
Limestone	1.57
Biofos 16/21p	1.57
Salt	0.47
Trace minerals	0.05
Vitamins	0.25

Table 12. Experimental design for commercial broilers challenged with *S.* Typhimurium, fed a diet with and without Diamond V Original XPC and exposed to either a normal or a heat stress environment.

	Treatment		
Variable	**CON**	**XPC**	**XPC 2**
Product[1]	N/A	Original S.	Original S.
Product Inclusion Rate	N/A	1.25 kg/MT	2.00 kg/MT
Salmonella Challenge	Yes	Yes	Yes
Heat Stress Pens	4	4	4
Non-Heat Stress Pens	4	4	4
Total Number of Birds	160	160	160

1. Diamond V, Cedar Rapids, IA 52404

Feed was weighed and recorded during daily visits to the growout facility when it was added. Upon change of diets from starter to grower, grower to finisher, finisher to withdrawal, feed from the previous diet left in the feeders was weighed back and recorded (Ohaus Champ CD-11, Pine Brook, NJ).

Blood draw. On Days 40-42, blood samples were collected from 10 birds per pen. The area around the jugular vein was sanitized with 70 percent alcohol, and in preparation, the inside of a 10 mL syringe was lined with a small amount of heparin. Between 2-3 mL of blood was collected from each bird, and a drop was used to prepare a blood-smear slide. The remaining blood was injected into a Vacutainer (BD 368056, BD, Franklin Lakes, NJ) containing the additives plasma separation gel and lithium heparin. Vaccutainers were temporarily stored in an ice bath. Once all samples were collected, the vaccutainers were spun down in a Beckman GS-6R centrifuge (Beckman Coulter, Brea, CA) for 15 min at 4000 RPM to separate the cells from the plasma. The plasma was drawn into 2 mL micro-centrifuge tubes and stored at -19°C until further

analysis. The blood-smear slides were stained using a hematology staining kit (Cat# 25034, Polysciences Inc., Warrington, PA), then air-dried.

Plasma corticosterone concentrations were measured using a commercially available ELISA kit (Enzo Life Sciences, ADI-901-097, Farmingdale, NY). Plasma HSP70 concentrations were measured using a commercially available ELISA kit (Enzo Life Sciences, EKS-715, Farmingdale, NY). Intra and Inter CV were less that 0.05.

Heterophil/Lymphocyte ratio were measured by taking the blood smear slides prepared earlier and observing them under 1000X magnification (10X eyepiece, 100X oil emersion lens) using an Omax DCE-2 microscope (Kent, WA). An area of the slide that had moderate cell density (no overlapping cells) was chosen, and the numbers of both heterophils and lymphocytes were counted until the total observed number reaches 100 (Campo, et al., 2000). A keystroke counter was used to accurately keep track of the number of cells observed.

Physical asymmetry of each marked bird was measured at 41 and 43 days of age before processing, following the protocol outlined in Archer and Mench (2013). Using a calibrated Craftsman IP54 Digital Caliper (Sears Holdings, Hoffman Estates, IL), the middle toe length, metatarsal length, and metatarsal width were measured for both the right and left legs. The composite asymmetry score was calculated by taking the sum of the absolute value of left minus right of each trait, then dividing by the total number of traits. Thus the formula for this trial would be (|L-R|MTL+|L-R|ML+|L-R|MW)/3= composite asymmetry score.

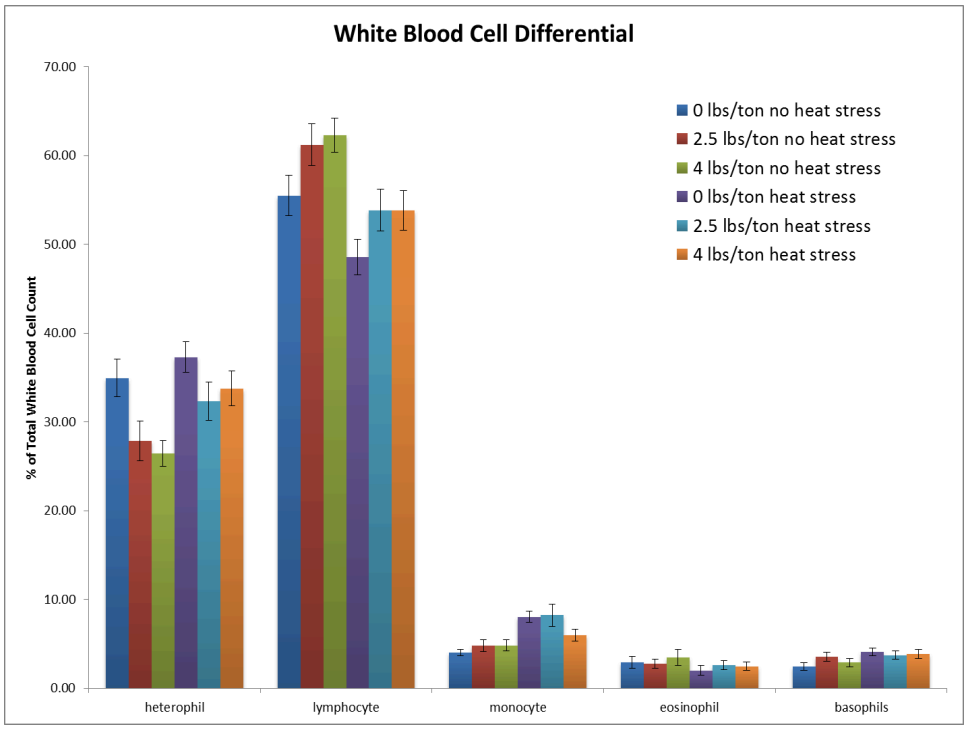
Welfare assessment. Footpad dermatitis scoring occurred post processing and involve inspecting the footpads of all birds individually and noting any dark dermatitis lesions present. They were scored on a scale of 0 to 4. A score of 0 indicates no dermatitis is present, 1 and 2 indicated minimal evidence of dermatitis is present, and 3 and 4 indicated that noticeable evidence of dermatitis is present (Butterworth, 2013).

Hock burn scoring occurred post processing and involved examining all individual birds for the presence of dermatitis on the back of the hock caused by contact with the litter. The scores were on a scale of 0 to 4. A score of 0 indicates no hock burn is present, 1 and 2 indicate minimal evidence of hock burn is present, and 3 and 4 indicate that noticeable evidence of hock burn is present (Butterworth, 2009).

Statistical Analysis

Stress measures, growth, feed conversion and mortality were all analyzed using the GLM procedure in Minitab 17.1.0. The model used was y = level of XPC, heat stress, and level of S. Cerevisiae fermentation product x heat stress. Significant differences were considered at $P > 0.05$. Mean separation was performed using the LSD post hoc procedure. The footpad and hock scores were analyzed using the Kruskal-Wallis procedure in Minitab 17.1.0. Significant differences were considered at $P > 0.05$.

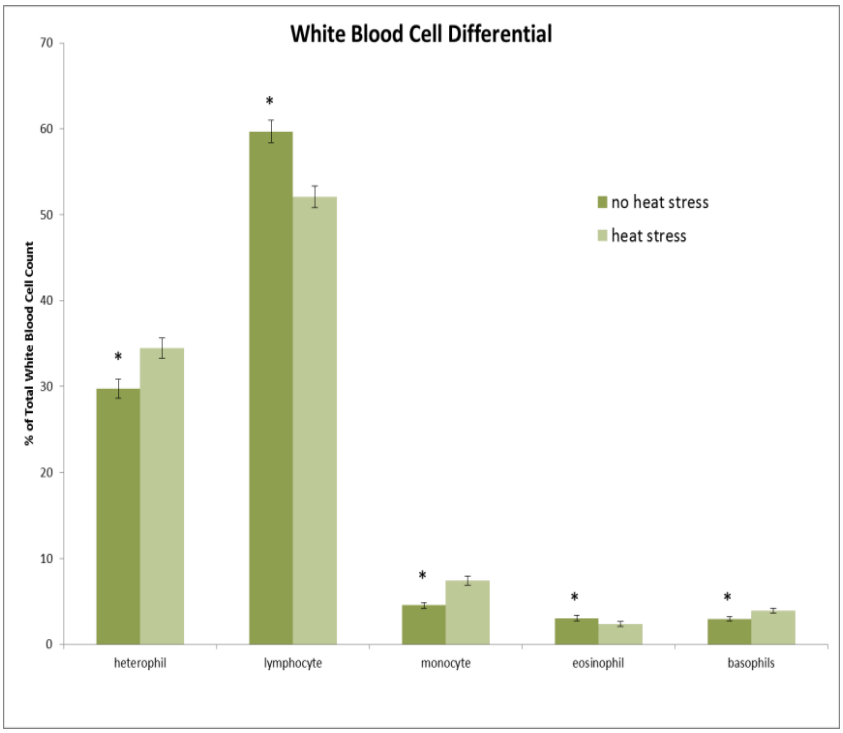
Stress. The addition of XPC to the diet altered the heterophil and lymphocyte populations (Figure 3), and the overall WBC counts (P = 0.04; 0 lbs/ton, 36235 ± 1402 WBC; 1.25 KG/MT, 32417 ± 1304 WBC; and 2.00 KG/MT ,31914 ± 1273 WBC). However, the white blood cell populations of the broilers in this study were changed mainly by heat stress (Figure 4). The heterophil/lymphocyte ratio was greater (P < 0.01) in those birds not fed S. Cerevisiae fermentation product (0.81 ± 0.05) when compared to those fed XPC (1.25 KG/MT, 0.62 ± 0.05; 2.00 KG/MT, 0.61 ± 0.05). The heat stress also resulted in higher (P < 0.001) heterophil/lymphocyte ratios (heat stressed, 0.78 ± 0.04 vs non-heat stressed, 0.58 ± 0.05).



