PROBIOTIC SUPPLEMENTATION EFFECTS ON PERFORMANCE, BODY COMPOSITION, AND ENERGETIC EFFICIENCY OF BROILERS UNDER THERMO-NEUTRAL AND HEAT STRESS CONDITIONS

By

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Abstract: Supplementation effect of 3 Oklahoma State University (OSU) *Bacillus* strains were examined on broilers' performance, body composition, metabolic parameters, and respiration rate. The experiment was conducted in 2 experimental phases, 0 to 41 d of age and 21 to 41 d of age, fed diets with and without probiotic supplementation, and exposed to thermo-neutral (TN) and cyclic heat stress (HS), respectively. During phase I, a total of 432 d-old Cobb-500 chicks were transported to the study site and randomly divided into 72 floor pens and fed 2 diets, control (no probiotic) and treatment (probiotic) to 6 wk of age. On d 21, phase II of the experiment was initiated with 52 broilers, fed with and without the OSU probiotic. The broilers were randomly selected from the floor pens and transferred to the OSU Poultry Metabolic Chambers. The birds were subjected to 4 treatments, no probiotic at TN (CTN), probiotic at TN (PTN), no probiotic at HS (CHS), and probiotic at HS (PHS). The HS birds were exposed to 32 ± 1°C from 1800 to 2100 h. Phase I probiotic-fed birds showed an increase ($P < 0.05$) in their BW, body composition, and metabolizable energy retained during the first 2 wk of age. However, mixed results were noted after wk 2. Furthermore, in phase II, PHS showed an improvement ($P < 0.05$) in their wk 6 BW when compared to CHS. Weekly average respiration per min was lower ($P < 0.05$) for PHS when compared to CHS, indicating a limit in these birds ability to dissipate heat and maintain homeostasis. Based on obtained results, the fed probiotic improved performance in the early life of broilers. In addition, broilers exposed beyond optimal ambient temperatures found relief when the diet included OSU probiotics. These results suggest continuous probiotic supplementation in a TN environment has no economic merits. Probiotics may give more benefits to broilers when supplemented the first few days during ration changes and in stressful management scenarios, such as HS. Further research is needed to investigate the effect of probiotic supplementation for the first few days of each diet change.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Statement of the problem

There has been an increase in the supplementation of probiotics in human and animal nutrition. The use of probiotics is said to be an alternative solution for the use of sub-therapeutic antibiotics (Verstegen and Williams, 2002). Sub-therapeutic antibiotics are typically used to prevent disease and aid in BW gain (Dibner and Richards, 2005). Some consumers view sub-therapeutic antibiotics negatively. This is because of growing evidence that antibiotic resistance genes could be transmitted from animals to humans (World Health Organization, 2000; Greko, 2001). Therefore, less are fed today. Today, many fast food chains and restaurants state that they do not accept meat from chickens grown with antibiotic growth promoters (AGP) (Dibner and Richards, 2005). Furthermore, the Food and Drug Administration (FDA) is putting more rules in place for medically important products used in both human and animal nutrition (FDA, 2012, 2016, 2017). While the use of AGP's definitely plays a role in poultry production and phasing out their use can decrease finishing weights, this coupled with, the negative effects of heat stress (HS) can decrease production substantially.

Heat stress can be detrimental in tropic and subtropical regions of poultry production (Lin et al., 2006). In the United States alone, HS results in an estimated total

annual economic loss of $128 to $165 million for the poultry industry. The livestock industry suffers a total annual loss between $1.69 and $2.36 billion (St-Pierre et al., 2003). Eleven states in the United States are considered subtropical. Out of those 11 states, 10 are in the top 15 for United States broiler production. These 10 states make up 73% of total broiler production in the United States (National Chicken Council, 2010). Probiotics have shown many beneficial properties with the ability to improve immunity, intestinal architecture, and gut barrier function in broilers. These factors can improve digestion and absorption, which ultimately can increase performance results during HS (Al-Zenki et al., 2009; Larsson et al., 2012). There have been several studies using probiotics in animal nutrition, but very few studies focused on the effects of probiotics in the broiler's diet, as an alternative for AGP's, and as a tool to alleviate consequences of HS.

Purpose of the study

The objectives of this study were:

1. Investigate probiotic supplementation effects on performance, body composition, and metabolic parameters of broilers from 1 to 6 wk of age.
2. Investigate probiotic supplementation effects on performance, body composition, metabolic parameters, and respiration rate of broilers raised during cyclic HS from 3 to 6 wk of age.

Hypotheses

The null hypothesis of this study is as follows:

1. There is no significant effect on performance, body composition, or metabolic parameters with the addition of a *Bacillus* based probiotic compared to the control treatment of Cobb 500 broilers from 1 to 6 wk of age.
2. There is no significant effect on performance, body composition, metabolic parameters, or respiration rate with the addition of a *Bacillus* based probiotic compared to the control treatment of Cobb 500 broilers raised under cyclic HS from 3 to 6 wk of age.

The alternative hypothesis of this study is as follows:

If null hypotheses are rejected, then the effect of a *Bacillus* based probiotic added to a commercial broiler diet will be explained by increased performance data from 1 to 6 wk of age. In addition, differing levels of protein and fat accretion, or improved levels of efficiency, will be explained by metabolizable energy consumed (MEC), metabolizable energy retained (MER), and heat production (HP) for broilers raised in an ideal management situation. Furthermore, the effect of a *Bacillus* based probiotic added to the diets of 3 to 6 wk broilers during cyclic HS will be explained in terms of improved levels of performance variables. Improved levels of protein accretion, fat accretion, and metabolic parameters will explain the effect the *Bacillus* based probiotic has on efficiency and providing more energy for expenditure towards production.

Assumptions

The alternative hypothesis will use the following assumptions to explain conclusions from the study. The first phase of the study expects to show that probiotic supplementation will aid broilers in performance data such as BW, feed intake (FI), feed conversion ratio (FCR), and average daily gain (ADG). The second phase of the experiment expects that broilers raised under HS conditions and supplemented with probiotics will exhibit improvement in their performance, which may be comparable to both treatments raised under thermo-neutral conditions with and without probiotic supplementation. This outcome would indicate supplementation of broilers with probiotics during acute HS conditions might help improve performance of broilers. This information is important to poultry farmers and managers in making managerial decisions when raising broilers during warmer environmental conditions.

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CHAPTER II

REVIEW OF LITERATURE

Introduction

In poultry, as well as other intensively managed livestock, the nutritional requirements of animals are met by supplementing limiting nutrients in a concentrated form to the feed. The addition of feed additives used directly with available nutrients from the feed allow for a higher quality ration, which the animal may be able to use more efficiently (Luis, 2003). Productivity of farm animals is not only influenced by nutritional factors such as the nutrient content of the feed, but also by palatability and digestibility of the feed and presence of non-nutritional factors. Moreover, non-nutritional factors such as hygiene, processing of feed ingredients, ambient temperature, animal health, and genetic makeup have an impact on animal performance (Jacob, 2015). The poultry industry has achieved tremendous progress in their production system during the last 50 years through improvements in genetic makeup, proper management, and advancements in nutritional science. The use of feed additives has increased and contributed to the success achieved in current broiler production (Fanelli, 2012).

Feed additives are generally considered materials used to enhance the effectiveness of nutrients and exert their effects in improving animal performance in healthy livestock. There are a number of feed additives used in animal feeds such as antibiotics, probiotics, oligosaccharides, enzymes and organic acids (Windisch et al.,

2008). They are included in the diet of animals for promoting animal growth through their potential effect in increasing feed intake (FI) (Demir et al., 2003). In addition, low levels of additives in animal feed can contribute to increase in production of animal protein for human consumption, which in some instances can decrease the cost of animal production (Walsh et al., 1993).

History and health benefits of fermented foods

Humans have consumed food with live microbial activity for thousands of years. Most likely, the first fermented food consumed was milk. However, the intentional practice of eating fermented foods, which contain microorganisms to produce beneficial properties, started during the 20th century (Morelli and Capurso, 2012). Today, yogurts are a popular source of probiotics and the public sees them as a benefit to a healthy lifestyle (Lourens-Hattingh and Viljoen, 2001).

The first investigator in the area of fermentation and probiotics was Eli Metchnikoff who worked at the Pasteur Institute in Paris. He reported the existence of increased human longevity by drinking large amounts of soured milk in Bulgarian peasants. This strengthened Metchnikoff's belief that the lower gut and overall health would be affected by microbes from the soured milk. Following this realization, he tested cultures of milk that were fermented by the *Lactobacillus* genus. For instance, *Lactobacillus bulgaricus* later became the strain popular for fermenting yogurt (Fuller, 1992).

Probiotics are considered live microbial feed supplements that can benefit the animal, otherwise known as the host. The word 'probiotic' means 'for life' and originated

from the Greek language (Fuller, 1992). Other sources have broken the word down more extensively, stating the pro stem is of Latin origin, meaning *in favor of*. The bios portion, which means *life*, was derived from the Greeks too (Morelli and Capurso, 2012). The meaning of probiotics have changed over the years (Fuller, 1992). In 1953, Werner Kollath offered the scientific community the term 'probiotika.' His definition of the term stated live microorganisms are essential for healthy development of the gut for life. In 1965, Lilley and Stillwell redefined probiotics. They described probiotics as microorganisms which would aid in the growth of other beneficial microorganisms in the gut (Vila et al, 2010). They are given the majority of credit for today's meaning. This definition caused it to have the opposite meaning of today's antibiotic (Fuller, 1992). Antibiotics inhibit the growth of bacteria by introducing a chemical substance (Waksman, 1947). Guarner and Schaafsma described probiotics as the consumption of sufficient live microorganisms with the ability to contribute health benefits to the host (Morelli and Capurso, 2012). This added even more refinement on the term probiotic.

Effectiveness of probiotic supplementation can be attributed to the species of microbes and the form of supplementation used, such as wet or powdered (Food and Agricultural Organization and World Health Organization, 2001). Furthermore, scientific experts concluded that properties, benefits, and purposes of identified probiotics are individualized and specific to each strain. Also, unique strains ingested by the host have induced effects which may cause other reactions in the body (Morelli and Capurso, 2012). For instance, bifidobacteria can release metabolic end products, such as acetate and lactate, which can decrease both gram-positive and gram-negative pathogenic

microbes. More research needs to be completed to learn about metabolic effects that are induced by bacteria like bifidobacteria (Gibson and Roberfroid, 1995).

Sources of probiotics vary but they can be isolated from milk, fermented foods, feces, or the gut microbiota of different animals (Fontana et al., 2013). The main two sources of probiotics isolated from traditional fermented products are species of lactic acid bacteria and bifidobacteria, but many other probiotic sources can be identified and used commercially (Morelli and Capurso, 2012). Species of lactic acid bacteria have become popular for human use because they can improve the ability to digest lactose if the individual is lactose intolerant. These lactic acid species have other proposed benefits, but none have been completely proven. Still, suggested benefits include prevention of certain cancers, decreased intestinal infections, and decreasing serum cholesterol levels. Furthermore, species of lactic acid bacteria have been utilized to improve health and growth of food animals (Gilliland, 1990). Bifidobacteria has health promoting functions which include lowering blood cholesterol levels, attacking malignant cells, decreasing blood ammonia levels, and producing many B vitamins (Gibson and Roberfroid, 1995), which can directly affect metabolism of proteins, carbohydrates, and lipids.

The technological use of fermentation to produce final probiotic products has made it possible to produce large scale quantities for commercial companies (Ghani et al., 2013). The bacterial strain *Bacillus licheniformis* under aerobic conditions can produce a natural polypeptide antibiotic called bacitracin (Kayalvizhi and Gunasekaran, 2008; Anthony et al., 2009). *Bacillus licheniformis* also has the ability to produce bacitracin under anaerobic conditions and can thrive with little oxygen (Pattnaik et al., 2001). Aerobic strains of *Bacillus subtilis* can reproduce anaerobically when they use nitrate or nitrite as

an electron acceptor. The other mode of anaerobic proliferation is by fermentation (Zhang et al., 2002; Feng et al., 2003; Hmidet et al., 2009).

Prebiotics

Prebiotics are used in both human and animal nutrition (Grajek et al., 2005). They are non-digestible food ingredients with potential of stimulating and increasing existing host microbes, already residing in the gut or colon, such as a probiotic bacterium (Gibson et al., 1995; Crittenden and Playne, 1996). Prebiotics are often used in conjunction with probiotics, which is referred to as synbiotics. These fibrous feed ingredients (prebiotics) that are low in digestibility will have their effect throughout the gastrointestinal tract (GIT) and encourage growth of beneficial microbes such as bifidobacteria. Some examples of prebiotics include galacto-oligosaccharides, lactulose, lactosucrose, fructo-oligosaccharides, palatinose (isomaltulose) oligosaccharides, glucosyl sucrose, malto-oligosaccharides, isomalto-oligosaccharides, cyclodextrins, gentio-oligosaccharides, soybean oligosaccharides, and xylo-oligosaccharides (Crittenden and Playne, 1996).