* indicates that 0 KG/MT differed from 2.5 and 2.00 (P<.01).

Figure 3. White blood cell differential of broilers fed XPC at different levels.

The birds fed XPC at either 1.25 or 2.00 KG/MT were seen to have lower stress levels as indicated by both plasma corticosterone concentrations (Figure 4, $P < 0.001$)) and physical asymmetry scores (Figure 5, $P < 0.001$). Heat stress increased corticosterone concentrations ($P < 0.001$) but not physical asymmetry scores ($P > 0.05$).



* indicates that Heat Stress differed from the Non-Heat Stressed ($P < 0.02$).

Figure 4. White blood cell differential of broilers heat stressed or not.

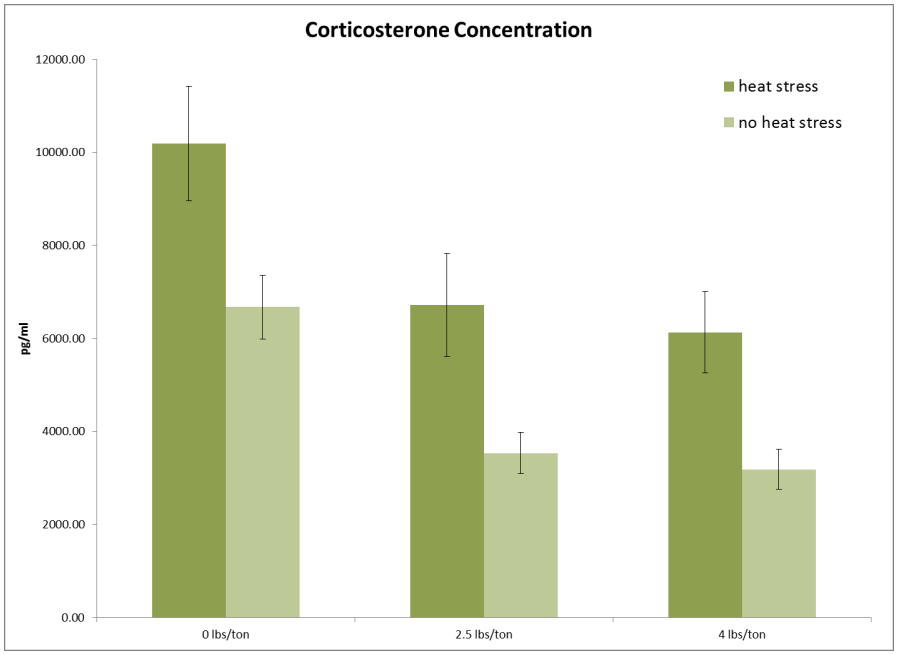


Figure 5. Plasma corticosterone concentrations of broilers fed XPC at different levels and exposed to heat stress or no heat stress

There were no observed differences between broilers feed differing levels of XPC in plasma HSP70 concentrations (Figure 6, $P > 0.05$); However, heat stress did increase the plasma HSP70 concentrations compared to non-heat stressed birds (Figure 7, $P < 0.001$).

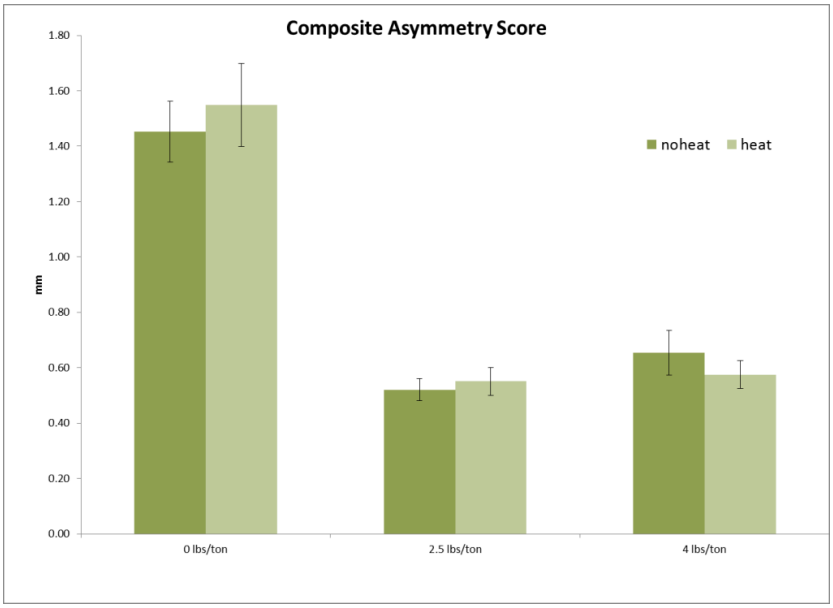


Figure 6. Composite asymmetry score of broilers fed XPC at different levels and exposed to heat stress or no heat stress

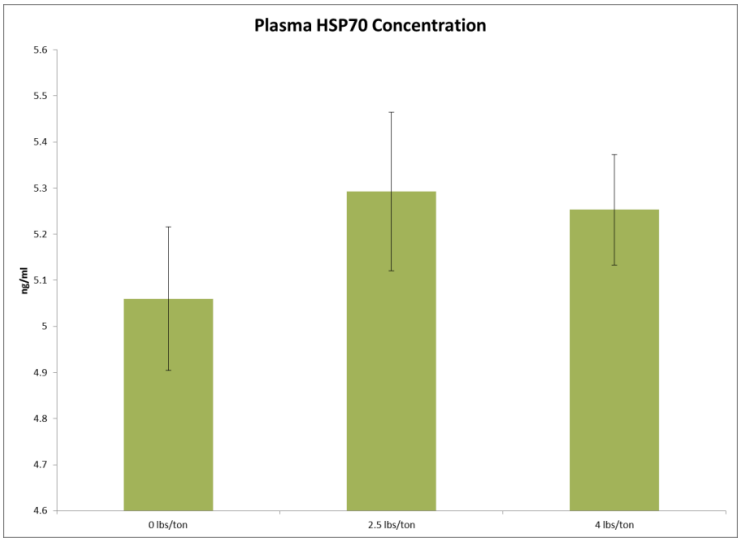


Figure 7. Plasma HSP70 concentrations in broilers fed XPC at different levels.

Welfare scores, growth, and feed conversion. Feeding XPC at different levels did not affect Footpad scores, Hock scores, body weights, mortality, feed consumption, or FCR (Table 13, $P > 0.05$). Heat stress affected Footpad scores, body weights, feed consumption and mortality ($P < 0.01$) but not Hock scores or FCR ($P > 0.05$).

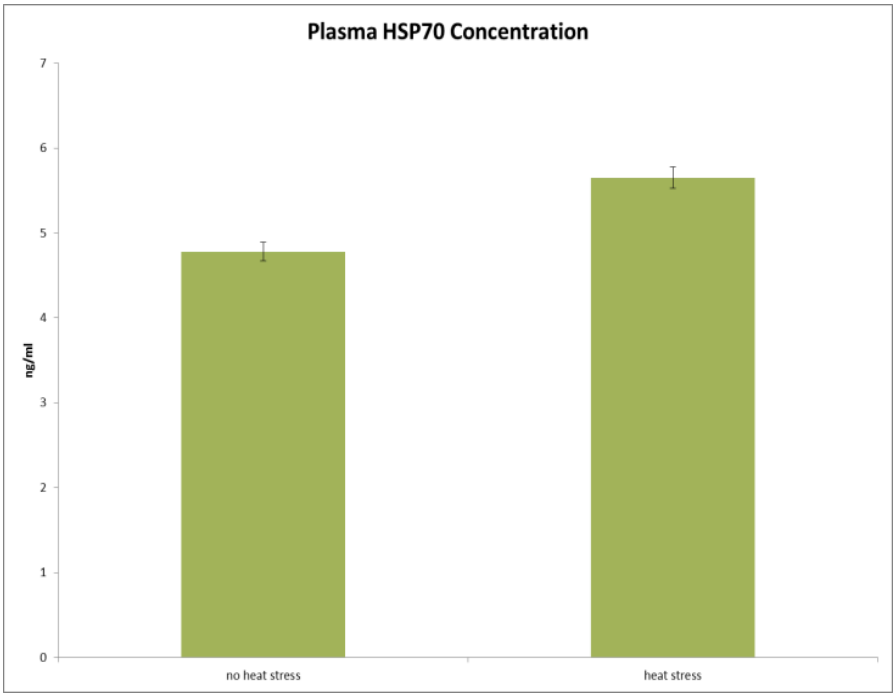


Figure 8. Plasma HSP70 concentrations in broilers exposed to heat stress or no heat stress.

Table 13. Effect of XPC and heat stress on footpad scores, hock scores, 42 d body weight, mortality, feed consumption and FCR.

Heat Stress	S. Cerevisiae fermentation product	FOOT PAD	HOCK	42D BW	MORT	FEED CONSUMED	FCR
NO Heat Stress	0lbs/T	1.25	.53	2.86	17.5	123.98	1.97
	2.5lbs/T	1.39	.36	2.85	13.8	121.31	1.89
	4lbs/T	.97	.55	2.83	10.0	115.74	1.79
Heat Stress	0lbs/T	1.25	.33	2.32	35	75.65	1.88
	2.5lbs/T	.53	.41	2.28	33.8	78.48	1.86
	4lbs/T	.97	.65	2.41	46.3	76.78	1.84
	SEM	.08	.05	.03	3.4	4.77	.02
				KG	percent	KG	

Results and Discussion

To effectively determine XPC's impact as a potential stress reduction measure, it was first necessary to ensure that the model used did in fact elicit a stress response from the birds. The parameters measured in this study included HSP70, CORT, H/L, feed consumption, mortality, bilateral asymmetry, footpad scoring, and hock scoring. These measures are associated with stress and/or fear in broilers, as well as welfare (Zulkifkli, 2009, Al-Aqil, 2009, Gross, 1983, Camp, 2000). The expression of HSP70 measured in blood serum as well as serum CORT was markedly increased in birds exposed to heat stress in this study. Additionally, feed consumption was decreased, mortality was increased, H/L ratios were increased, and asymmetry scores increased in birds exposed

to heat stress. Hock scores and footpad scores did not show a difference, but there was sufficient evidence that the manufacture of heat stress in this model did in fact affect the birds stress response. It can be concluded that the model used would provide acceptable measurement of XPC's potential as a stress reduction and welfare improvement measure.

HSP70 has been shown to offer protective benefits to birds experiencing an environmental stress, as it assists in inhibiting lipid peroxidation. Gu (2012) showed a negative correlation between HSP70 and CORT & H/L. The latter two stress measures were increased when HSP70 was inhibited. The elevation of antioxidant enzymes superoxide dismutase and glutathione peroxidase and increase in antioxidant capacity help to protect the intestinal environment during HSP70 expression under heat stress. There is a decrease in lactic dehydrogenase when HSP70 is expressed, which helps to protect the intestinal mucosal barrier (Gu, 2012). The present study did not observe any interaction between dietary treatment and HSP70 expression. It has been proposed that hsp70 homologue can assist with nascent protein transport across the endoplasmic reticulum, offering another benefit and necessity for its presence in stressful environments (Chirico, 1988).

CORT has been shown to decrease adrenal gland responsiveness, measured by CORT production, after a challenge with adrenocorticotropic hormone (ACTH) (2003). Heat stress can lead to CORT increase from activation of the hypothalamic-pituitary-adrenocortical axis (Rimoldi, 2015). The importance of this axis in stress is evident, as it leads to a rapid release of corticotropin-releasing hormone (CRH) and

adrenocorticotropic hormone from the cells located in the hypothalamus and pituitary, respectively. ACTH stimulates the synthesis and release of steroids from the adrenal cortex by promoting the uptake of cholesterol and its enzymatic conversion to the glucocorticoid hormone CORT (Jones et al., 1988; Fraisse and Cockrem, 2006; Rimoldi, 2015). If this occurs and too much CORT is present, it can retard the longitudinal growth of bones by depressing the proliferation and differentiation of chondrocytes in broilers. According to Zhang's (2012) work it depressed the longitudinal growth of the long bones by inhibiting the proliferation and differentiation of chondrocytes in growth plate in the birds (Zhang, 2012). CORT is one of the stress indicator glucocorticoids that has shown to rise in many occasions of stress, and is shown to have an impact on other measures of stress and welfare. In this study with the increase in CORT also came an increase in asymmetry of heat stressed birds.

Heterophil/Lymphocyte (H/L) ratio is commonly measured indicator of susceptibility to stress. These measurements as stress indicators trace back to Davison and Rowell (1983) who described how dietary cortisone could induce lymphocytopenia and granulocytosis and so affect the granulocyte:lymphocyte ratio. They suggested the granulocyte:lymphocyte ratio might provide a convenient measure of adrenal-corticoid hyperactivity. Gross (1989) indicated that social stress, chilling, and injected Escherichia coli affect the H/L ratio (Gross, 1989). Gross (1983) also measured H/L ratio as a response to corticosterone in feed. Lymphocytes in chicken blood samples decreased and the number of heterophils increased in response to stressors and to increasing levels of corticosterone in the chicken feed (Gross, 1983).

Morphological or physical asymmetry is an additional measure of stress that was added to this experiment as the combination of HSP70, CORT, H/L, and some physical measurements such as bilateral asymmetry and footpad/hock scores offers a more comprehensive look at susceptibility to stress. Any challenge immunologically can alter nutrient absorption efficiency in broilers and thus challenges result in physiological changes. Numerous challenges will be present throughout commercial broiler development and they begin to show their response to these challenges in the form of asymmetry. Deviations from perfect symmetry represent asymmetry and may be estimated by the frequency distribution of left minus right (L-R) for a given bilateral trait. There are 3 forms of bilateral asymmetry; fluctuating asymmetry (FA), directional asymmetry (DA) and antisymmetry (AS) (Palmer and Strobeck, 1992). The definition of fluctuating asymmetry is random deviations from perfect growth symmetry that is generally expected in certain body parts when morphological development is successfully controlled, and it is the result of both genetic factors and environmental conditions. Prieto (2010) showed the addition of lactic acid or CORT resulted in greater fluctuating asymmetry of wing length of heat-stressed chicks, further evidencing the effects of stress hormones on physiological development (Prieto, 2010). The effects of IBV and NDV vaccination on commercial broilers have been shown to affect growth rates due to an expected immune response (Wang, 2015). These effects on the immune system of respiratory virus exposure can be seen as a stressor contributing to the asymmetrical development as the birds body focuses energy on immune response. S. Cerevisiae fermentation product has been shown to balance the immune system and

improve respiratory vaccine response for NDV, ILT, and IBV (H.Toro, Auburn University, Auburn, AL personal communication). This could perhaps explain the ability of XPC fed birds to overcome stress easier and thereby improve asymmetry scores.

The measurements included in this experiment for footpad and hock scoring were as an analysis of XPC's potential to improve welfare through mitigating footpad burns or hock blisters. Reduced walking or standing ability often leads to breast blisters and hock burns as the bird has to spend a long time crouching on poor quality litter (Naas, 2008). The welfare concerns from lameness causing behavioral restriction and pain has been reported by Reiter & Kutriz, 2001; Weary et al., 2006. While it has been seen that physical properties of collagen are altered during growth, leading to weak legs and gait problems, with a consequent reduction in feed intake and productivity (Onyango et al.,2003), leg lesions are also a culprit in the causation of limited mobility and the resulting lack of feed intake. Baracho (2011) found there was a positive correlation between back toe and outer toe asymmetry with the presence of leg lesions (Baracho, 2011). XPC has been shown to have a beneficial effect on bird environments during periods of heat stress as shown by Zhou & Zhen (2005), where it was observed that XPC fed birds had 9 percent less ammonia content (mg/100g) in feces sampled. This could be attributed to the change in microflora of XPC treated birds, as they also exhibited higher counts of bifidobacteria in feces sampled (Zhou & Zhen, 2005). This is an important area of focus for animal welfare as reduced ammonia content could certainly lead to fewer birds exhibiting hock and footpad burns. This in turn could lead to fewer birds laying down to stay off their feet and thereby less incidence of breast blisters.

The effects of heat stress itself have been discussed and shown to exhibit responses in several of the parameters measured. The inclusion of XPC in treatment diets showed measurable effects in several measured parameters as well. H/L ratios were affected by both XPC fed at 1.25kg/MT and XPC fed at 2.00kg/MT, as birds given these treatments exhibited decreased ratios. For both treatments, heterophils were decreased in comparison to CON, and lymphocytes were increased compared to CON. Basophils and monocytes were also increased in birds fed either treatment of XPC. XPC fed birds also exhibited significant reduction in serum CORT in both heat stress and non heat stress environments. This is noteworthy as the results indicate XPC's potential to reduce CORT and its effects thereby alleviating stress in times of heat stress challenge, as well as in normal conditions. While CORT was reduced by XPC treatment in this study, HSP70 was not affected. Bensi (1990) and Beutler (1989) have shown that HSP70 can actually inhibit the expression of cytokines interleukin-1 (IL) and tumor necrosing factor-alpha (TNF-a). XPC has been shown to upregulate IL & TNF-a in gene extraction studies (Park, 2015). This may further indicate the importance of HSP70 as a protective mechanism in environmental heat stress conditions, but further study on XPC's influence of different immunological genes could reveal why it did not have an effect on HSP 70. Composite asymmetry scoring was improved in birds fed either treatment of XPC compared to CON, and this held true in both heat stress and non heat stress environments as well. This was a second stress measure in which XPC fed birds displayed reduction in comparison to CON.