Often, prebiotics are used in conjunction with probiotics in order to increase the activity of probiotic bacterium. The main products produced from these short chain carbohydrates (prebiotics) are short chain fatty acids (SCFA). These SCFAs, composed of acetate, butyrate, and propionate, are potential substrates, involved in energy creation during their metabolism in both animals and humans (Grajek et al., 2005).

Macfarlane et al. (2006) indicated that prebiotics are cheaper, less risky, and easier to incorporate into common animal diets compared to probiotics. Prebiotics are more easily applied in feed without having to worry about survivability of

microorganisms ingested by the host to provide benefits, like probiotics. Furthermore, production is cheaper because isolation of prebiotic sources from plant sources is relatively easy (Macfarlane et al., 2006).

Factors affecting GIT balance and probiotic success

In children, there are several factors which have direct effect on the function and structure of the gut microbiota including: exposure in early infancy, route of delivery during birth, gestational age, and levels of antibiotics taken by the mother during the prenatal period. In livestock species, factors might include feeding practices, composition of the ration formulated, stress, and management. All of these factors influence the microbial balance in the gut of animals (Gareau et al., 2010).

There is growing evidence that feeding certain species of probiotics will have very different results. Moreover, a number of factors will influence results in probiotic research trials, including administration level, species of livestock, application method, age of livestock, environmental stress factors, and diet formulation. Some of these are similar to the influential variables listed that might have an effect on the microbial balance of the gut in animals. Scientists speculate to feed probiotics and have a beneficial effect in the GIT requires in vitro and in vivo studies to be fully successful. Researchers should take isolations from the microflora of an animal's healthy gut to understand the types of microbes residing in the small intestine. This is hypothesized to have a better impact when feeding a direct-fed microbial (DFM) (Mountzouris et al., 2007). Probiotic species isolations from different parts of the GIT have been performed in poultry, pigs, and rats (Fontana et al., 2013).

Use of antibiotics as growth promoters in farm animals

Antibiotics fed at sub-therapeutic levels to improve growth and, ultimately, efficiency in commercially raised animals has been practiced for more than 50 years. Studies in the early 1950's showed improved performance (Dibner and Richards, 2005). Starr and Reynolds (1951), as well as Barnes (1958) reported almost immediately on the possibility of resistance after their initiated use in animal production. These studies concluded an association with resistance towards the antibiotics Streptomycin in turkeys and Tetracycline in broilers. Concerns about antibiotic resistance towards human pathogens arose and discussion to ban the sub-therapeutic use of antibiotics started around 1969 (Dibner and Richards, 2005).

It should be noted that by 1997 farming in Europe was the second largest user of antibiotics after human medicine. Thirty three percent of those antibiotics came from the sub-therapeutic or supplemental category of animal feeding (Hong et al., 2005). In 2006, the European Union banned the use of sub-therapeutic antibiotics (Franz et al., 2010; Huyghebaert et al., 2011). Antibiotics in the United States are more highly regulated today than they were in the 20th century (Hong et al., 2005).

Antibiotic resistance genes can be transmitted from animal to human microbiota (Greko, 2001). Suggestions in the industry include improved animal health management to avoid the use of antibiotics fed at low levels for prevention rather than treatment. Therefore, World Health Organization (WHO) (2000) stated producers should take responsibility to keep detailed records of antibiotic use, increase hygiene and disinfection of facilities, increase bio-security measures, change in stocking rate if necessary, and

increase implementation of vaccination protocols. Furthermore, WHO voiced that antibiotics should be limited and dispensed by prescription only. These ideas are based on the potential for bacterial populations, like enterococci, to become resistant in food animals, which might be transferred to humans (World Health Organization, 2000). As production will have to change if antibiotic growth promoters (AGP) are phased out, the use of feed technologies like prebiotics, probiotics, or a mixture of both, known as synbiotics, (Hong et al., 2005) will probably also increase.

Medically important products used in both animals and humans include Penicillins, Cephalosporins, Quinolones, Fluoroquinolones, Tetracyclines, Macrolides, Glycopeptides, and Sulfas (Table 2 and Table 3) (FDA, 2012; FDA, 2016). All of these listed antibiotic products now require a veterinary feed directive (VFD). The VFD rule was modified January 1, 2017. A VFD is a prescription written by a veterinarian for a producer for the use of regulated feed additives in rations. This encourages a strong veterinary client patient relationship (VCPR) and will aid in decreasing bacterial resistance to medically important products used in both human medicine and animal production (FDA, 2012; FDA, 2016). All VFD drugs are classified as category II animal drugs requiring a medicated feed mill license (MFML) for inclusion of Type A medicated feed in rations. Category II drugs have a withdrawal period and are regulated on a "no-residue" basis. This is due to the fact they are of carcinogenic concern. In contrast, category I drugs require no withdrawal period for minimum supplementation levels in each major species (FDA, 2016; FDA, 2017).

Gut health and antibiotics

Animal performance and feed efficiency are closely linked to the microbial health in the animal's gut (Huyghebaert et al., 2011). The intestinal walls morphology contributes to gut health, as well as the activity and strength of the immune system. The gut mucosa in the digestive system is made up of digestive epithelial cells, gut-associated lymphoid tissues, and the mucosal lining that is arranged on top of the epithelium. All of these components, coupled with commensal and transient bacteria, should cooperate with one another to produce an equilibrium within the gut that ensures a well-working digestive tract (Conway, 1994; Van Dijk et al., 1999). For instance, gut-associated lymphoid tissues are an important line of defense between the external environment of the intestine and substances permitted to pass (Targan, 1992; Brom, 2010). Feed and additives can stabilize or create disorder for the microflora, which will affect the structure and function of the gut (Conway, 1994Van Dijk et al., 1999). This stabilization or disorder ultimately can contribute to absorption, because it primarily occurs in the mucosa of the small intestine (Turk, 1982).

Antibiotics can have effects on the physiology of the gut, some, which might be considered positive, and others negative. For example, AGP's increase the uptake of nutrients because of a thinner intestinal wall barrier associated with fed antibiotics (Francois, 1962). Antibiotics can also directly reduce microbes in the gut, which require energy and protein. Additionally, indirect effects include production of metabolites like aromatic phenols, ammonia, and bile degradation products, which can cause less intestinal inflammation. Both indirect and direct effects can increase feed conversion (Gaskins et al., 2006). Antimicrobial growth promoters are an asset to make meat cheaper

in conventional farming of many livestock species (Huyghebaert et al., 2011). On the downside, when gut lining thins from extensive and repeated antibiotic treatment it can compromise gut health and create microflora disturbances (Brom, 2010).

Alternatives for antibiotics

There are several current technologies being marketed as a source to improve FI and efficiency without sub-therapeutic antibiotics (Allen et al., 2013). Some of these technologies include organic acids, probiotics, enzymes, prebiotics, etheric oils, and immunostimulants (Huyghebaert et al., 2011). Below are multiple studies which include these technologies.

Organic acids have shown to lower the cases of necrotic enteritis (Timbermont, 2009). Necrotic enteritis is a common problem in the poultry industry and is caused by *Clostridium perfringens.* A report by Garrido et al. (2004) found that a mixture of sodium lignosulfonate, formic acid, and propionic acid sprayed on the litter decreased ($P < 0.05$) *Clostridium perfringens.*

Another study compared treatments in broilers fed diets containing Flavomycin, thyme, garlic, an enzyme complex, enzyme and flavomycin mixture, enzyme and thyme mixture, and enzyme and garlic mixture to identify its impacts on growth, carcass traits, total plasma cholesterol concentration, intestinal traits, and the dry matter excreta. Overall hot carcass yield was the highest in birds fed the enzyme and flavomycin mixture, but still similar to the basal diet, flavomycin, and the enzyme and thyme mixture. The combined addition of flavomycin and enzyme complex resulted in the lowest ($P < 0.05$) *E. coli.* concentration from small intestine samples compared to all

other treatments. The poorest performing treatment was the thyme supplemented diet, which may indicate that herbs may need to be supplemented with a mixture of enzymes or an antibiotic to reap the benefits in performance data (Sarica et al., 2005).

The effect of probiotics, (Protexin – A mixture of Lactobacillus *spp*., Bifidobacterium *spp*., Enterococcus *spp*., Streptococcus *spp*., and Aspergillus *spp*.) organic acids (Genex - propionic, formic acid salts, vegetable essential oil, mineral salts) or antibiotics (Flavomycin) supplemented feed on broiler performance were evaluated by Denli et al. (2003). Supplementation with probiotics (Protexin) resulted in the highest intestinal weight, but also the highest intestinal length in broilers reared to 42 d of age. However, a combination of antibiotics (Flavomycin) and organic acids (Genex), resulted in the highest numerical increase for performance (BW gain, FI, and carcass weight). The study also found that carcass yield, liver weight, and intestinal pH were not significant across all treatments (Denli et al., 2003).

Enzymes as an alternative source for antibiotics

Enzymes have become popular in the feed industry during the last 20 years (Choct, 2006). Common enzymes used in the poultry industry include amylase, protease, lipase, phytase, non-starch polysaccharides (NSP) degrading enzymes, and cellulase (Modyanov and Zel'ner, 1983; Kirk, 2007). Using enzymes in animal production is used to improve feed efficiency (Modyanov and Zel'ner, 1983; Odetallah, 2000). While animals have the ability to naturally produce enzymes for digestion, the use of supplemental enzymes has been prevalent in cereal-based feed for monogastric animals, such as hogs or chickens in order to improve digestion. These animals, contrary to the

ruminant, are unable to efficiently utilize plant based feeds that are high in cellulose and hemicellulose content (Kirk, 2007). However, there is still much to be understood about what benefits enzymes can induce in the host. When enzymes are supplemented in feeds they have shown a reduction in intestinal viscosity, which is a major factor limiting growth and performance in broilers (Bedford and Morgan, 1996).

Pentosanases

Today, pentosanases (NSP enzyme) are commonly used in poultry and swine diets that contain wheat, barley, rye, and oats (Fischer and Classen, 2000; Choct, 2006). When pentosanases first became popular, nutritionists were using enzymes to improve efficiency, overall digestibility, and absorption of nutrients (Campbell and Bedford, 1992; Lei and Stahl, 2000). Pentosanases have also shown the ability to influence the intestinal microflora towards a more balanced state (Fischer and Classen, 2000). According to the report by Fischer and Classen (2000), broilers fed a wheat-based diet and supplemented with xylanase were lower in their bacterial count taken from the small intestine than birds that received no supplementation. Enzymes have the ability to reduce microbial population in the small intestine, which changes the entire balance of the gut (Choct et al., 1995; Dunn, 1996).

Glycanases

Enzymes, specifically glycanases (Choct, 2006), have beneficial properties to remove anti-nutritive components of NSP, like arabinoxylans and β-glucans (Campbell and Bedford, 1992). Glycanases hydrolyze polysaccharides, specifically polysaccharides that make up glycoproteins. Put simply, glycanases are enzymes that degrade

carbohydrate sources (Choct, 2006). This positively effects growth by increasing digestion.

Phytase

Phytase, an NSP-enzyme, increases the utilization of organic phosphorus from the plant (Lei and Stahl, 2000). This is because phytase increases the ability to digest phytate by 25 – 40%. Phytate irreversibly chelates divalent cations and can decrease amino acid absorption in the GIT of birds (Odetallah, 2000). Phytase also decreases the indigestible nutrients found in excreta. Including phytase in the diet can reduce problems associated with wet droppings, including increased dirty eggs from the layer industry, increased ammonia production, and increased fly and rodent population in the house. Phytase is utilized by the swine and poultry industry for its environmental properties of reducing the amount of phosphorus in the excreta. These properties made phytase addition popular in other feeds including corn, soybean and sorghum (Choct, 2006).

Amylase

This NSP-enzyme is primarily used to improve starch digestibility and is fed in corn and soybean meal based diets (Odetallah, 2000). Young, rapid growing animals in swine or broiler production may reap more benefits from the addition of this enzyme when the pancreas is lower in its production of enzymes and there is less amylase acting in the small intestine. Enzyme production from the pancreas can be decreased due to weaning and as age of the animal rises enzyme productions increases due to tissue weight and enzyme activity per gram of tissue (Lindemann et al., 1986). However, reports have been mixed in the effectiveness of the particular enzyme. There have been studies

reporting the use of crude amylases supplemented to growing pigs increasing growth, but there is some doubt this was the only enzyme having an effect, as it is likely the ration also contained β-glucanase (Campbell and Bedford, 1992). The importance of amylase supplemented to monogastric diets is still debatable, because it seems the amylase secreted in the small intestine is substantial compared to what is offered in the feed (Lindemann et al., 1986).