The effects on CORT and asymmetry scores observed in XPC fed birds did not appear to be specific to either level of dietary inclusion. The effects were significantly different for both levels on in comparison to CON, not in comparison to each other. Therefore further study is needed to be able to infer if there is any benefit in increased dosage of S. Cerevisiae fermentation product. Feed consumption, while reduced in birds experiencing heat stress, was not significantly different for XPC treatments vs CON. There was certainly a numerical trend toward improvement in FCR for birds fed increasing levels of XPC, though the trial could be improved by utilizing larger numbers of pens to achieve more statistically significant results. The goal of this experiment was not to measure FCR or performance, but rather chemical and physiological parameters of stress and welfare.

CHAPTER V

CONCLUSION

Footpad and hock scoring for birds fed XPC treatments did not exhibit any difference in comparison with CON or between treatments or challenges. This can be attributed to a couple of potential factors. First, the facility used for growout in this experiment consisted of concrete floors and solid brick walls with one pass airflow and thermostat controlled environments. Commercial field conditions and conditions for most conventionally housed research facilities normally experience more temperature fluctuation and potential humidity that contributes to poor litter quality. This in turn exacerbates paw and hock quality with higher ammonia and hotter litter. Conditions were much improved comparatively in this facility. Secondly, as the heat stress challenge mortality was observed to be high, welfare concerns by USDA ARS veterinarians necessitated that fresh litter be brought in to ensure that birds who spent more time on their breasts in the litter would not suffer breast blistering. This change in litter quality occurred at the peak of heat stress, and thus potentially reduced the opportunity for hock and footpad scores to begin to show differences among treatments.

The changes attributed to XPC inclusion in CORT, H/L, and asymmetry scores give support to the hypothesis that XPC could be used to reduce stress and improve bird welfare. Past work has shown that XPC can improve immune system function during stress (Fathi, 2012, Al-Homidan, 2011). This experiment showed that effects on the immune system are measurable and may be correlated with other measures of stress.

More work is needed to examine areas such as other heat shock proteins and differences in basophils and monocytes, as these measures were affected by both levels of XPC.

The significant increase in wing in higher levels of S. Cerevisiae fermentation product was offset by the significant decrease in *Salmonella* prevalence in ground breast meat at 42 days by XPC fed at 1.25kg/MT. Previous work has shown that XPC fed at 1.25kg/MT is an optimum dose and increased levels do not exhibit significant further pathogen reduction (Smith, 2011). The reduction in *Salmonella* prevalence in ground breast meat is noteworthy as this is the furthest point in the processing of the meat at which *Salmonella* would be present and is also the point at which *Salmonella* is often seen to increase due to a number of potential factors such as surface agitation through grinding and cross contamination. Prior work with XPC has shown that it can reduce the ability of *Salmonella* to invade host cells and diminish virulence (Carlson, 2015). Based on this more work is needed to further investigate if it is possible that while counts & prevalence did not decrease significantly in the ceca and correlate with the reduction in ground meat 42 day prevalence, the *Salmonella* present in cecal samples at 42 days could have been diminished in virulence enough to allow for reduction by the time the meat was ground. Regardless of dietary treatment, heat stress did not affect the *Salmonella* counts in breast meat, but heat stress did increase the counts in ceca while reducing the counts in ground breast. Heat stress increased the *Salmonella* counts in both wing and carcass for broilers fed the higher inclusion rate of Original XPC compared to their non-heat stressed counterparts. Moreover, on Day 55, the addition of Original S. Cerevisiae fermentation product at either inclusion rate in the diet did not reduce the

Salmonella counts of big-size challenged broilers for any of the parts measured following re-infection at 50d. For this reason, it is concluded that additional study is needed with the utilization of a known virulent strain of *Salmonella* spp. The results indicated birds may have been overcoming the initial challenge, and this would seem to warrant more investigation into what effect S. Cerevisiae fermentation product treatments may have had on virulence and at what point in production and processing would this effect begin. Based on the observations of this study, feeding XPC has the potential to affect broiler welfare in heat stress and non-heat stress environments based on a variety of stress measures. This potential effect could address both performance and quality conerns in high stress growing environments, but also serve as a measure to satisfy the growing consumer demand for welfare-enhanced broiler conditions. Feeding XPC also showed some potential to reduce *Salmonella* prevalence in ground breast meat, but the effects need to be further evaluated to understand the mode of action. A preharvest intervention that leads to reduction in *Salmonella* prevalence in ground meat could help greatly in improving consumer safety and FSIS compliance among broiler processing plants.

REFERENCES

Abe J, Okuda M, Huang Q, Yoshizumi M, Berk BC. 2000. Reactive oxygen species activate p90 ribosomal S6 kinase via Fyn and Ras. J Biol Chem. 275:1739-48.

Abdollahi A, Najafipour S, Kouhpayeh SA, Meshkibaf M. 2011. Salmonlla Enterica:Serotyping, Drug Resistance & Extended Spectrum of B-Lactamase (ESBLs). J. Fasa Univ. Med.Sci. 1: 38-44.

Al-Homidan A, Fahmy MO. 2007. The effect of dried yeast (Saccharomyces cerevisiae) supplementation on growth, performance, carcass chemical analysis, immunity, ileum villi heights and bacterial counts of broiler chickens. Egypt. Poult.Sci. J., 27: 613-623.

Al-Mansour S, Al-Khalf A, I. Al-Homidan and M.M. Fathi. 2011. Feed Efficiency and Blood Hematology of Broiler Chicks Given a Diet Supplemented with Yeast Culture. International Journal of Poultry Science. 10: 603-607.

Al-Zenki S, Al-Nasser A, Al-Safar A, Alomirah H, Al-Haddad A, Hendriksen S, 2007. Aarestrup. Prevalence and antibiotic resistance of *Salmonella* isolated from a poultry farm and processing plant environment in the State of Kuwait. Foodborne Pathog Dis. 2007 Fall; 4(3): 367-73.

Andreopoulou M, Tsiouris V, Georgopoulou, I. 2014. Effects of organic acids on the gut ecosystem and on the performance of broiler chickens. J. Hellenic Vet Med Soc. 65: 289-302.

Angelotti R, Foter MJ, Lewis KH. 1961. Time-temperature effects on *Salmonellae* and Staphylococci in Foods. III. Thermal death time studies. Appl. Microbiol. 9: 308-315.

Ansari-Lari M, Shekarforoush S, Mehrshad S, Safari H. 2014. Prevalence and risk factors for *Salmonella* spp. colonization in broiler flocks in Shiraz, southern Iran. Veterinary Research Forum : An International Quarterly Journal, 5(1), 65–68.

Arjona, AA, Denbow DM, Weaver Jr. WD. 1988. Effect of heat stress early in life on mortality of broilers exposed to high environmental temperatures just prior to marketing. Poult Sci. 67(2):226-31.

Arsenault J, Letellier A, Quessy S, et al. 2007. Prevalence and risk factors for *salmonella* spp and campylobacter spp cecal colonization in broiler chicken and turkey flocks slaughtered in Quebec, Canada. Prev Vet Med. 81(4):250–264.

Bailey JS, Stern NJ, Fedorka-Cray P, Craven SE, Cox NA, Cosby DE, Ladely S, Musgrove MT. 2001. Sources and movement of *Salmonella* through integrated poultry operations: a multistate epidemiological investigation. J Food Prot. 64(11):1690-7.

Baracho MS, Nääs IA, Bueno LGF, Nascimento GR, Moura DJ. 2011. Broiler Walking Ability and Toe Asymmetry Under Harsh Rearing Conditions. Brazilian Journal of Poultry Science. 14(3):159-232.

Bartlett JR, Smith MO. 2003. Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress. Poult. Sci. 82:1580–1588.

Bauermeister LJ, Bowers JW, Townsend JC, McKee SR. 2008. The microbial and quality properties of poultry carcasses treated with peracetic acid as an antimicrobial treatment. Poult Sci. 11:2390-8.

Baumgard LH, Rhoads Jr. RP. 2013. Effects of heat stress on postabsorptive metabolism and energetics. Annu Rev Anim Biosci. 1:311-37.

Bensi G, Mora M, Raugei G, Buonmassa D, Rossini M, Melli M. 1990. An inducible enhancer controls the expression of the human interleukin-1β gene. Cell Growth Diff.1. 491–497.

Berghaus, RD, Thayer SG, Maurer JJ, Hofacre CL. 2011. Effect of vaccinating breeder chickens with a killed *Salmonella* vaccine on *Salmonella* prevalences and loads in breeder and broiler chicken flocks. J Food Prot. 5:727-34.

Berghaus RD, Thayer SG, Bibiana FL, Mild R, Hofacre CL, Singer RS. 2013. Enumeration of *Salmonella* and *Campylobacter* spp. in Environmental Farm Samples and Processing Plant Carcass Rinses from Commercial Broiler Chicken Flocks. Appl Environ Microbiol. 13: 4106–4114.

Berrang ME, Dickens JA. 2000. Presence and Level of Campylobacter spp. on Broiler Carcasses Throughout the Processing Plant. J Appl Poult Res. 9 (1): 43-47.

Berrong SL, Washburn KW. 1998. Effects of genetic variation on total plasma protein, body weight gains, and body temperature responses to heat stress. Poult Sci. 773:379-85.

Beutler B, and Cerami A. 1989. The biology of cachectin/TNF: a primary mediator of the host response. A. Rev. Immun.7. 625–655.

Bhatia TR, McNabb GD, Wyman H, Nayar GP. 1979. *Salmonella* isolation from litter as an indicator of flock infection and carcass contamination. Avian Dis. (4):838–847.

Bhatia TR, McNabb GD. 1980. Dissemination of *Salmonella* in broiler-chicken operations. Avian Dis. 3:616-24.

Broomhea J, Severenson D, Butler J, and Frank J. 2012. Effects of a *Saccharomyces* Cerevisiae fermentation product on volatile fatty acid production and growth of *salmonella* in a complex fecal microbial population in vitro. Poult.Sci. 2012. 91:132.

Betts J, Finlay BB. 1992. Identification of *Salmonella* Typhimurium invasiveness loci. Can J Microbiol. 1992. Aug;38(8):852-7

Bilgili, SF. 1988. Research Note: Effect of Feed and Water Withdrawal on Shear Strength of Broiler Gastrointestinal Tract. Poultry Science. 1988. 67 (5): 845-847.

Blivet D, Salvat G, Humbert F, Colin P. 1997. Evaluation of a new enrichment broth for the isolation of *Salmonella* spp. from poultry products. International Journal of Food Microbiology. 38 (2-3) 211–216.

Branton SL, May JD, Lott BD, Maslin WR. 1997. Various blood parameters in commercial hens acutely and chronically infected with Mycoplasma gallisepticum and Mycoplasma synoviae. Avian Dis. 41(3):540-7.

Broom DM. 2006. Behaviour and welfare in relation to pathology. Applied Animal Behaviour Science. 97 (1): 73-83.

Buhr RJ, Richardson LJ, Cason JA, Cox NA, Fairchild BD. 2007. Comparison of four sampling methods for the detection of *Salmonella* in broiler litter. Poult Sci. 86(1):21-5.

Butterworth AF, Haslam SM. 2009. A Lameness Control Strategy for Broiler Fowl. Welfare Quality Reports. No. 13.

Butterworth, AF, Mench JA, Wielebnowski N. 2013. Practical Strategies to Assess (and Improve) Welfare', in Appleby MC, Mench JA, Olsson IAS, Hughes, BO (Eds.). Animal Welfare. 200-214

Byrd JA, Corrier D, DeLoach J, Nisbet D. 1997. Comparison of Drag-Swab Environmental Protocols for the Isolation of *Salmonella* in Poultry Houses. Avian Diseases, 41(3), 709-713.

Byrd JA, Anderson RC, Callaway TR, Nisbet DJ. 2015. Effect of experimental chlorate product administration in the drinking water on *Salmonella* Typhimurium contamination of broilers. Poult. Sci. 82(9):1403-6.

Calefi, Atilio Sersun, Juliana Garcia da Silva Fonseca., Daniel Wagner Hamada Cohn*, Bruno Takashi Bueno Honda*, Carolina Costola-de-Souza*, Lucila Emiko Tsugiyama†, Wanderley Moreno Quinteiro-Filho*, Antonio J. Piantino Ferreira* and João Palermo-Neto. 2016. The gut-brain axis interactions during heat stress and avian necrotic enteritis. Poult. Sci. 95: 1005-14.

Chen X, Bauermeister LJ, Hill GN, Singh M, Bilgili SF, McKee SR. 2008. Efficacy of various antimicrobials on reduction of *salmonella* and campylobacter and quality

attributes of ground chicken obtained from poultry parts treated in a postchill decontamination tank. J Food Prot. 11:1882-8.

Calefi, AS, de Siqueira A, Namazu LB, Costola-de-Souza C, Honda BB, Ferreira AJ, Quinteiro-Filho WM, da Silva Fonseca JG, Palermo-Neto J. 2016. Effects of heat stress on the formation of splenic germinal centres and immunoglobulins in broilers infected by Clostridium perfringens type A. Vet Immunol Immunopathol. 171:38-46.

Campo JL, Da´vila SG. 2000. Changes in heterophil to lymphocyte ratios of heat-stressed chickens in response to dietary supplementation of several related stress agents. Arch. Geflu¨gelk. 66:80 – 84.

Carter JD, Hudson AD. 2009. Reactive arthritis: clinical aspects and medical management. Rheum Dis Clin North Am. (1):21-44.

Cengiz Ö, Köksal BH, Tatlı O, Sevim O, Ahsan U, Üner AG, Ulutaş PA, Beyaz D, Büyükyörük S, A.Yakan, Önol AG. 2015. Effect of dietary probiotic and high stocking density on the performance, carcass yield, gut microflora, and stress indicators of broilers. Poult Sci. 94:2395-403.

Chand N, Naz S, Khan A, Khan S, Khan RU. 2014. Performance traits and immune response of broiler chicks treated with zinc and ascorbic acid supplementation during cyclic heat stress. Int J Biometeorol. 58(10):2153-7.

Chen X, Bauermeister LJ, Hill GN, Singh M., Bilgili SF, McKee SR. 2014. Efficacy of various antimicrobials on reduction of *salmonella* and campylobacter and quality

attributes of ground chicken obtained from poultry parts treated in a postchill decontamination tank. J Food Prot. (11):1882-8.

Cheng HW, Muir WM. 2004. Chronic social stress differentially regulates neuroendocrine responses in laying hens: II. Genetic basis of adrenal responses under three different social conditions. Psychoneuroendocrinology 29 961–971.

Chirico WJ, Waters MG, Blobel G. (1988). 70K heat shock related proteins stimulate protein translocation into microsomes. Nature 332, 805-81 O.

Clifton-Hadley, FA, Breslin M, Venables LM, Sprigings KA, Cooles SW, Houghton S, Woodward MJ. 2002. A laboratory study of an inactivated bivalent iron restricted *Salmonella* enterica serovars Enteritidis and Typhimurium dual vaccine against Typhimurium challenge in chickens. Vet Microbiol. 89(2-3):167-79.

Cooper MA, Washburn KW. 1998. The relationships of body temperature to weight gain, feed consumption, and feed utilization in broilers under heat stress. Poult Sci. 77(2):237-42.

Corrier DE, Hinton Jr. A, Ziprin RL, DeLoach JR. 1990. Effect of dietary lactose on *Salmonella* colonization of market-age broiler chickens. Avian Dis. 1990 Jul-Sep;34(3):668-76.

Corry JE, Allen VM, Hudson WR, Breslin MF, Davies RH. 2002. Sources of *Salmonella* on broiler carcasses during transportation and processing: modes of contamination and methods of control. J Appl Microbiol. 2002;92(3):424-32.

Cotter PF. 2014. Atypical lymphocytes and leukocytes in the peripheral circulation of caged hens. Poultry Science 00:1–7.

Crum Cianflone NF. 2009. Salmonellosis and the GI Tract: More than Just Peanut Butter. Curr Gastroenterol Rep. Author manuscript; available in PMC 2009 Sep 28.

Cui S, Beilei G, Zheng J, Meng J. 2005. Prevalence and Antimicrobial Resistance of Campylobacter spp. and *Salmonella* Serovars in Organic Chickens from Maryland Retail Stores. Appl Environ Microbiol. 71(7): 4108–4111.

Davison TF, Rowell JG, Rea J. 1983. Effects of Dietary Corticosterone on peripheral blood lymphocytes and granulocyte populations in immature domestic fowl. Res Vet Sci. 34:236-239.

Dawkins MS 2004. Using Behaviour to assess animal welfare. Animal Welfare. 2004. 13:53-7.

Dias de Oliveira SF, Siqueira Flores LR, dos Santos, A. Brandelli. 2005. Antimicrobial resistance in *Salmonella* enteritidis strains isolated from broiler carcasses, food, human and poultry-related samples. Int J Food Microbiol. 97(3):297-305.

De Oliveira JE, van der Hoeven-Hangoor E, van de Linde IB, Montijn RC, van der Vossen JM. 2014. In ovo inoculation of chicken embryos with probiotic bacteria and its effect on posthatch *Salmonella* susceptibility. Poult Sci. 2014. 93:818-29

Department of Agriculture Food Safety and Inspection Service. 1998. Contents of HACCP Plans. 9 CFR Part 417. Fed Reg. 63: 4562-3.