Protease

This NSP-enzyme is most commonly added in corn and soybean diets (Odetellah, 2000). The addition of both protease and amylase, have shown significant improvements ($P < 0.05$) in efficiency and performance (Burrows et al., 2002; Greenwood et al., 2002). Greenwood et al. (2002) found a significant increase ($P < 0.05$) in BW for broilers at d 14 and d 42 that were fed an addition of protease, amylase, and xylanase. The diet was corn and soybean meal based. The addition of these enzymes were included in the starter diet, which agrees with Lindemann et al. (1986), who found the addition of enzymes for young, rapid growing animals has the most substantial effect.

Lipase

Lipase is a NSP-enzyme typically used in corn or soybean meal formulated diets (Odetallah, 2000). It is primarily utilized to aid in lipid digestion endogenously (Polin et al., 1980; Krogdahl and Sell, 1989). A report by Krogdahl and Sell (1989) found that turkeys fed a low fat diet compared with the control were significantly lower in their lipase activity ($P < 0.05$) in the intestinal contents after 28 d of age compared to the control fed a normal fat diet. Furthermore, as the bird matured lipase activity increased

most noticeably after d 14. However, in the finisher phase, lipase activity started to plateau prior to completion of the experiment (Krogdahl and Sell, 1989). These phases of production indicate that as the animal deposits more fat, endogenous lipase becomes more active as it is more extensively used in the digestive processes.

Cellulase

Cellulase acts to break down cellulose of the plant cell wall to glucose, cellobiose, or cellooligosaccharides (Murad and Azzaz, 2010). Cellulose is commonly associated with lignin and pentosans. Therefore, cellulase must gain access by permeating the layer of lignin before it can act on cellulose (Sears and Walsh, 1993). Cellulase can be produced by several different microorganisms, including bacterias and fungis (Suto and Tomito, 2001).

Challenges feedings enzymes

One of the challenges associated with using enzymes in feed is the pelleting process to be practical. Pelleting is performed at high temperatures in the feedmill, roughly 80°C. While pelleting is only performed for short periods of time, it still causes many of the enzymes to denature (Kirk, 2007).

Probiotics and their relationship to enzymes

Enzymatic production by different strains of bacteria has caused rapid growth and advancement in the field of probiotics. *Bacillus licheniformis* strains have been heavily used in the industry because of its ability to produce amylase, alkaline, protease, keratinase and B-mannanase (Zhang et al., 2002; Feng et al., 2003; Hmidet et al., 2009).

A study conducted by Sohail et al. (2011) utilized 250 broiler chicks under either thermo-neutral (TN) conditions or heat stress (HS) conditions. The birds were divided into 5 groups and after d 21, HS was administered up to 42 d to some of the treatments. The treatments included a TN basal diet, a HS basal diet, a HS basal diet supplemented with 0.5% MOS (Alltech, Lexington, KY), a HS basal diet supplemented with 0.1% PM (Probiotics International consisting of *Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus rhamnosus, Bifidobacterium bifidum, Streptococcus thermophilus, Enterococcus faecium, Aspergillus oryzae,* and *Candida pintolopesii*, with a minimum combined total of 6×10^7 colony forming units (cfu) /g of product), or a HS basal diet supplemented with combination of the prebiotic and probiotic, as a synbiotic. The TN, basal treatment was significantly the highest for the paraoxonase enzyme ($P < 0.05$) compared to all the HS treatments. However, the probiotic mixture and synbiotic group were numerically the highest in paraoxonase when compared back to the prebiotic and basal HS treatments, but it was not completely effective in increasing all enzyme levels measured. The study also measured total oxidants and antioxidants which were decreased ($P < 0.05$) with dietary supplementation, but did not affect enzyme levels. The study also concluded that some detrimental effects of HS could be reduced by the prebiotic, probiotic or synbiotic mixture (Sohail et al., 2011).

Another study by Mountzouris et al. (2007) raised 400 d-old Cobb broilers, which were separated into four treatments for a 6 wk long experiment. The purpose of the study was to research the total impact of a probiotic mixture on performance and cecal microbial ecology. The diet was corn and soybean meal based and the treatments

consisted of a basal diet, a probiotic in feed and water (administered at 1 g/kg of feed continuously and in water for scheduled periods for the first 4 wk), a probiotic in feed (fed continuously at 1 g/kg of feed for the first 4 wk), and an antibiotic (Avilamycin at 25 mg/kg of feed). The probiotic product (Biomin Poultry5Star) was composed of the following probiotic bacteria: *Lactobacillus reuteri*, *Enterococcus faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici,* and *Lactobacillus salivarius.* There was a total bacterial count of 2 x 10^{12} cfu/kg of product. The results of the experiment showed that β-Galactosidase enzyme activity from cecal digesta of 42-d-old broilers was numerically the highest for both probiotic treatments compared to the basal diet and significantly higher ($P < 0.05$) than the antibiotic treatment (Mountzouris et al, 2007).

Mode of action for antibiotics and alternatives

Antibiotics

Antibiotics can be utilized to improve performance while having several actions including decreased infections, reduced growth depressing microbial communities, reduced microbe use of nutrients, and a higher degree of nutrient uptake. Animals fed higher levels of antibiotics traditionally have a slender villus structure and less lymphoid elements (Gaskins et al., 2006). According to Reynolds (1989), vancomycin, a glycopeptide antibiotic, first increases precursors required for cytoplasm. Then, formation of the subunit required on a lipid develops. Finally, the subunit on the lipid is moved to the outer most surface of the membrane. This creates a growing glycan chain that attaches to a wall subunit by a reaction and is linked to a mature wall (Reynolds, 1989). This process will have a direct effect on the gut microbiota. Antibiotic growth promoters

can decrease competition for nutrients and microbial metabolites by eliminating other microorganisms (Viesk, 1978; Anderson et al., 1999). This reduction of microorganisms leaves more excess nutrients to be absorbed in the small intestine of the bird, which can be utilized toward net energy gain (Coates et al., 1955).

Organic Acid

Organic acids can be isolated from both plant and animal tissues (Timbermont, 2009). Organic acids can be obtained through fermentation of carbohydrates by taking caeca samples in birds (Van Der Wielen et al., 2000). Organic acids can diffuse into cell cytoplasm. The acid will dissociate within the cell's cytoplasm (pH of roughly 7), and it decreases bacterial cell enzymes like decarboxylases and catalases (Adams and Hall, 1988; Van Immerseel et al., 2006). Monogastric animals with bacterial probiotics in the gut can produce additional organic acids such as lactic and acetic acid. Strains of bacterial probiotics then can assist in a decreased pH in the gut. This can make the microbiome more favorable in its environment for some resident microorganisms, which also decreases pathogen colonization. Furthermore, strains are competitive in nature and have characteristics to exclude pathogenic bacteria (Chaucheyras-Durand and H. Durand, 2010; La Ragione et al., 2003, 2004).

Probiotics and prebiotics

Probiotics are ingested by the animal and create physiological changes in the intestinal tissue structure; this causes immunological variations in the GIT. These immunological changes enhance the animal's resistance to pathogenic bacteria. Probiotics may be able to produce short organic fatty acids and metabolites with

antimicrobial activity. These metabolites may activate receptor sites to stimulate the immune system (Madsen et al., 2001; Sherman et al., 2009). Rolfe (1991) summarized four major factors that induce the development of a microflora which prefers beneficial microorganisms. These bacteria allow for the expression of several mechanisms which decrease the amount of pathogens from inhabiting the intestinal tract. These factors include: (a) development of an intestinal ecosystem that is antagonistic to other bacterial species, (b) removal of existing receptor sites, (c) secretion of antimicrobial metabolites, and (d) competition for nutrients (Rolfe, 1991).

Prebiotics have selective activation to grow intestinal microbials like *Bifidobacteria* and *Lactobacillus spp*. A prebiotic will not be degraded by enzymes or absorbed in the upper part of the GIT. This feed additive will act as a substrate to induce growth of beneficial bacteria, creating luminal or digestive effects that are positive to the animal's health. Prebiotics indirectly provide the host with metabolic substrates and micronutrients because of their ability to stimulate microbial growth (Gibson and Roberfroid, 1995).

Probiotics, acidity, and pH

Certain strains of probiotics have the unique ability to survive extreme environments in their hosts. They are able to travel through the GIT and remain viable when exposed to particularly acidic environments such as stomach acid and bile (Smith, 2014). This is challenging as the stomach pH of many animals ranges from 1.5 to 3.0. Even more, there are bile salts and several gastric, intestinal enzymes, which cause breakdown of the microbes (Fontana et al, 2013). Vegetative cells of probiotic bacteria

stand little chance passing through the stomach because of its extreme environment. However, current evidence shows that spores germinate and survive throughout the GIT. Bacteria can adhere to feed particles to help protect it in its passage through the animal's body. Re-sporulation is the easiest method of transit for bacteria to survive transit throughout the animal's body. The diet of the animal seems to affect the ability of the spore to germinate and proliferate as the spores depend on plentiful nutrients to flourish (Hong et al., 2005).

Spore formers

Spore formers are known for their ability to germinate, produce more bacteria and then re-sporulate. They have the ability to reproduce and survive even during nutrient limitation. Spore-forming bacteria, like *Bacillus*, have the ability to survive transit into the gut and proliferate. The field of microbiology is still researching to understand whether the vegetative probiotic cell produces the beneficial effect, or the actual spore created by the vegetative cell (Hong et al., 2005).

Firmicutes are a phylum of bacteria which are mostly categorized as gram positive in their cell wall structure. *Bacillus* are firmicutes with round cells and a rod-like form. Many different Firmicutes are known for their production of endospores, which are often resistant to dry conditions where water is minimal. They are known to survive extreme conditions and can be found in many different environments (Whitman, 2009).

Spore formers with probiotic effects include species of *Sporolactobacillus*, *Brevibacillus*, and *Bacillus* (Sanders et al, 2003). These have become more utilized by the animal industry as they can undergo more intense processing methods. For instance,

Bacillus is easier to distribute as a DFM as it is tougher and can withstand a larger temperature range at the feed mill. Additionally, it offers a longer shelf life (Chaiyawan et al., 2010). In production practices, this is vital in the ability to produce large amounts of probiotic supplements that will survive when fed to the animal (Nguyen et al., 2015). According to Nguyen et al. (2015), 7 different strains that had high sporulation efficiency of more than 90% could undergo heat treatment of 80°C for 20 minutes. Seven tested *Bacillus* strains were able to hydrolyze starch rapidly and metabolize glucose after this heat treatment (Nguyen et al., 2015).

It has been determined that wild type and laboratory strains of *Bacillus subtilis* (spore formers) are of different origins, but are still within the same species. This can cause a difference in the response from the DFM. Reports have confirmed that origins have effects on biological functions. For instance, the activity of antimicrobials and susceptibility to antibiotics can be heavily affected just by the origin difference in two distinct *Bacillus* strains (Chaiyawan et al., 2010).

Probiotics and the GIT

A desired characteristic of a DFM is that it is nonpathogenic and can increase the number of beneficial colonies in their host (Smith, 2014). This is important because it creates either a commensal or symbiotic relationship. Commensal relationships refer to the interaction between the nonpathogenic bacteria and host coexisting, but obvious benefits are not always apparent. A symbiotic relationship is between two different species, where at least one species benefits without causing a negative effect to the other partner (Hooper and Gordon, 2001). For example, oral inoculation of Lactobacillus

plantarum induced significant levels of tetanus toxin fragment C specific immunoglobulin G, which caused immune responses in respect to the expressed antigen, thus causing a symbiotic relationship (Shaw et al., 2000). In contrast, sometimes probiotics can cause GIT disorder and infections in immunocompromised people. Hata et al. (1988) reported that *bifidobacterium* caused a meningitis case in an infant child. Thus, not all probiotics are neutral or positive in their effects and must be selected carefully.

The GIT plays a significant role in the birds' success in health and growth during its estimated 45-d production phase. Microbial communities in the gut are essential for host nutrition and performance (Sohail et al., 2015). For instance, *Bacillus subtilis* strains of live microorganisms or probiotics have been fed to poultry to improve gut health, secretions of IgA's from the duodenum and improved feed conversion ratio (FCR) (Amerah et al., 2013). Amerah et al. (2013) fed two diets with and without probiotics. These included a basal diet and a supplemented diet with three *Bacillus subtilis* strains (BS8, 15AP4 and 2084; Enviva Pro 202 GT, Danisco Animal Nutrition). On d 21, the probiotic supplemented treatment that received pelleting temperatures at 85 or 90° C increased IgA's by 61 and 51%, respectively. On d 42, FCR for probiotic-supplemented birds was 2.3% higher than the control (Amerah et al., 2013).

Nyguyen et al. (2015) reported *Bacillus* species identified in the intestinal tract of the chicken included *Bacillus subtilis, Bacillus pumilis, Bacillus firmus* and *Bacillus cereus*. Furthermore, feces collection indicated a wide array of species can be found in the gut environment. Some isolations from fecal samples consisted of *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus licheniformis, Bacillus pumilus* and *Bacillus megaterium*. Those listed strains have illustrated positive activity in the reduction of

Salmonella Typhimurium. This was indicated specifically from the crop and ceca samples of the broiler anatomy (Nguyen et al., 2015).These samples illustrate the high percentage of the Bacillus genus associated with the bird and its ability to increase health by decreasing pathogenic bacteria.