Del Vesco AP, Gasparino E, Grieser Dde O, Zancanela V, Soares MA, Neto AR. 2015. Effects of methionine supplementation on the expression of oxidative stress-related genes in acute heat stress-exposed broilers. Br J Nutr. 113:549-59.

Dominguez SA, Schaffner DW. 2009. Survival of *salmonella* in processed chicken products during frozen storage. J Food Prot. 72(10):2088-92.

Erickson JW, Gross CA. 1989. Identification of the sigma E subunit of Escherichia coli RNA polymerase: a second alternate sigma factor involved in high-temperature gene expression. Genes Dev. (9):1462-71.

FASS. 2010. Guide for the Care and Use of Agricultural Animals in Research and Teaching. Third Edition. Jan 2010.

Fathi M, Al-Mansour S, Al-Homidan A, Al-Khalaf A, Al-Damegh M. 2011. Effect of yeast culture supplementation on carcass yield and humoral immune response of broiler chicks. Vet. World, 2012, Vol.5(11): 651-657.

Feberwee A, deVries TS, Hartman EG, deWit JJ, Elbers ARW, deJong WA, 2001. Vaccination against *Salmonella* Enteritidis in Dutch commercial layer flocks with a vaccine based. on a live *Salmonella* Gallinarum 9R strain: Evaluation of efficacy, safety, and performance of serologic *Salmonella* tests. Avian Diseases. 45, 83-91.

Feddes JJR, Emmanuel EJ, Zuideft MJ. 2002. Broiler performance, body weight variance, feed and water intake, and carcass quality at different stocking densities. Poult. Sci. 81:774–779.

Fehlhaber K, Kruger G. 1998. The study of *Salmonella* enteritidis growth kinetics using rapid automated bacterial impedance technique. J. Appl, Microbiol. 84: 945-949.

Flower FC, Weary DM. 2006. Effect of hoof pathologies on subjective assessments of dairy cow gait. J. Dairy Sci., 89. 2006. pp. 139–146.

Fraisse F, Cockrem JF. 2006. Corticosterone and fear behaviour in white and brown caged laying hens. Br Poult Sci. 47(2):110-9.

Fravalo P, Hascoet Y, Le Fellic M, Queguiner S, Petton J, Salvat G. 2003. Convenient Method For rapid and quantitative assessment of *Salmonella* enterica contamination: the mini MSRV-MPN technique. Journal of Rapid Methods and Automation in microbiology. 11(2):81-88.

Foodsafety.gov. *Salmonella* food poisoning. 2015. Accessed Jan 2016. http://www.foodsafety.gov/poisoning/causes/bacteriaviruses/salmonella/

Hanna MO, Stewart JC, Carpenter ZI, and Vanderzant C. 1977. Effect of heating, freezing, and pH on Yersinia enterolcoliticalike organisms from meat. J. Food Protect. 40: 689-692.

Hofacre C, Berghaus R, McIntyre D, Smith D. 2015. Field studies: Preharvest *Salmonella* control using the immune modulator Original XPC in broilers and commercial turkeys. Int. Poult. Sci. Forum Abs. Book. Accessed at http://www.southernpoultrysciencesociety.org/pdfs/2015AbstractBook.pdf.

Galarneau KD, Bailey RH, Wills RW. 2015. Agreement of 3 carcass rinse sampling methods (split carcass, repeat rinse, and adjacent pair) on the detection of *Salmonella* contamination in broiler carcasses. Poult Sci. 94(3):461-6.

Gao J, Zhang HJ, Wu SG, Yu SH, Yoon I, Moore D, Gao YP, Yan HJ, Qi GH. 2009. Effect of *Saccharomyces* Cerevisiae fermentation product on immune functions of broilers challenged with Eimeria tenella. Poult Sci. 88:2141-51.

Geraert PA, Padilha JC, Guillaumin S.1996. Metabolic and endocrine changes induced by chronic heat exposure in broiler chickens: biological and endocrinological variables. Br J Nutr. 75:205-16.

Ghareeb K, Awad W, Sid-Ahmed O, Bohm J. 2014. Insights on the Host Stress, Fear and Growth Responses to the Deoxynivalenol Feed Contaminant in Broiler Chickens. Plos One. 9(1).

Ghazi S, Amjadian T, Norouzi S. 2015. Single and combined effects of vitamin C and oregano essential oil in diet, on growth performance, and blood parameters of broiler chicks reared under heat stress condition. Int J Biometeorol. (8):1019-24.

Gorbach SL, Bartlett JG, Blacklow NR. 2003. Infectious Diseases. 3rd Edition. LWW.

Greene JA, McCraken RM, Evans RT. 1985. A contact dermatitis of broilers—Clinical and pathological findings. Avian Pathol.14:23–38.

Grimaldi E, Scopacasa F. 2000. Evaluation of the Abbott CELL-DYN 4000 hematology analyzer. Am J Clin Pathol. (4):497-505.

Gu XH, Hao Y, Wang XL. 2012. Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 2. Intestinal oxidative stress. Poult Sci. 91(4):790-9. doi: 10.3382/ps.2011-01628.

Habibian M, Ghazi S, Moeini MM, Abdolmohammadi A. 2014. Effects of dietary selenium and vitamin E on immune response and biological blood parameters of broilers reared under thermoneutral or heat stress conditions. Int J Biometeorol. 58(5):741-52.

Hangalapura B, Nieuwland MGB, De Vries Renlingh G, Buyse J, Van Den Brand H, Parmentier HK. 2005. Severe Feed restriction enhances innate immunity but suppresses cellular immunity in chicken lines divergently selected for antibody responses. Poult. Sci. 84: 1520-1529.

Higgins R, Malo R, Rene-Roberge E, and Gauthier R. 1982. Studies on the dissemination of *Salmonella* in nine broiler-chicken flocks. Avian Dis. 26, 26–33.

Hofacre CL, Mathis GF, Miller SH, LaVorgna MW. 2007. Use of Bacitracin and Roxarsone to Reduce *Salmonella* Heidelberg Shedding Following a Necrotic Enteritis Challenge Model. The Journal of Applied Poultry Research Volume 16, Issue 2Pp. 275-279.

Hosseini-Vashan SJ, Golian A, Yaghobfar A. 2015. Growth, immune, antioxidant, and bone responses of heat stress-exposed broilers fed diets supplemented with tomato pomace. Int J Biometeorol. 60(8):1183-92.

Humphrey T, Allen V. 2002. Food Standards Agency project ZB00034: Biosecurity on the broiler farm as an anti-*Salmonella* control measure.

Ilhak O, Guran HS. 2014. Combined Antimicrobial Effect of Thymol and Sodium Lactate against Listeria monocytogenes and *Salmonella* Typhimurium in Fish Patty. Journ. Food Safety. 34(3): 211-17.

Jones, GW, Richardson LA, Uhlman D. 1981. The invasion of HeLa cells by *Salmonella* typhimurium: reversible and irreversible bacterial attachment and the role of bacterial motility. J Gen Microbiol. 127(2):351-60.

Jensen AN, Sørensen G, Baggesen DL, Bødker R, Hoorfar J. 2003. Addition of novobiocin in pre-enrichment step can improve *Salmonella* culture of modified semisolid Rappaport-Vassiliadis. J. Microbiol. Methods 55:249-255.

Jones RB, Beuving G, Blokhuis HJ. 1988. Tonic immobility and heterophil/lymphocyte responses of the domestic fowl to corticosterone infusion. Physiol Behav. 42(3):249-53.

Kestin SC, Su G, and Sørensen P. 1999. Different commercial broiler crosses have different susceptibilities to leg weakness. Poultry Sci. 78:1085–1090.

Khosravinia H. 2015. Effect of dietary supplementation of medium-chain fatty acids on growth performance and prevalence of carcass defects in broiler chickens raised in different stocking densities. J Appl Poult Res. 24 (1): 1-9.

Kim KH, Lee GY, Jang JC, Kim JE, Kim YY. 2013. Evaluation of Anti-SE Bacteriophage as Feed Additives to Prevent *Salmonella* enteritidis (SE) in Broiler. Asian-Australas J Anim Sci. 26(3): 386–393.

Kingston DJ. 1981. A comparison of culturing drag swabs and litter for identification of infection with *Salmonella* spp. in commercial chicken flocks. Avian Dis. 25:513–516.

Kotula KL, and Pandya Y. 1995. Bacterial contamination of broiler chickens before scalding. J. Food Prot. 58:1326–1329.

Kregel KC. 2002. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol (1985). 2002 May;92(5):2177-86.

Lan PT, Sakamoto M, Benno Y. 2004. Effects of two probiotic Lactobacillus strains on jejunal and cecal microbiota of broiler chicken under acute heat stress condition as revealed by molecular analysis of 16S rRNA genes. Microbiol. Immunol. 48(12): 917-929.

Latimer KS, Tang KN, Goodwin MA, Steffens WL, Brown J. 1988. Leukocyte changes associated with acute inflammation in chickens. Avian Dis. 32(4):760-72.