Moreover, probiotics effect in the GIT can increase absorption of nutrients, which yields more energy to be potentially available for net energy of production. This increased net energy can improve egg production in layers. Kurtoglu et al. (2004) used 480 27-wk-old Brown-Nick layers, which were divided into four different treatments. The treatments included a basal diet supplemented with 0, 250, 500 or 750 mg/kg^{-1} of probiotics (BioPlus 2B) over a 90 d period. Each 1 g of BioPlus 2B included at least 3.2×10^9 cfu of *Bacillus licheniformis* (CH 200) and 3.2×10^9 cfu *Bacillus subtilis* (CH 201) spores. Egg production for the 250, 500, and 750 mg/kg^{-1} supplementation was increased over the control (83.1%) to 85.8, 86.1 and 86.7%, respectively (Kurtoglu *et al*., 2004; Chaucheyras-Durand and H. Durand, 2010).

Competitive exclusion (CE)

Bacteria are naturally competitive and because of that they attempt to eliminate pathogenic bacteria which might negatively affect the intestinal tract. This is often referred to as CE, bacterial antagonism or bacterial interference (Nurmi and Rantala, 1973; Lloyd et al., 1974; Fuller, 1989). Probiotics, prebiotics and synbiotics all have CE properties (Callaway et al, 2008). The establishment of bacteria resistant to pathogenic strains in young chicks through administration of intestinal microorganisms became

known as the Nurmi concept, which later developed into the CE concept (Nurmi et al., 1992).

Intestinal infections are caused by pathogens dominating adhesion sites or mucosal surfaces, thus disrupting the microbiota balance in the intestines (Fontana et al., 2013). A beneficial characteristic of probiotics is their ability to adhere to the intestinal epithelium lining, thus increasing the amount of time the probiotic resides in the GIT. Increased reproduction of probiotic bacteria will take up more gut space which excludes pathogens through competition. This results in increased uptake of nutrients by the bird. Competitive exclusion is generally thought of occurring in the intestines or caeca of the bird (Mead, 2000).

La Ragione and Woodward (2003) reported a strain of *Bacillus subtilis* and its ability to improve bird health. Samples taken in broilers received a pre-dose of *Bacillus subtilis* PY79^{hr} at 1×10^9 cfu compared to those that received no pre-dose. Both treatments (with and without probiotic) of birds were challenged with 1×10^5 cfu of *Clostridium pefringens*. The results showed a significantly decreased level of *Clostridium pefringens* from the spleen ($P < 0.01$) and duodenum ($P < 0.03$) in birds receiving *Bacillus subtilis*.

In this same study by La Ragione and Woodward (2003), *Salmonella Enteritidis* was recovered from tissue samples of the liver, spleen, duodenum, jejunum, ileum, colon and caeca of chicks. Both treatments (with and without probiotic) of birds were challenged with 1×10^5 cfu of *Salmonella Enteritidis*. Broilers that received a pre-dose of *Bacillus subtilis* PY79^{hr} at 1×10^9 cfu were lower in *Salmonella Enteritidis* from the ceaca ($P < 0.035$) than those chicks that did not receive the pre-dose. However, both of these

significant differences disappeared in samples taken 24 h later (La Ragione and Woodward, 2003). The oral administration of spores, mostly of the genus *Bacillus*, can help the host fight off infectious disease and is a form of CE.

Development of probiotics through microbiology techniques

Using microbiology techniques and performing in vitro studies improves the understanding of probiotics and industry application. This section will discuss the makeup of probiotic bacteria, isolation strategies, and identification of specific strains. The outermost layer of the vegetative cell wall of different probiotic bacteria can include a crystalline S-layer. This S-layer is resistant to phagocytosis and 18 species of *Bacillus* are known to have S-layers. It is important for bacterium to be able to cross the mucosal epithelium of the small intestine. Once this occurs, the bacterium can have its effect on target tissues and organs, eventually giving it the chance to reproduce (Hong et al., 2005).

Bacteria can be collected and isolated from portions of the animal's desired anatomical location such as the trachea, intestines, ceca, and colon to develop probiotics to promote a healthy organ or system (Sohail et al., 2015). Using this method allows these strains to have the highest and most lucrative chance in survival and reproduction in their host (Nguyen et al., 2015).

After collection, samples are exposed to DNA extraction, followed by a polymerase chain reaction denaturing gradient gel electrophoresis known as PCR-DGGE or pyrosequencing. This procedure can reveal several phylas, classes, orders, families and genera (Sohail et al., 2015) of bacteria, protozoa, fungi, and archea (Chaucheyras-Durand and H. Durand, 2010). Taxonomic classification is the process of documenting

biodiversity to the microbial population by analyzing both phenotypic and genotypic methods. In the past sugar fermentation and general fermentation of products were relied on to perform taxonomic classification. However, the primary approach today is using the 16S RNA gene analysis (Fontana et al, 2013).

Bacteria are adaptive to their environments. Therefore nutrient rich media, nutrient poor media, and pH will affect it accordingly and should be taken into account during in vitro analysis (Fontana et al., 2013).Following species identification, the bacteria can be applied to different mediums to be grown and heated for different lengths of times in order to reproduce (Sohail et al., 2015). During in vitro studies, the probiotic can be incubated in gastric or intestinal juices which range in their pH from 2.0 to 4.0 to determine their susceptibility to bile. They typically incubate for one to three hours. The same process should be performed for enzymatic media at a pH of 1.5 to 3.0 for one to four hours. Bile salts aid in digestion but are also an antimicrobial influencer and have effect on the intestinal microbiota balance (Fontana et al., 2013).

Gram-negative and gram-positive bacteria can be identified using a 3% solution of potassium hydroxide on various bacterial strains. Gram staining is a useful test to identify unknown bacteria and provides information about the bacteria's cell morphology, size and genus class. Gram negative and gram positive is differentiated based upon a violet color reaction of bacteria cells. *Bacillus* are generally gram positive species (Gregersen, 1978).

Foodborne illnesses

Salmonella from ingested poultry meat has caused a raise in foodborne illnesses. Annually, 10% of consumers become ill from *Salmonella* and 25% of all global diarrheal diseases are caused by *Salmonella* cases. Resistant serotypes of *Salmonella* are a growing concern to the public and an issue for food safety (WHO, 2006).

Live beneficial bacteria like probiotics might be able to alleviate and overcome this challenge. Not only do probiotics show promising results for a healthier consumer, but also increased performance and an increased immunity for the broiler (Higgins et al., 2007). Specifically, in broilers, research has concluded probiotics both live yeast and bacteria, can increase resistance to *Salmonella*, *Escherichia coli*, or *Clostridium perfringens* infections.

La Ragione et al. (2001) show the avian intestine can house *Bacillus subtilis* for 36 d when given a dose of spores (strain PY79) at 2.5 X 10^8. These broilers also showed a greater resistance to the pathogen *Escherichia coli* O78:K80. These birds were dosed orally at 36 h of age with 10^5 cfu *E. coli* O78:K80 *nal*^{r} suspended in 0.1 ml PBS. The study indicated that the pathogen had a substantial decrease ($P < 0.01$) in colonization of the spleen, caeca and liver (La Ragione et al., 2001). A study conducted by Higgins et al. (2008) reported a Lactobacillus based probiotic culture given at 10^6 or 10^8 cfu was able to significantly reduce *Salmonella Enteritidis.* However, when given at 10^4 cfu no significance was found (Higgins et al., 2008). This shows that dosing amount is essential to probiotics success in decreasing pathogens.

Heat stress (HS)

Stress is defined as a condition in an animal that results from the action of one or more stressors that may be of either external or internal origin (Von Borell, 2001). A stressor often disrupts standard physiological balance or homeostasis impacting an animal's health and performance. For example, during summer and winter seasons, farm animals are exposed to environmental stress due to ambient temperature fluctuation beyond the TN zone. Poultry are homeotherms and under mild temperature fluctuation they will try to maintain relatively constant body temperatures by balancing heat loss and HP in their bodies through behavioral and physiological adaptation. However, balancing body temperature through adaptation becomes difficult for birds when temperatures and humidity increases beyond the critical levels, which can be defined as HS (Lara and Rostagno, 2013).

Bird exposure to higher ambient temperature deviation will result in a HS condition where behavioral and physiological adaptations will no longer help the birds maintain their body temperature (Soleimani et al., 2011.) A number of factors could cause HS, including rise in ambient temperature, increased relative humidity, harshness of the sun, and airflow rate (Beker and Teeter, 1994; Lara and Rostagno, 2013). Unless management interference is made, loss in production and increased mortality will occur (Lara and Rostagno, 2013; Butcher and Miles, 2015).

Certain management practices have been utilized to minimize detrimental effects of HS. Some management techniques that were used include provision of cold water in houses, use of increased ventilation rate, feeding the birds in the morning and night when

temperatures are lower, and supplementation with KCl to encourage water intake (Beker and Teeter, 1994). Increased water intake will ultimately lower body temperature, since it serves as a heat sink and improves bird survivability (Butcher and Miles, 2015).

When temperatures are high, broilers want to maintain their body temperature in a certain range. When they respond to HS, they first protect their visceral organs. Heat stress response can start in the hypothalamo-pituitary adrenal (HPA) axis. Heat stress also affects the orthosympathetic nervous system, which is highly sensitive to high heat temperatures (Quinteiro-Filho et al., 2015). The central nervous system seems to be activated by HS, which will cause poor development of the GIT and affect intestinal homeostasis of the broiler (Calefi, et al., 2014). Heat stress has the potential to activate the HPA axis which may release hormones, such as cortisol releasing hormone. These hormones may act as neurotransmitters to increase central nervous system activity (Minton, 1994). Additionally, corticosterone release increased by HS might lower the general immunity of commercial broilers. Ultimately, this reduces their resistance towards pathogens like coccidia, which could in turn develop into necrotic enteritis (Calefi et al., 2014).

When HS is consistent, mortality will increase, feed consumption decreases, as well as, BW gain and meat quality. Over the years, growth rate and feed efficiency have been high in the selection category for broilers. Both high growth rate and increased breast meat yield are encouraged in the broiler production industry (Lin et al., 2006). However, these traits are at an even higher susceptibility to HS.

Heat stress has caused physiological challenges, which include systemic immune dysregulation, endocrine disorders and electrolyte imbalances (Teeter at el., 1985; Sohail

et al., 2010; Sohail et al., 2012). Some reports have also noticed that HS significantly destroys the intestinal mucosa and microbiota (Burkholder et al., 2008; Quinteiro-Filho et al., 2010).

Many factors including HS can effect an animal's microbiome and community of healthy bacteria. Heat stress, disease, and diet can negatively influence this environment of bacterial colonization (Hume et al, 2012; Sohail et al., 2012; Suchodolski et al., 2012; Ursell et al., 2012). Heat stress can cause the increase of pathogen colonization, which will inevitably aid in shedding of the intestinal lining and an increased risk of food safety (Traub-Dargatz et al, 2006). Heat stress can expose the bird to immunosuppression, which promotes onset of both infection and disease (Cheville, 1979; Mulder, 1995). When HS causes damage to the microbiome it has a devastating effect on intestinal morphology often because of pathogenic bacteria increases. For instance, changes in the villus-crypt structures are observed in the highest amount when birds are in heat stressed environments (Sohail et al., 2012).

HS effects energy

Heat stress has serious consequences on health and performance of all species of livestock; however, it seems consequences are more severe in poultry, which are mostly raised in confinement. This is due to more energy wastage for thermo-regulatory adaptions that take place for the bird to overcome the stress condition and gain weight (Lara and Rostagno, 2013). Under mild ambient temperature deviation from the TN zone, poultry make both behavioral and physiological adaptations to maintain their body temperature. Lying still in their pens, spreading their wings to increase their body surface,

increase in water consumption and panting to increase evaporative cooling, decrease in feed consumption to lower metabolic heat production (HP) as well as shunt blood to body surface, along with vasodilation of the blood vessels to increase heat dissipation are common observations (Butcher and Miles, 2015). All these adaptation processes demand energy.

Energy consumed by poultry is utilized for maintenance of vital body functions and growth or production. Of the energy consumed, maintenance energy has to be satisfied first before allocation for weight gain or production. Factors that increase the maintenance need of birds will adversely affect energy left for production and, consequently, impact bird energetic efficiency and cost of production (Lara and Rostagno, 2013; Butcher and Miles, 2015). Among the many factors that affect the maintenance need of birds (age, BW, ambient temperature, feed type, activity, health status, etc.), ambient temperature plays a major role. Temperature variation outside the comfort zone will cause an increase in HP, which will inversely impact energy left for growth or production purposes. In most commercial farms, birds are raised under controlled environments (Zhai et al., 2014). Adverse climatic conditions that occur with annual seasonal changes will dispose birds to temperature fluctuations outside of their comfort zone especially during winter and summer seasons impacting their health and performance (Lara and Rostagno, 2013; Butcher and Miles, 2015).