Ledesma A, de Lacoba MG, Rial E. 2002. The mitochondrial uncoupling proteins Genome Biol. 3:3015. Epub 2002 Nov 29.

Lee CA, Falkow S. 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proc Natl Acad Sci U S A 87:4304–4308. doi:.10.1073/pnas.87.11.4304.

Li H, Decuypere E, Buyse J. 2004. Oxidative stress induced by corticosterone administration in broiler chickens (Gallus gallus domesticus) 2. Short-term effect. Comp Biochem Physiol B Biochem Mol Biol. 139:745-51.

Lu Q, Wen J, Zhang H. 2007. Effect of chronic heat exposure on fat deposition and meat quality in two genetic types of chicken. Poult Sci. 86(6):1059-64.

Luangtongkum T, Morishita TY, Ison AJ, Huang S, McDermott PF, Zhang Q. 2006. Effect of conventional and organic production practices on the prevalence and antimicrobial resistance of Campylobacter spp. in poultry. Appl Environ Microbiol. 72(5):3600–3607.

Luh, SP, Kuo PH, Kuo TF, Tsai TP, et al. 2007. Effects of thermal preconditioning on the ischemia-reperfusion-induced acute lung injury in minipigs. Shock 28: 615-622.

Magwood SE, Rigby C, Fung PH. 1967. *Salmonella* contamination of the product and environment of selected Canadian chicken processing plants. Can J Comp Med Vet Sci. 31(4):88-91.

Malorny BC, Löfström M, Wagner N, Krämer, Hoorfar J. 2008. Enumeration of *salmonella* bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. 2008. Appl Environ Microbiol. 74(5):1299-304.

Mansoub NH, Karim R, Leila M, Mohammad AMN, Seyede LZ, Mehdi MK. 2011. Effect of different level of butyric acid glycerides on performance and serum composition of broiler chickens. World J Zool. 6(2):179–182.

Mashaly MM, Hendricks GL, Kalama MA, Gehad AE, Abbas AO, and Patterson PH. 2004. Effects of heat stress on production parameters and immune responses of commercial laying hens. Poult. Sci. 83:889–894.

Maxwell MH, Robertson GW, Mitchell MA, Carlisle AJ. 1992. The fine structure of broiler chicken blood cells, with particular reference to basophils, after severe heat stress. Comparative Haematology International. 2: 190.

Maxwell MH. 1993. Avian blood leucocyte responses to stress. World's Poult. Sci. J. 49:34–43.

McCormick PH, Chen G, Tlerney S, Kelly CJ, Bouchier-Hayes DJ. 2003. Clinically relevant thermal preconditioning attenuates ischemia-reperfusion injury. J Surg Res. 109:24–30.

McIntyre D, Broomhead DJ, Mathis G, Lumpkins B. 2013. Effects of feeding Original XPC and Salinomycin during a coccidia challenge in broilers. Poult. Sci. 92 (E-Suppl. 1):59-60.

Mitchell MA, Kettlewell PJ. 1998. Physiological stress and welfare of broiler chickens in transit: solutions not problems! Poult Sci. 1998 Dec;77(12):1803-14.

Mitchell MA, Sandercock DA. 1992. Creatine kinase isoenzyme profiles in the plasma of the domestic fowl (Gallus domesticus): effects of acute heat stress. Research in Veterinary Science. 59, 30-34.

Møller AP, Shykoff JA. 1999. Morphological developmental stability in plants: patterns and causes. Int J Plant Sci 160(suppl): S135–S146.

Moore MM, Feist MD. 2006. Real-time PCR method for *Salmonella* spp. targeting the stn gene. Journ Appl Microbiology. 102(2): 516-530.

Morita VS, Almeida VR, Matos Junior JB, Vicentini TI, Van Den Brand H, Boleli IC. 2016. Incubation temperature alters thermal preference and response to heat stress of broiler chickens along the rearing phase. Poultry Science. 2016. 95(9).

Morris GK, Wells JG. 1970. *Salmonella* Contamination in a Poultry-Processing Plant. Appl Microbiol. 19(5): 795–799.

Murphy LB, Preston AP. 1988. Time-budgeting in meat chickens grown commercially. Br. Poult. Sci. 29:571–580.

Nääs I, Miragliotta M, Baracho M, Moura D. 2007. Aerial environment in broiler housing: dust and gases. Engenharia Agricola, 27, 326-335.

Nagel GM, Bauermeister LJ, Bratcher CL, Singh M, McKee SR. 2013. *Salmonella* and Campylobacter reduction and quality characteristics of poultry carcasses treated with various antimicrobials in a post-chill immersion tank. Int J Food Microbiol. 165(3):281-6.

Najafi P, Zulkifli I, Jajuli NA, Farjam SA, Ramiah SK, Amir AA, O'Reily E, Eckersall D. 2015. Environmental temperature and stocking density effects on acute phase proteins, heat shock protein 70, circulating corticosterone and performance in broiler chickens. Int J Biometeorol. 59(11):1577-83.

Northcutt JK, Jones DR. 2004. A survey of water use and common industry practices in commercial broiler processing facilities. J. Appl. Poult. Res. 13:48–54.

EM, Hester PY, Stroshime R. 2003. Bone densitometry as an indicator of percentage tibia ash in broiler chicks fed varying dietary calcium and phosphorus levels. Poult Sci. 82:1787-1791.

Palmer AR, Strobeck C. 1992. Fluctuating asymmetry as a measure of developmental stability: Implications of non-normal distributions and power of statistical tests. Acta Zool. Fenn., 191, pp. 55–70.

Park JH, Kim IH. 2014. Supplemental effect of probiotic Bacillus subtilis B2A on productivity, organ weight, intestinal *Salmonella* microflora, and breast meat quality of growing broiler chicks. Poult Sci. 93(8):2054-9.

Parker D, Hofacre CL, Mathis GF, Quiroz MA, Dibner J, Knight C. 2011. Organic acid water treatment reduced *Salmonella* horizontal transmission in broiler chickens. Centre for Agricultre and Bioscience international. WPSA http://www.cabi.org/Uploads/animal-science/worlds-poultry-science-association/WPSA-italy-2006/10272.pdf

Post J, Rebel JM, ter Huurne AA. 2003. Physiological effects of elevated plasma corticosterone concentrations in broiler chickens. An alternative means by which to assess the physiological effects of stress Poult Sci. 82:1313-8.

Prieto MT, Campo JL. 2010. Effect of heat and several additives related to stress levels on fluctuating asymmetry, heterophil:lymphocyte ratio, and tonic immobility duration in White Leghorn chicks. Poult Sci. 89(10):2071-7.

Proudfoot FG, Hulan W, Ramay DR. 1979. The effect of stocking density on broiler carcass grade, the incidence of breast blisters, and the other performance traits. Poult. Sci. 58:791–793.

Puron D, Santamaria R, Segavra JC, Alamilla JL. 1995. Broiler performance at different stocking densities. J. Appl. Poult. Res. 4:55–60.

Quinteiro-Filho WM, Gomes AVS, Pinheiro ML, Ribeiro A, Ferrazde-Paula V, Astolfi-Ferreira CS, Ferreira AJP, & Palermo-Neto J. 2012. Heat stress impairs performance and induces intestinal inflammation in broiler chickens infected with *Salmonella* Enteritidis, Avian Pathology, 41:5, 421-427

Rasschaert G, Michiels J, Tagliabue M, Missotten J, De Smet S, Heyndrickx M. 2016. Effect of Organic Acids on *Salmonella* Shedding and Colonization in Pigs on a Farm with High *Salmonella* Prevalence. J Food Prot. 79:51-8.

Rimoldi S, Lasagna E, Sarti FM, Marelli SP, Cozzi MC, Bernardini G, Terova G. 2015. Expression profile of six stress-related genes and productive performances of fast and slow growing broiler strains reared under heat stress conditions. Meta Gene. 6:17-25.

Reiter K, Kutriz B. 2001. Behavior and leg weakness in different broiler breeds. Arch. Geflugelkd. 65:137–141.

Riddell C, 1992. Non-infectious skeletal disorders of poultry: an overview. Bone Biology and Skeletal. Disorders in Poultry. 119–145.

Russell JB, Diez-Gonzalez F. 1998. The effects of fermentation acids on bacterial growth. Adv Microbial Physiol. 39: 205–234.

Rychlik I, Barrow PA. 2005. *Salmonella* stress management and its relevance to behaviour during intestinal colonisation and infection. FEMS Microbiol Rev. (5):1021-40.

Ryu ST, Park BS, Bang HT, Kang HK, Hwangbo J. 2016. Effects of anti-heat diet and inverse lighting on growth performance, immune organ, microorganism and short chain fatty acids of broiler chickens under heat stress. J Environ Biol. 37(2):185-92.

Sachse C, Jahns-Streubel G, Henkel E. 1998. First clinical evaluation of the Cell Dyn 3200 haemalology analyser. Clin. Lab. Haematol. 20:333–340.