Animals have the ability to adapt in situations of energy scarcity, which can be an issue when birds go off feed during HS. Energy scarcity or an excess of caloric energy has changes on the microbial diversity in the gut. The microflora in the small and large intestine can also affect whole-body metabolism by affecting total energy (Bäckhed et al.,

2004; Chou et al., 2008). Throughout evolution, animals have evolved in times of scarcity to maximize the use of calories from various foods or when energy demand was higher than normal. An example of this would be cold exposure. It has been found that cold exposure changes the composition of the microbiome. The host is able to increase the intestinal absorptive surface area, resulting in a significant increase in the length of villa and microvilli (Chevalier et al., 2015). This may be reason to believe that HS could induce microbial changes and gut transformations as well. From what is already known about the ability for probiotics to provide benefits to animal performance and GIT health, this feeding strategy seems applicable to decrease negative properties of HS.

Modern-bred chickens will suffer the worst effects of HS. Similarly, commercial broilers that did well in the spring often underperformed during the summer. Fast growing broilers have a higher heat output, thus HS is more pronounced. Birds that grow faster also seem to drink less water in high temperatures, which may result in decreased feed consumption and ultimately lower BW (Zhai et al., 2014). Chronic HS causes a decrease in protein synthesis and increase protein breakdown, to ultimately reduce protein deposition. Decreased protein synthesis cannot be restored with high dietary protein from the diet. Protein has a high heat increment, but low levels of amino acids cause for poor feed efficiency and lowered BW gain. Chickens tend to consume more food to meet their protein requirement in a low protein ration, which results in increased fat deposition and a higher heat output (Lin et al., 2006).

Dietary strategies to reduce effects of HS

In order to alleviate HS in broilers, additivities like electrolytes have been added to water to increase water intake. Some of these include 1% NH_4CL or 0.5% $NaHCO_3$. The inclusion of 0.5% $NaHCO_3$ in the diets of chronically HS birds increased BW gain by 9%. The addition of 1% NH_4CL improved BW gains by 25%. It also decreased blood pH by 7.2% (Teeter et al., 1985). Chromium, zinc and vitamin A fed as supplements all showed some alleviation of HS on the performance effects (Lin et al., 2006). For instance, vitamin A was fed to layers at 2 different levels consisting of a control treatment fed at NRC (1994) recommendation of 3,000 international units (IU) of vitamin A and a supplemented diet of 9,000 IU of vitamin A. Feed intake was significantly improved by 5.8% in the vitamin A group supplemented with 9,000 IU, compared to the control group. Egg yield and egg weight was also significantly improved ($P < 0.05$) by 11.1% and 1.0%, respectively (Lin et al., 2002).

A study conducted by Tadtiyanant et al. (1991) fed wetted-down feed which increased consumption by 38% compared to dry feed in a HS environment of 33.3°C. Therefore, this overcame some of the HS effects, while also having positive performance results (Tadtiyanant et al., 1991). Habibian et al., (2015) indicated that broiler feed supplemented with selenium decreased negative effects of HS. This might be attributed to the fact that selenium is vital in metabolism and many aspects of the immune system (Arthur et al., 2003). A deficiency can cause a reduction in CD_4+ T-helper cells, which are vital for recognition of viral antigens on infected cells. These CD_4+ T-helper cells also release cytokines that initiate a cellular response to become more resistant to infection (Look et al, 1997).

Other feeding strategies did not alleviate HS factors. Supplementation with orange and lemon peels to broiler's diet showed no statistical differences on performance variables or thyroid plasma hormones for broilers reared in HS. Lemon and orange peels are believed to be anti-inflammatory and can have antioxidant activity; however, these benefits could not overcome the negative consequences of HS when compared back to the control (Akbarian et al., 2015).

Conclusion of literature review

Probiotics have been used extensively over the past 15 years to improve nutritional and bird health status. Chaucheyras-Durand and Durand (2010) reported that inclusion of defined amounts of probiotics in the diet offered health benefits to different species of chickens. Consumption of the probiotic regulates and balances microbiota in the gut minimizing pathological conditions (Chaucheyras-Durand and Durand, 2010). Current research reports on broilers showed supplementation with probiotics of certain strains of bacteria or yeast increased performance and well-being. Additionally, health benefits such as the bird's ability in fighting infections of *Salmonella, E.Coli and Clostridium perfringens* were reported (La Ragione et al., 2003, 2004; Banjeree and Pradhan, 2006; Higgins et al., 2007, 2008).

Probiotics may fight off pathogens, as they have an extremely competitive nature to find space, adhesion sites and nutrients, which in turn could eliminate pathogens. This is referred to as CE. When this happens, the probiotics are able to release their antibacterial substances like bacteriocins or volatile fatty acids. Usually, the spore former *Bacillus* has exhibited the most antibacterial activity against pathogens like *Escherichia coli, Salmonella, Clostridium,* and *Listeria moncytogenes*. When probiotics were

administered they decreased the chronic inflammation of the gut by altering microflora and increasing the mucosal immune response in patients (Arsi, et al., 2014).

Heat stress has become difficult to manage due to increased genetic performance and feed conversion efficiency. Furthermore, a large proportion of broilers are raised in tropic and subtropical locations, thus giving a larger threat to high temperatures and humidity. Factors such as air temperature, humidity, heat, and airflow all influence the level of HS. However, HS can be alleviated or increased based upon the breeding strain, feathering, nutrition, nutritional supplements and management systems (Lin et al., 2006). More research on feed additives may alleviate symptoms of HS and be used as an alternative to AGP.

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CHAPTER III

PROBIOTIC SUPPLEMENTATION EFFECTS ON PERFORMANCE, BODY COMPOSITION, AND ENERGETIC EFFICIENCY OF BROILERS UNDER THERMO-NEUTRAL AND HEAT STRESS CONDITIONS

INTRODUCTION

Increase in world population is eminent and as current population trends exponentially rise, projections state more than 9 billion people will inhabit the earth by 2050 (Holechek, 2013). Today's consumer eats a higher proportion of meat and animal products. These demands for more meat and dairy products may require agricultural food production to increase by 60-110% by 2050 (Conforti, 2011; Tilman et al., 2011; FAO, 2012). In addition, today's consumers also voice their preferences on how meat-producing animals should be managed, fed, and raised. In today's conventional animal production system, sub-therapeutic level supplementation of antibiotics in food animals for prevention of infection rather than treatment is receiving heavy criticism from consumers, as it has contributed to the rise of antibiotic-resistant bacteria (Anomaly, 2009). The use of antibiotics to maintain animal well-being, promote growth, and improve efficiency has been practiced for more than 50 years. However, as early as the 1950's researchers identified concern on development of resistance bacteria for the antibiotics streptomycin and tetracycline used in turkeys and broilers, respectively (Dibner and Richards, 2005). These findings laid the groundwork for agricultural officials to impose stricter regulatory parameters on the use of antibiotics in animal feeds.

The Europeans were the first to ban antibiotic growth promoters (AGP) in animal feed in 2006, except in treating sick animals (Huyghebaert et al., 2011). Medically important products now require a veterinary feed directive (VFD) in the U.S. The VFD ruling was altered January 1, 2017 (FDA, 2012; FDA, 2016). It is possible that the US will discontinue all use of antibiotics fed at sub-therapeutic levels in the near future, because of consumer pressure (Dibner and Richards, 2005). This makes sustainable production for a growing population challenging. Therefore, to satisfy increased demand for animal protein by the growing population, alternatives to antibiotic use for food animals to promote growth and efficiency must be identified (Allen et al., 2013).

Probiotics are live microorganisms included in the diet of animals as feed additives or supplements. Commonly known as a direct-fed microbial (DFM), probiotics provide beneficial properties to the host, primarily through action in the gastrointestinal tract (GIT) of the animal (Agarwal et al., 2002; Fuller, 1992; Morelli and Capurso, 2012). Supplementation of probiotics in the diet have the ability to increase animal health and performance; through contributions to gut health and nutrient use (Agarwal et al., 2002; Ahmad, 2006; Mountzouris et al., 2007). For instance, supplementation of probiotics have been demonstrated to benefit farm animals in immune modulation, structural modulation and increased cytokine production, which positively affect the intestinal mucosal lining against pathogens (Rajput and Li, 2012). *Bacillus subtilis* has been a popular bacterium used within the industry and was shown to improve intestinal villi height (Pluske et al., 1996). Increasing the villa height and architecture of the crypts in the GIT, allows for improvement of nutrient digestion and absorption (Ahmad, 2006). Maintenance of tight junctions of intestinal epithelial cells decreases the chances of leaky

gut, which increases animal health and performance. Tight junctions maintain important defenses against pathogenic bacteria and cellular homeostasis (Sakaguchi et al., 2002).

In certain regions of the nation and the world, heat stress (HS) can be a major environmental challenge during broiler production. In the summer months, when temperatures rise above 32°C and 90% humidity, birds could be subjected to chronic HS (Traub-Dargatz et al, 2006). Heat stress causes the bird to fluctuate its internal core temperature beyond their comfort zone. To overcome such challenges, broilers will attempt to balance their HP and dissipation through behavioral and physiological adaptation mechanisms (Lara and Rostagno, 2013). Some of these mechanisms include increased respiration or panting, decreased feed intake, shunting blood to body surface, elevated water consumption, and spreading of their wings for increased body surface (Butcher and Miles, 2015). Any stress condition disrupts physiological homeostasis, which could decrease growth and jeopardize animal health (Lara and Rostagno, 2013). Systemic immune dysregulation, endocrine disorders and electrolytes imbalances are all common outcomes of HS (Teeter at el., 1985; Sohail et al., 2010; Sohail et al., 2012). Moreover, due to the selection of genotype for increased breast meat yield, broilers today are more susceptible to HS than ever before (Lin et al., 2006). Additionally, stocking density in broiler houses is generally high, which contributes to increased humidity and HP throughout the house (Feddes et al., 2002). Therefore, timely management intervention must be made to minimize bird mortality and economic losses. Some current intervention techniques practiced by producers include increased ventilation rate, providing cold drinking water in houses, feeding the birds before sunrise or after sunset and supplementation of drinking water with KCl to increase water intake (Beker and

Teeter, 1994). However, even though these practices were effective to a certain extent, none of them completely prevent HS. Recently, researchers reported that probiotics alleviate stress conditions in farm animals. Some studies in broilers have reported benefits of probiotics for broilers raised under HS conditions and challenged with Newcastle disease and infectious bursal disease virus. Broilers raised with probiotic supplementation increased their levels of antibodies against the respective viruses compared to those birds with no supplementation during HS periods (Sohail et al., 2010). Therefore, the purpose of this study was to investigate supplementation effect of an OSU developed probiotic mixture on broilers performance during their grow-out, and the probiotics ability in alleviation of the negative consequences of HS during the grower and finisher phases.

MATERIALS AND METHODS

Probiotic preparation

The isolation and characterization of the Bacillus *spp*. probiotics used in this study were described by Penaloza-Vazquez et al. (2017). The procedure was used as described without modifications. In brief, source of the probiotics were healthy 2 d broilers obtained from Cobb-Vantress Siloam Springs, AR. Birds were used from the starter phase because typically increased health and performance during the first 2 wk will affect the finisher period positively. Compatibility streaked tests were utilized to determine relationships of species. The growth in the crosses indicated compatibility where the species touched after an incubation period of 18 h at 39°C. If no growth in the crosses was assessed, then the species were not compatible. All three selected strains

were compatible. Additionally, the enzymatic properties of the strains were evaluated, which included alpha-amylase, protease, phytase, and cellulase activity. Finally, the 3 strains to be used in the mixture were finalized and a concentration dose of 1 X 10^6 colony forming units (cfu)/gram of feed was confirmed. A 10 L bioreactor was used to produce one strain at a time where the endospores were obtained and further collected. The broth provided from the bioreactor was further centrifuged. The Bacillus *spp*. endospores were isolated using heat treatment at 80°C for 40 min to kill the vegetative cells, and isolate surviving endospores. Enumeration was done with standard dilutions and plate counting.

Phase I trial – Floor pen study

The study protocol was approved by the Oklahoma State University Institutional Animal Care and Use Committee (IACUC), protocol AG-14-12 (Appendix 1).

House and treatment diets preparation

Prior to arrival of the birds at the Oklahoma State University (OSU) Poultry Research Center, a broiler house with 72 floor cages was prepared. The house floor was swept, washed, and disinfected to lower the microbial load from previous flocks. Feeders were washed, and disinfected. Nipple drinkers were flushed and set up to the appropriate height for chicks prior to their arrival. Following cleaning, and set up of the cages, the cage floor was bedded with approximately 12.7 cm of wood shavings. Drinkers height were reset every 2 to 3 d to appropriate height to accommodate water intake. A commercial basal diet was formulated for optimal growth and performance to mirror typical industry managerial practices (Table 4 and 5). Preparation of the control and

treatment diets was performed by dividing the basal diet into 2 batches. The basal diet (control) remained in its feeding bin without probiotic supplementation. The other half of the basal, treatment diet (probiotic) was placed into a grain mixer and a liquid probiotic mixture was top dressed over the ration. A dose of 1,000,000 live microorganisms per gram was calculated to be used in the treatment diet prepared. This was mixed for approximately 15 min until the probiotic was evenly dispersed throughout the feed. The dosing rate was confirmed using a sample of the prepared feed post mix. Similar procedure was followed in the preparation of the grower and finisher diets prior to feedings. Prepared treatment diets, starter, grower, and finisher were stored in feed bins throughout the study and provided to the birds as needed. Any leftover feed during the respective feeding phases was removed and properly disposed of.

Management of birds

Four hundred thirty two day old male Cobb chicks were obtained from a commercial hatchery in Siloam Springs, AR. Upon arrival at OSU, the chicks were weighed, individually identified with a wing band number and randomly allocated to 72 floor cages arranged in 6 rows with 12 cages in each row, and 6 chicks in each cage. Each floor cage had an area of 11.6 m^2. This floor cage area meets both industry and IACUC guidelines. Feed and water were provided for *ad libitum* consumption during the study which took place over 41 d period. Lighting and ambient temperature provision were as provided to the chicks according to the breeding company guidelines (Cobb-Vantress, Siloam Springs, AR Cobb Broiler Management Guide, 2013).

The phase I trial was set up as a completely randomized block design. Each row was randomly labeled as either the control or the probiotic treatment. The cages' feeder was fitted with a wire mesh covering to minimize feed wastage or litter entering into the feeder. Mortality was monitored and recorded as it happened.

Variables monitored

During the entire study period, individual BW and group feed intake (FI) was recorded on a weekly basis to assess the following variables: cumulative feed intake (CFI), cumulative body weight gain (CBWG), feed conversion ratio (FCR), average daily gain (ADG), body composition, and metabolic parameters. Body weight was taken on an individual basis using wing bands to clearly identify each bird. Feed added to each floor cage was recorded per pen basis. Remaining feed at the end of each week was weighed and recorded as feed "weigh back" so that feed consumption for each pen could be determined. Cumulative FI and CBWG were determined by summing the weekly intake and BW, respectively. Feed conversion ratio was determined as a ratio between CFI and CBWG. Average daily gain was determined as a ratio between CBWG and the number of experimental days.

Performance variables were quantified using the following equations:

CBWG = cumulative final BW – cumulative initial BW

CFI = cumulative feed offered – cumulative feed refusal

Cumulative FCR = cumulative feed consumed/cumulative weight gained

ADG = finish weight – start weight / age (d)

Body composition

Throughout the experiment, birds were randomly selected during weigh days on a weekly basis for body composition analysis, with the exception of wk 1 and 5. A regression equation was developed using the data points collected in this phase of the study to estimate missing body composition data in wk 1 and 5. Selected birds were humanely euthanized using a carbon dioxide chamber following the AVMA Guidelines for the Euthanasia of Animals (2013). Birds were packed in double Ziploc bags, labeled and transported to a -40°F freezer until needed for analysis. Body composition analysis (protein, fat, ash, and water; g) were assessed using QDR 4500 Elite X-ray Bone Densitometer (Marlborough, MA, Hologic, Inc.) at OSU Department of Nutritional Sciences.

The following equations developed by OSU poultry research team for Cobb birds to convert X-ray bone densitometer (DEXA) composition data to proximate analysis body composition (AOAC,) were implemented. This conversion from DEXA to proximate analysis can be referenced by McKinney (2005).

Bird Protein = -6.13349 + 0.1119*Fatg + 0.00003567*$Fatg^2$ + 0.18308*Lean g – 0.00000370*$Leang^2$ + 0.00004728*Leang*Fatg – 1.252E – 11*$LeanFatg^2$

R^2=0.99

Bird Fat = -5.6813 + 0.03129*Fatg + 0.00006536*$Fatg^2$ + 0.10041*Leang + 0.00002336*$Leang^2$ + 0.000096*LeanFatg – 1.2042E – 11*$LeanFatg^2$

R^2=0.97

Bird Water = 5.79504 + 0.76994*Fatg – 0.00003797*$Fatg^2$ + 0.68501*Leang – 0.00001373*$Leang^2$ – 0.00015077*Leang*Fatg + 2.43437E – 11*$LeanFatg^2$

R^2=0.99

Bird Ash = -1.6675 + 0.01579*BMCg + 0.02658*Leang + 0.02434*Fatg – 0.00000395*LeanBMCg – 0.00000254*FatBMCg + 0.00000144*LeanFatg

R^2=0.99

Equation Abbreviations:

Fatg = fat mass in grams

Leang = lean mass in grams

$LeanFatg^2$ = leangfatg x leanfatg

BMCg = bone mass content in grams

LeanBMCg = leang x BMCg

FatBMCg = fatg x BMCg

Metabolic variables

The following equations were used to determine metabolic parameters, weekly metabolizable energy consumed (MEC), metabolizable energy retention (MER), heat production (HP), and efficiency of metabolizable energy use (EMEU).

Starter period

MEC kcal/g wk 1 and wk 2 = CFI*2.988 kcal/g

Grower period

MEC kcal/g wk 3 and wk 4 = CFI*3.082 kcal/g

Finisher period

MEC kcal/g wk 5 and wk 6 = CFI*3.177 kcal/g

MER kcal/g = protein mass retained in g*5.65kcal/g + fat mass retained in g*9.3kcal/g.

HP kcal = MEC kcal – MER kcal.

EMEU = MER kcal/MEC kcal.

Phase II trial – Metabolic chamber study

The study protocol was approved by the Oklahoma State University Institutional Animal Care and Use Committee (IACUC), protocol AG-14-12 (Appendix 1).

Chamber preparation

Prior to birds being moved to the phase II study site, metabolic chambers were cleaned, and disinfected to lower bacterial load. Nipple drinkers were flushed; feeders and fans inside the chambers were cleaned, and disinfected. The treatment diets used in this experiment were the same as the grower and finisher diets used in phase I of the study and received the same mixing protocol referenced earlier in the house and treatment diets preparation, Phase I – thermo-neutral treatment section.

Management of birds

On d 21, fifty-two broilers from Phase I trial, were randomly selected from the 2 treatment groups (control n=28, and probiotic n=24), individually weighed, and transferred to the OSU Poultry Metabolic Chambers housed in three separate rooms (Room X, Y, and Z). Each room was composed of 20 metabolic chambers (12-broilers size (8.3 m^2) and 8-turkey size (17.1 m^2). The area of the chambers met both industry and IACUC guidelines. Four treatment combinations, two ambient temperatures (TN, HS) x two feed (control, probiotic) were arranged and assigned to chambers in the three rooms. Room X was used as thermo-neutral (TN) ambient temperature while Room Y and Z

were used as HS chambers. The 12 broiler chambers in Room X housed broilers subjected to TN ambient temperature and probiotic supplemented feed (PTN). The remaining 16 broilers were housed in the 8 turkey chambers in the same room and were subjected to TN ambient temperature and control feed (CTN). In room Y and Z, only 12 of the chambers from each room were used. Room Y birds were subjected to HS environment and control diet (CHS) while Room Z birds were subjected to HS and probiotic feed (PHS). The HS room birds were subjected to cyclic HS at 32°C ± 1 and maintained from 1800 to 2100 h every night until d 41. Lighting and ambient temperature provision were as provided for the CTN and PTN treatments according to the breeding Co. guidelines. The CHS and PHS treatment were exposed to these guidelines when cyclic HS was not administered (Cobb-Vantress, Siloam Springs, AR Cobb Broiler Management Guide, 2013). Feed and water were provided to the birds for *ad libitum* consumption. Mortality was monitored and recorded as it happened.

Variables monitored

Data points collected in phase II were similar to phase I. Initial BW at d 21, BW and FI were recorded on a weekly basis to assess the following variables: Weekly FI, Weekly BW, and weekly FCR. The data points were determined as follows:

Weekly weight gain = weekly final BW – weekly initial BW
Weekly FI = weekly feed offered – weekly feed refusal
Weekly FCR = weekly FI/weekly weight gain.

Body composition

Similar to phase I, all remaining broilers from their respective groups were euthanized to provide body composition samples at the conclusion of the experiment (d 41). A regression equation was developed for phase II broilers to estimate missing body composition data points from wk 4 and 5. Randomly selecting birds in those weeks (wk 4 and 5) of the chamber study would have weakened statistical power for other measured variables. Therefore, on d 41 birds were humanely euthanized using a carbon dioxide chamber following the AVMA Guidelines for the Euthanasia of Animals (2013). Dead birds were packed in double Ziploc bags, labeled and stored at -40°F freezer until needed for analysis. Body composition analysis (protein, fat, ash, and water; g) were assessed using QDR 4500 Elite X-ray Bone Densitometer (Marlborough, MA, Hologic, Inc.) at OSU Department of Nutritional Sciences. Then, DEXA data was converted to proximate analysis (McKinney, 2005) using the equation developed by OSU poultry research center for Cobb strain birds indicated above.

Metabolic variables

A similar procedure as Phase I was followed to determine metabolic parameters considered, weekly MEC, weekly MER, weekly HP, and weekly EMEU and can be referenced earlier in the metabolic parameters, Phase I – thermo-neutral treatment section.

Respiration

Due to failure in data acquisition system controller, gas exchange data was manually recorded only during the HS period. Daily respiratory samples were taken from each chamber between 1800 to 2100 h every night until d 41. Panting of birds was counted on representative sample birds in 15-s time intervals every night between 1800 and 2100 h. Initiation of data sampling respective to room was randomly changed every night. Additionally sampling of respective birds started on the left or right every other day. Once number of pants was counted the number was multiplied by 4 to obtain panting rate per min.

Average respiration per min = Number of breaths per 15 s*4.

Statistical analysis

Data for all response variables from Phase I trial was analyzed as a completely randomized block design using the General Linear Models procedure of SAS (SAS 9.4, 2012) . The statistical model included the effects of diet (basal diet or supplementation with probiotic), block effect and their interactions. Data was expressed as means. When the F-test was significant (P,0.05), treatment means were separated using least significant difference (Steel and Torrie, 1960). Similarly, data for all response variables from Phase II trial was analyzed as a completely randomized design using the General Linear Models procedure of SAS (SAS 9.4, 2012) . The statistical model included the effects of temperature (TN or HS), diet (basal diet or supplementation with probiotic), and their interactions. Data were expressed as means. When the F-test was significant (P,0.05), treatment means were separated using least significant difference

(Steel and Torrie, 1960). The relationships between FI, BW gain and FCR was established by regressing ME intake on BW gain and FCR values measured.

RESULTS

Phase I – Floor pen study

Performance

Growth performance of broiler chicks fed a commercial basal diet (Table 4 and Table 5) with and without OSU probiotics during phase I of the study is depicted in Figure 1 and Figure 2. Compared to the control group, OSU probiotic treatment showed an increase ($P < 0.05$) in CFI during the starter period (wk 1 and 2) and wk 3 of the experiment with an improvement of 4.4, 3.7 and 7.0%, respectively. However, mixed probiotic supplementation effects on CFI were observed during the grower (wk 3 and 4) and finisher periods (wk 5 and 6). Probiotic supplementation impact on CFI was insignificant ($P > 0.05$) during wk 6, but significantly higher ($P < 0.05$) in wk 5.

Similar results for other performance variables, CBWG and ADG were noted in Table 6. Chicks fed the OSU probiotic supplemented feed showed an increase ($P < 0.05$) in both CBWG and ADG, during the starter period. The probiotic supplemented broilers had a 7.1 and 8.7% increase in CBWG and 6.9, and 8.6% in ADG relative to the control, respectively.

Similarly, probiotic treatment showed an improved ($P < 0.05$) FCR during the starter period (Table 6). However, mixed probiotic supplementation effects on FCR were noted post starter period. An increased ($P < 0.05$) FCR during wk 3 seems to be a reflection of increased appetite observed in birds supplemented with probiotics.

Body composition

Data of protein, fat, ash, and water mass of whole body was measured using QDR 4500 Elite X-ray Bone Densitometer (Marlborough, MA, Hologic, Inc.) in OSU Nutritional Sciences, for wk 2, 3, 4, and 6 on broilers raised with and without OSU probiotics during phase I of the experiment. Both, wk 1 and 5 were estimated with regression equations previously mentioned in the Materials and Methods. As shown in Table 7, OSU probiotic supplemented birds in wk 2 of the trial had higher *(P* < 0.05) protein, fat, water, and ash mass than the control group. However, OSU probiotic supplementation effects on the broilers disappeared post starter period (*P* > 0.05) in all body composition variables.