Sahin K, Sahin N, Kucuk O, Hayirli A, Prasad AS. 2009. Role of dietary zinc in heat-stressed poultry: a review. Poult Sci. 88(10):2176-83.

Sandercock DA, Hunter RR, Nute GR, Mitchell MA, Hocking PM. 2001. Acute heat stress-induced alterations in blood acid-base status and skeletal muscle membrane integrity in broiler chickens at two ages: implications for meat quality. Poult Sci. 80(4):418-25.

Sharma CS, Ates A, Joseph P, Soni KA. Evaluation of antimicrobial effects of lauric arginate on reduction of *Salmonella* spp. in ground chicken. Int. Journ. Food Sci Techn. 48(7):1410-15.

Shini S, Kaiser P. 2009. Effects of stress, mimicked by administration of corticosterone in drinking water, on the expression of chicken cytokine and chemokine genes in lymphocytes. Stress. 12(5):388-99.

Siegel PB, Dunnington EA. 1987. Selection for growth in chickens. CRC Crit. Rev. Poult. Biol. 1:1–24.

Singer RS, Cox Jr LA, Dickson JS, Hurd HS, Phillips I, Miller GY. 2007. Modeling the relationship between food animal health and human foodborne illness. Prev Vet Med. 2-4:186-203. Epub 2007 Jan 30.

Slader J, Domingue G, Jørgensen F, McAlpine K, Owen RJ, Bolton FL, Humphrey TJ. 2002. Impact of transport crate reuse and of catching and processing on Campylobacter and *Salmonella* contamination of broiler chickens. Appl Environ Microbiol. 68(2):713-9.

Sohail MU, Ijaz A, Yousaf MS, Ashraf K, Zaneb H, Aleem M, Rehman H. 2010. Alleviation of cyclic heat stress in broilers by dietary supplementation of mannan-oligosaccharide and Lactobacillus-based probiotic: dynamics of cortisol, thyroid hormones, cholesterol, C-reactive protein, and humoral immunity. Poult Sci. 89(9):1934-8.

Sohail MU, Hume ME, Byrd JA, Nisbet DJ, Ijaz A, Sohail A, Shabbir MZ, Rehman H. 2012. Effect of supplementation of prebiotic mannan-oligosaccharides and probiotic mixture on growth performance of broilers subjected to chronic heat stress. Poult Sci. 2012 Sep;91(9):2235-40.

Sohail MU, Hume ME, Byrd JA, Nisbet DJ, Shabbir MZ, Ijaz A, Rehman H. 2015. Molecular analysis of the caecal and tracheal microbiome of heat-stressed broilers supplemented with prebiotic and probiotic. Avian Pathol. 44(2):67-74.

Soleimani AF, Zulkifli I, Hair-Bejo M, Omar AR, Rahal AR. 2011. The role of heat shock protein 70 in resistance to *Salmonella* Enteritidis in broiler chickens subjected to neonatal feed restriction and thermal stress. Poult.Sci. 91:340-345.

Spratt CD, 1985. Effect of mould inhibitor treated high moisture corn on performance of poultry. M.Sc. Thesis, University of Guelph, Canada.

Sridhar S, Steele-Mortimer O. 2016. Inherent Variability of Growth Media Impacts the Ability of *Salmonella* Typhimurium to Interact with Host Cells. PLoS One. 11(6).

Syafwan SK, Kwakkel RP, Verstegen MWA. 2011. Heat stress and feeding strategies in meat-type chickens. World's Poultry Science Journal 67:653-673.

Tadesse WM, Cízek A. 1994. The isolation of *salmonella*e from poultry carcasses and equipment in the poultry processing plant by means of two procedures. Vet Med (Praha). 1994;39(6):315-20.

Tan GY, Yang L, Fu YQ, Feng JH, Zhang MH. 2010. Effects of different acute high ambient temperatures on function of hepatic mitochondrial respiration, antioxidative enzymes, and oxidative injury in broiler chickens. Poult Sci. 891:115-22.

Thaxton JP, Dozier WA, Branton SL, Morgan GW, Miles DW, Roush WB, Lott BD, Vizzier-Thaxton Y. 2006. Stocking density and physiological adaptive responses of broilers. Poult Sci. 85(5):819-24.

Teeter R. 1993. Effect of Yeast culture in broilers under heat stress and nonspecific antigen challenge. Department of Animal Science Oklahoma State University, Stillwater, OK. Yeast culture poultry research report 2.

Totton SC, Farrar AM, Wilkins W, Bucher O, Waddell LA, Wilhelm BJ, McEwen SA, Rajić A. 2012. The effectiveness of selected feed and water additives for reducing *Salmonella* spp. of public health importance in broiler chickens: a systematic review, meta-analysis, and meta-regression approach. Prev Vet Med. 106(3-4):197-213.

USDA ERS Broiler production area. Accessed Dec. 2015. http://www.ers.usda.gov/topics/animal-products/poultry-eggs/background.aspx

USDA FSIS Draft Compliance Guide: *Salmonella* and Campylobacter Verification Program for Raw meat & poultry products. 2015. Accessed Feb. 2016.

http://www.fsis.usda.gov/wps/wcm/connect/6732c082-af40-415e-9b57-90533ea4c252/Controlling-*Salmonella*-Campylobacter-Poultry-2015.pdf?MOD=AJPERES.

Van Immerseel F, Boyen F, Gantois I, Timbermont L, Bohez L, Pasmans F, Haesebrouck F, Ducatelle R. 2005. Supplementation of coated butyric acid in the feed reduces colonization and shedding of *Salmonella* in poultry. Poult Sci. 12:1851-6.

Varasteh S, Braber S, Akbari P, Garssen J, Fink-Gremmels J. 2015. Differences in Susceptibility to Heat Stress along the Chicken Intestine and the Protective Effects of Galacto-Oligosaccharides. PLoS ONE 10.

Veerkamp CH. 1986. Fasting and yield of broilers. Poult. Sci., 65: 1299-1304.

Wang X, Zhou Q, Shen J, Yao J. 2015. Effect of difference doses of Newcastle disease vaccine immunization on growth performance, plasma variables and immune response of broilers. J Anim Sci Biotechnol. 6(1): 20.

White PL, Baker AR, James WO. 1997. Strategies to control *Salmonella* and Campylobacter in raw poultry products. Rev Sci Tech. 2:525-41.

Wabeck, CJ. 1972. Feed and water withdrawal time relationship to processing yield and potential fecal contamination of broilers. Poult. Sci., 51: 1119- 1121.

Wideman N, Bailey M, Bilgili SF, Thippareddi H, Wang L, Bratcher C, Sanchez-Plata M, Singh M. Evaluating best practices for Campylobacter and *Salmonella* reduction in poultry processing plants. Poult Sci. 95:306-15.

Willemsen H, Swennen Q, Everaert N, Geraert PA, Mercier Y, Stinckens A, Decuypere E, Buyse J. 2011. Effects of dietary supplementation of methionine and its hydroxy analog DL-2-hydroxy-4-methylthiobutanoic acid on growth performance, plasma hormone levels, and the redox status of broiler chickens exposed to high temperatures. Poult Sci. 90:2311-20.

Yang A, Dunnington EA, Siegel PB. 1997. Developmental stability in stocks of white leghorn chickens. Poult. Sci. 76:1632-36.

Young SD, Olusanya O, Jones KH, Liu T, Liljebjelke KA, Hofacre CL. 1997. *Salmonella* Incidence in Broilers from Breeders Vaccinated with Live and Killed *Salmonella*. J. Appl. Poult. Res. 16:521–528.

Zeferino CP, Komiyama CM, Pelícia VC, Fascina VB, Aoyagi MM, Coutinho LL, Sartori JR, Moura AS. 2016. Carcass and meat quality traits of chickens fed diets concurrently supplemented with vitamins C and E under constant heat stress. Animal. 10(1):163-7.

Zhang H, Lin L, Zeng C, Shen P, Huang YP. 2007. Cloning and characterization of a haloarchaeal heat shock protein 70 functionally expressed in Escherichia coli. FEMS Microbiol Lett. 275(1):168-74.

Zhang H, Zhou Z, Luo J, Hou J. 2015. Effects of corticosterone on the metabolic activity of cultured chicken chondrocytes. BMC Vet Res. 11: 86.

Zhu YZ, Cheng JL, Ren M, Yin L, Piao XS. 2015. Effect of γ-Aminobutyric Acid-producing Lactobacillus Strain on Laying Performance, Egg Quality and Serum

Enzyme Activity in Hy-Line Brown Hens under Heat Stress. Asian-Australas J Anim Sci. 28(7):1006-13.

Zulkifli I, Dass RT, Che Norma MT. 1999. Acute heat-stress effects on physiology and fear-related behaviour in red jungle fowl and domestic fowl. Can. J. Anim. Sci. 79: 165–170.2015. FSIS baseline and proposed standards. FSIS. Accessed June 2016. http://www.fsis.usda.gov/wps/portal/fsis/topics/datacollection-and-reports/microbiology/baseline/baseline.