Metabolic parameters

Metabolic parameters measured are presented in Table 8. Broilers supplemented with OSU probiotics showed an increase *(P* < 0.05) in MEC during wk 1 through wk 3. The increase for the probiotic treatment compared to the control for observed MEC during wk 1, 2, and 3 were 3.9, 3.7, and 7.0% higher, respectively. Improved probiotic supplementation effects on MEC disappeared post 3 wk of age. Similarly, MER was higher *(P* < 0.05) during some portions of the starter period (wk 2, Table 8). However, MER showed a non-significant difference among treatments post-starter period.

There were several weeks HP was significantly lower in the control treatment *(P* < 0.05), including wk 1, 3, and 4. The decrease in HP for the control during wk 1, 3, and 4 were 42.0, 12.4, and 14.3%, respectively.

While EMEU showed mixed results in both the grower and finisher phase, there were multiple significant differences *(P* < 0.05) (Table 8). These include an increase *(P* < 0.05) in efficiency for the control treatment during wk 1 and 3. Conversely, the opposite effect was shown in wk 2, where the OSU probiotic treatment showed an advantage in efficiency *(P* < 0.05) when compared to the control. Week 4 through wk 6 showed no significant differences in efficiency.

To examine the interaction between ME intake, body weight gain, and FCR, 3-dimensional plots were constructed (Figure 1 and 2). As feed intake of the birds increased with age, weight gain also increased proportionally. However, an inverse relationship was noted with the FCR in both the control as well as probiotic supplemented broilers.

Phase II – Metabolic chamber study

Performance

Growth performance of broiler chicks fed a commercial basal diet with and without OSU probiotic and raised under either a TN condition or HS scenario during phase II of the study is depicted in Table 9. Weekly BW resulted in no significance differences after wk 4 where periods of HS, as well as TN conditions were monitored. However, after the end of wk 5, data tended towards a significant difference in weekly BW. The CHS treatment was numerically lower than all other treatments and there was a 6.4% increase in weekly BW for PHS birds. Finally, during wk 6 the CHS group was significantly lower *(P*<0.05) in weekly BW than all other treatments. When comparing

PHS and CHS in wk 6, there was an 11.8% increase in weekly BW for broilers that were raised with the OSU probiotic during daily cyclic HS intervals.

Similarly, broilers showed no differences for weekly gain during wk 4 (Table 9), their first wk in this phase of the study. In wk 5, weekly gain was significantly higher *(P* < 0.05) for broilers reared in CTN treatment compared to all other treatments. The PTN birds showed a decrease in weekly gain by 14.4% compared to CTN. Still, PHS resulted in a weekly gain increase of 14.3% during wk 5 when compared to CHS. The final week measuring gain for broilers had similar results to wk 5 and the data for wk 6 of weekly gain was significantly different (*P* = 0.052). In contrast to wk 5 weekly gain data, wk 6 data showed PTN to have the highest numerical increase compared to all other treatments. Control heat stress had the poorest performance and lowest weekly gain for the second consecutive week of phase II.

In addition, weekly FI was the most consistent among the performance data when analyzing phase II and can be referenced in Table 9. Week 4 through wk 6 all showed to be highly significant *(P* < 0.05) in FI. In wk 4, weekly FI was comparable among all groups of birds besides the CHS group. The CHS broilers had a decrease *(P* < 0.05) in weekly FI by 26.8% compared to PHS, where PHS was similar to both TN treatment birds during wk 4. In wk 5 and wk 6, both TN groups showed to be similar but significantly different *(P* < 0.05) from both HS groups, in regards to improved FI.

Furthermore, the final variable assessed was weekly FCR (Table 9), which was significantly improved *(P*<0.05) in the CHS treatment during wk 4. Therefore, while BW and gain was lower for CHS, there was an advantage made by the CHS treatment in the

FCR variable measured. However, this advantage did not continue in wk 5 or wk 6, resulting in no significant differences.

Body composition

The data for body composition is illustrated in Table 10. Body composition was measured using QDR 4500 Elite X-ray Bone Densitometer (Marlborough, MA, Hologic, Inc.) at OSU Nutritional Sciences for wk 6 analysis. However, birds were not pulled for sampling during wk 4 and 5 of phase II of the experiment to increase statistical power for performance and energetic data. Therefore, wk 4 and wk 5 were both estimated using a regression equation as previously mentioned in the Materials and Methods. No statistical differences were reported for protein, fat, ash, or water for wk 4 and wk 6.

Nevertheless, during wk 5 the CTN treatment birds were higher in protein, fat, ash, and water, when compared to PTN and CHS ($P<0.05$). The PHS treatment birds were similar to CTN, PTN and CHS during wk 5.

Metabolic parameters

Metabolic parameters of Phase II are reported in Table 11. Significant differences in weekly MEC were seen during wk 4, 5, and 6. In wk 4, CHS birds were lower ($P < 0.05$) than all other treatment groups, and when compared to PHS group, the CHS broilers had a loss of 26.8% in their appetite. Similar results were seen during wk 5 and 6. Weekly MEC was higher ($P < 0.05$) for both CTN and PTN, when compared to PHS and CHS. The CHS group showed the lowest weekly MEC value.

In terms of weekly MER (Table 11), there was no significant difference observed during wk 4 or wk 6. However, weekly MER in wk 5 resulted in a significant difference ($P < 0.05$). Both, CTN and PHS birds showed an increase ($P < 0.05$) in their weekly MER (wk 5) compared to PTN and CHS. The PTN and CHS groups were similar in their weekly MER (wk 5). The PHS group resulted in an increase of 16.5% MER over CHS. While, the CTN group showed an increase of 15.1% in MER over the PTN group.

The weekly HP is reported in Table 11. During wk 4, weekly HP was noticeably lower ($P < 0.05$), for CHS. When compared to the PHS group, CHS produced 78.8% less in terms of HP. The CTN and PHS broilers showed similar weekly HP during wk 4, but CTN group was the highest numerically in HP. The CTN birds showed a 15.7% increase in weekly HP more than PTN.

The final analyzed variable for the metabolic parameters for phase II of the trial was weekly EMEU (Table 11). The CHS birds were significantly improved ($P < 0.05$) in ME efficiency compared to all other treatments during wk 4. Furthermore, the PTN group was also more efficient than both CTN and PHS ($P < 0.05$). No significant differences were noted for weekly EMEU during wk 5 and 6.

Respiration

An increase in respiration rate for the CHS birds was observed among all weeks of phase II and can be seen in Table 12 and Figure 3. Furthermore, during wk 4, 5, and 6, PTN showed the lowest respiration rate ($P < 0.05$). In wk 5, the PHS was lower in respiration per min when compared to CHS, ($P < 0.05$). The respiration increase per min for the CHS treatment during wk 5 was 16.69% greater compared to the PHS birds.

Finally, the average respiration per week (ARW) for wk 4 through 6 was the highest *(P* < 0.05) for CHS when compared to PHS and on average the CHS group was 12.7% higher in their breaths per min.

DISCUSSION

Phase I – Floor pen study

Performance

In this study, feeding broilers under an ideal management or TN situation with probiotics seemed to only benefit the chicks during the starter period (Table 6). These findings are consistent with Yeo and Kim (1997), Zulkifli et al. (2000), and Bai et al. (2013). However, a study by Khaksefidi and Rahimi, (2005) found that probiotics enhanced their broilers' performance during the finisher stage, but was not significantly different in the starter phase. The study by Khaksefidi and Rahimi (2005) used 6 different strains not similar in type to this respective study's probiotic mixture utilized. Therefore, this might imply that strain type, as well as preparation, can have an effect on the bird's physiology in different stages of maturation, which makes probiotic research challenging.

Furthermore, because newborn chicks receive no contact with adult birds at the hatchery or the grower house, the litter upon placement in the growing house is their first major experience with diverse microbial organisms. One-d-old birds are the most vulnerable to infections (Pivnick and Nurmi, 1982; Olnood et al., 2015). Additionally, growers often line the house floor with new litter, but other instances top dress with used litter, even when rearing a new flock of birds. Under these conditions, bacteria from previous flocks or additional bacteria can establish in the GIT of the chick, which could cause a negative effect. This negative effect is attributed to new microorganisms that may

cause disturbances of the intestinal microbiota, which can increase incidences of infection (Patterson and Burkholder, 2003). However, probiotics have the ability to increase resistance to infection (Starvic and Kornegay, 1995; Rolfe, 2000). In this study, chicks fed the probiotic supplemented feed showed an increase in their FI, which contributed to a significantly higher BW in wk 2. Additionally, these birds had an improved FCR. Thus, it seems that probiotic supplemented chicks were more effective in lining their intestinal mucosa with the DFM, which improved their digestive health, gut integrity, and enabling the chicks to improve their performance. Therefore, administration of probiotics may improve gut microbial balance in a competitive exclusion (CE) act, during initial placement in the house, with fresh litter circumstances (Al-Zenki et al., 2009). The study conducted by Larsson et al. (2012) supplemented probiotics in the diet, which improved immunity, intestinal architecture, and intestinal barrier function. These factors would have contributed to increased nutrient absorption, energy metabolism, and performance. Based on the conclusions from Larsson et al. (2012), it seems our experiment may have received similar benefits.

However, constant supplementation through the grower and finisher phases showed no benefit to the bird during phase I of the trial in terms of performance. One possible explanation is that probiotics supplemented in the diet may have saturated the lining surface of the GIT and the benefit reached a plateau determined by the biological and biochemical functions of the broilers. As a result, the continuously supplemented DFM has nowhere to go and is excreted, causing all previously observed benefits to disappear. A study conducted by Walsh et al. (2008) utilized swine and measured the number of probiotics excreted on d 4 and d 28, post-weaning. The results showed that

some strains seemed to have a better chance being utilized in the GIT than others and excretion of strains is variable. Although this study was conducted in a different animal species, it suggests that the benefits of different probiotic strains are not the same.

Future experiments might supplement probiotics for strategic feeding days during ration changes or stressful implications, like transportation to the processing plant. Probiotic supplementation during these transitions in production may be beneficial and cause a difference in performance results. These transitional points are the most stressful time and can result in shifting the balance of the bacterial culture in the GIT (Apajalahti et al., 2004). Traditionally, commercial feed is pre-mixed and is delivered to the grower house in bulk. Supplementations of probiotics could be applied strategically, for an interval of days, via the feed hopper in the grower house, in water administration, or spraying probiotics directly on the litter (Olnood et al., 2015). This would eliminate the constant supplementation effect, saving the producer inputs. This strategy allows the probiotics to have the most influential effect on the GIT as supplementation to the diet seems to be time sensitive to when probiotics have the most benefit.

In addition to the barrier effect of probiotics in the GIT, the DFM also produces its own enzymes capable of digesting fibrous feed particles, which will aid in absorption (Ghani et al., 2013). This increased digestion and absorption may have resulted in an increased FI and nutrient availability. Typically, increases in FI noted during the first weeks of the broiler's life may have improved the development of the digestive system as well as growth for the grower and finisher phases. After week 3 mixed results are seen on FI. The OSU probiotics may increase palatability, which resulted in this improved FI. Probiotics improve the palatability of feedstuffs (Dhama et al., 2008; Nahanshon et al.,

1992, 1993) and increasing FI has been seen in the poultry studies by Tortuero (1973), Francis et al. (1978), and Yeo and Kim (1997).

Body composition

Body composition data of Phase I birds is displayed in Table 7. During wk 1, chicks will seek nutrients from the yolk of the egg post-hatch. The yolk is absorbed to the abdominal cavity, which may influence body composition data in wk 1 (Cobb Broiler Management Guide, 2013). The probiotic supplemented chicks showed a decrease in fat ($P < 0.05$) compared to the control. This may be because fat was used as a major source of energy, enabling lean tissue accretion to significantly increase in the probiotic group during wk 1. A study conducted by Khaksefidi and Rahimi (2005), found that probiotics fed in a broiler study also improved the amount of protein, ash, and water in both leg and breast meat. However, probiotic supplementation decreased the percent fat for these same organs. During wk 2 of this study, a significant increase ($P < 0.05$) was observed for protein, fat, ash, and water in the body of chicks. In this phase I of the experiment, all improvement in tissue accretion disappeared after wk 2 of the trial. Another study by Pietras (2001) reported significantly higher protein content for the probiotic supplemented birds but a numerical decrease in crude fat and total cholesterol.

Metabolic parameters

Metabolizable energy retained was significantly higher ($P < 0.05$) in wk 2 for probiotic-supplemented chicks. The microbiome in the small and large intestine can affect total energy (Bäckhed et al., 2004; Chou et al., 2008). Birds also were significantly lower ($P < 0.05$) in their HP during this week. Heat production is part of maintenance

energy. The lower the HP, the more energy that will be available for broilers to grow or accrete tissue. This might have contributed to increased protein and fat accretion observed and previously mentioned. However, an increased HP noted during wk 3 and 4 for the probiotic birds, might have given the control birds an opportunity to catch up in their performance during the grower and finisher phases. The control birds also showed an improvement ($P < 0.05$) in their EMEU in wk 3 due to less HP and a lower MEC.

Phase II – Metabolic chamber study

Performance

Weekly BW showed no improvement until the final week of the experiment. The PHS birds showed similar weekly BW compared to CTN and PTN. The CHS birds had the lowest weekly BW. This might mean that probiotic supplemented birds, find alleviation of HS via the supplement. Furthermore, a report by Dowd et al. (2007) reported results on the microflora of pigs weighed daily, an activity typically accepted as low stress handling. The study reported that handled pigs were significantly different in their microflora compared to the control. Thus, submitting the birds to daily HS could have affected their gut microflora, as this is considered a large stressor with major production consequences. Therefore, the probiotics used in this study might be an aid in balancing the microflora and increasing performance.

Probiotics might be able to benefit the intestinal architecture that typically is negatively affected by HS. Probiotics have been known to increase ileal villus height (Samli et al., 2007) and jejunal villus height (Chichlowski et al., 2007) in broiler studies under TN conditions. However, a study conducted by Song (2014) found that HS

significantly shortened villus height and induced significantly deeper crypt depths compared to broilers in a TN environment. It is postulated that the probiotic mixture provided in this study may have aided in increasing the villa height, decreasing crypt width, and providing tighter junctions of epithelial cells. This could have decreased leaky gut syndrome, which ultimately will aid performance (Ilan, 2012; Seki and Schnabl, 2012). While this effect was not seen in the finisher phase for phase I during TN conditions, it seems probiotics are more useful and beneficial for birds in challenged environments, such as HS. Thus, it had a more beneficial effect in the finisher stage of production during phase II of the experiment.

Weekly BW gain was significantly higher ($P < 0.05$) for the CTN group in wk 5 compared to all other treatments. In wk 6, BW gain was similar among the CTN, PTN, and PHS groups, only the CHS birds were significantly lower ($P < 0.05$) in their BW gain. Furthermore, in general, FI was expected to decrease in the cyclic HS environments (Lara and Rostagno, 2013; Butcher and Miles, 2015). The only exception was wk 4 where the PHS birds were significantly higher in their FI than the CHS treatment. The PHS treatment was also similar to the PTN and CTN treatments. A possible explanation might be HS acclimation was greater for birds fed probiotics and raised in HS conditions, initially.

Acclimation is described as the birds increased ability to survive at temperatures fluctuating well beyond the TN zone (Altan et al., 2000). However, this effect is lost in the following weeks. There are numerous reports, and it is well documented, that birds exposed to some form of HS prior to cycling acute HS periods found some relief and had lower mortality in the house (Hutchinson and Sykes, 1953, Reece et al., 1972; May et al.,

1986). While the CHS and PHS birds in this study were exposed to cycling HS at exactly the same time, the PHS birds still showed a higher FI than the CHS group. This might suggest that probiotics enable the bird to find some relief, which increases their appetite, because the DFM has the ability to acclimate birds to HS, at least initially. Furthermore, Teeter et al. (1992) suggested the reduction of feed consumption and HS acclimation may be beneficial to birds in lowering their heat load, which increases the chance of their survival during HS. Finally, FCR was improved for the CHS treatment in wk 4, however one must take into consideration that these birds were the lowest numerically in BW and FI, which contributed to their increased efficiency.

Body composition

Interestingly, body composition was not significantly different in protein, fat, ash, or water in wk 4 or 6. However, during wk 5, the CTN group was significantly higher ($P < 0.05$) in these measured parameters. Furthermore, the PHS group showed a numerical improvement compared to PTN and CHS, and was similar to the CTN treatment. This might be attributed to a ration change that occurred at the beginning of wk 5, in conjunction with the cyclic HS effects. The change in nutritional composition can be referenced in Table 4. The additions of HS and no supplementation to the bird may have been enough to cause the CHS birds to be the lowest in their protein, fat, ash, and water. While the probiotics provided some relief to the birds subjected to the ration change and HS (PHS), which may be the cause for the numerical increase observed in protein, fat, ash, and water.

Metabolic parameters

As HS occurs, the bird will attempt to achieve thermo-regulatory adaptations, which causes more energy wastage (Lara and Rostagno, 2013). Some physiological and behavioral changes adopted include lying still in their pens, spreading their wings and panting as a cooling mechanism to attain thermos-balance (Butcher and Miles, 2015). All these factors require additional energy from the bird, which could cause less energy to be directed towards net energy production use. Weekly MEC was similar in the respective temperature environments for the TN or HS birds. The only exception was during wk 4, where the CHS birds showed the lowest weekly MEC. Again, similar to FI during this week, initially the PHS treatment may have received some acclimation effects, allowing them to be more tolerant to their environment (Altan et al., 2000) given by the fed probiotic, which increased FI for the respective week, which then contributed to weekly MEC. Regardless, MER for the PHS treatment was similar to CTN and outperformed both PTN and CHS in wk 5 of phase II. Moreover, broilers that experience HS, require more energy to be used to adapt to the challenging physiological state. An example of this is panting more per minute.

Respiration

The CHS treatment showed the most negative physiological consequences in terms of breaths per minute. The respiration rate for CHS was consistently the highest numerically across all weeks and significantly the highest ($P < 0.05$) in wk 5, as well as, the 4-6 ARW. This might suggest that that probiotics have an effect on the bird's hypothalamo-pituitary adrenal (HPA) axis, because when the HPA axis is upregulated by stress conditions, a higher proportion of cortisol and adrenocorticotropic hormones are

released and found in the blood (Eutamene and Bueno, 2007). Probiotics may affect biochemical pathways in the hypothalamus regulation causing it to reset itself in terms of homeostasis (Bienenstock and Collins, 2010). For instance, according to Bienenstock and Collins (2010) the gut microbiota, intestinal tract, immune system, central and peripheral nervous systems all interact with one another. Epithelial cells and immune cells might be directly affected by intestinal microbes, which might produce bioactive compounds and neurotransmitters to modify resistance to stresses via the gut-brain axis. Some of these compounds might include histamine, gamma-aminobutyric acid (GABA), and short-chain fatty acids (SCFAs) (Hemarajata and Versalovic, 2012). This process results in signaling to the central nervous system, which can alter hormone status. Some of these altered hormones delegated by the gut-brain axis include corticotropin releasing hormone, adrenocorticotropic hormone, indoleamine-pyrrole, and 2,3-dioxygenase (Bienenstock and Collins, 2010). These possible hormone alterations might be enabling the bird to feel more relaxed in its challenging HS environment and thus might have caused the bird exhibit fewer pants per minute. Therefore, these possible hormonal changes may have allowed the bird to feel somewhat more comfortable than the CHS group and thus pant less per minute. However, more research is needed to develop clarity on how probiotics affect the HPA axis.

CONCLUSION

Modern day broilers are raised in confinement for a number of reasons. Modern-style houses enable easier management of broilers and decrease the number of days required prior to harvesting. This makes a more economical, affordable product for the consumer. Even though management has improved, the effects of HS and their negative

properties on production have not completely disappeared in the broiler industry. Therefore, feeding a 3-strain combination of *Bacillus* in this study showed promising results to help alleviate some HS factors and ultimately improve final BW during the finisher phase, when birds were subjected to HS for multiple weeks. Furthermore, the physiological repercussions of HS, like increased panting, also seem to be relieved by addition of *Bacillus* probiotics to the diet. However, feeding continuous supplementation of probiotics in phase I of the study, under a TN environment, showed no economic merit. In this part of the trial (Phase I), there were no significant differences seen at the conclusion of the experiment (grower and finisher), even though some were seen in the starter phase, such as performance data, body composition, MEC, MER, and EMEU.

There have been several studies conducted using probiotics in the human and animal industry. However, very few studies in the poultry industry have analyzed the effects of feeding a DFM under HS parameters. Still, researching probiotics is challenging due to the many factors that influence results. Some of these factors include animal species and breed, probiotic combination of species, concentration of dose, application method, continuous or strategic feeding, stress factors, and many more.

In this study, GIT samples were taken from 2-d-old Cobb chicks to identify the most viable probiotics to be fed to growing broilers in order to have a significant effect in performance and health. However, since the microflora changes extensively based on feed and possibly ration changes, it is reasonable to suggest that the microorganisms sourced originally in the chick are not near as practical in the grower and finisher bird. Thus, in phase I of the study they had the most significant effect only in the starter phase. Therefore, a recommendation for future trials to is take GIT samples from the starter,

grower, and finisher birds to be more specific in finding out what species and strains of probiotics are most useful to improve growth and health.

Regardless, more research needs to be done using different challenge models, as well as management conditions. Some management strategies to be further researched include intermittent feeding specifically during stressful production periods like ration changes and pre-transportation to the plant. This will help us further understand the total direct impact probiotics could have on animal feeding, animal health, and ability to improve the producer's bottom line.

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Table 1: Abbreviations for manuscript

Term	Item
Antibiotic growth promoters	AGP
Average daily gain	ADG
Average respiration per week	ARW
Body weight	BW
Colony forming units	cfu
Competitive exclusion	CE
Control heat stress	CHS
Control thermo-neutral	CTN
Cumulative body weight gain	CBWG
Cumulative feed intake	CFI
Direct-fed microbial	DFM
Efficiency of metabolizable energy use	EMEU
Feed conversion ratio	FCR
Feed Intake	FI
Food and Drug Administration	FDA
Gamma-aminobutyric acid	GABA
Gastrointestinal tract	GIT
Heat production	HP
Heat stress	HS
Hypothalamo-pituitary-adrenocoritcal	HPA
International units	IU
Metabolizable Energy Consumed	MEC
Metabolizable Energy Retained	MER
Non-starch polysaccharide	NSP
Probiotic heat stress	PHS
Probiotic thermo-neutral	PTN
Short chain fatty acids	SCFA
Thermo-neutral	TN
Veterinary client patient relationship	VCPR
Veterinary feed directive	VFD
World Health Organization	WHO
Dual Energy X-ray Absorptiometry	DEXA

Table 2: Growth-promoting antibiotics organized by their class, trade name, and generic name[1]

Class	Trade Name	Generic Name
Diterpene	Tiamulin	Tiamulin
Glycopeptide	Avotan	Avoparcin
Lincosaminides	Lincomix	Lincomycin
Macrolide	Tylan Spira 200	Tylosin Spiramycin
Oligosaccharide	Maxus	Avilamycin
β-lactam	Penicillin	Penicillin
Peptides	Bacitracin Zn Bacitra	Bacitracin Bactitractin
Streptogramin	Stafac	Virginiamycin
Phosphoglycolipid	Flavomycin	Bambermycin
Polyether	Salocin Monteban	Salinomycin Nerasin
Quinoxalines	Mecadox Bayonox	Carbadox Olaquindox
Sulfonamides	Sulfamethazine Sulfa thizole	Sulfamethazine Sulfathiazole
Tetracycline	Aureomycin Terramycin	Chlortetracycline Oxytetracyline

[1]Gaskins et al. (2006).

Table 3: Growth-promoting antibiotics, their spectrum, and their antibacterial mode of action[1]

Class	Spectrum	Mechanism of Action
Diterpene	$Gram^+$	Protein synthesis inhibition
Glycopeptide	$Gram^+$	Cell wall synthesis inhibition
Lincosaminides	$Gram^+$	Protein synthesis inhibition
Macrolide	$Gram^+$	Protein synthesis inhibition
Oligosaccharide	$Gram^+$	Protein synthesis inhibition
β-lactam	$Gram^+$	Cell wall synthesis inhibition
Peptides	$Gram^+$	Cell wall synthesis inhibition
Streptogramin	$Gram^+$	Protein synthesis inhibition
Phosphoglycolipid	$Gram^+$	Cell wall synthesis inhibition
Polyether	$Gram^+$	Membrane alterations
Quinoxalines	Broad	DNA synthesis inhibition
Sulfonamides	Broad	Metabolic inhibition
Tetracycline	Broad	Protein synthesis inhibition

[1]Gaskins et al. (2006).

Table 4. Basal ingredient and calculated nutritional composition of experimental diets during the study period

Ingredients	Starter (%) 1 – 2 wk	Grower (%) 2 – 4 wk	Finisher (%) 5 – 6 wk
Corn, yellow	52.88	57.18	60.46
Soybean Meal	39.66	34.85	30.71
Fat, Soybean Oil	3.46	4.21	5.18
Dical Phos 18.5%	2.04	1.81	1.68
Bag Limestone	1.06	0.97	0.95
Salt 96%	0.48	0.48	0.43
Methionine, DL	0.16	0.53	0.20
Choline Cl-60%	0.08	0.09	0.08
Nutra Blend Mix[a]	0.08	0.25	0.23
Threonine 98%	0.05	0.07	0.07
Total	100	100	100
Nutritional Analysis			
ME[b] (kcal/Kg)	2987.60	3082.20	3176.80
CP[c] (%)	21.5	19.61	18.00

[a]Nutra Blend Mix = reported in Table 5. [b]ME = metabolizable energy. [c]CP = crude protein.

Table 5. Nutra Blend vitamin mix guaranteed analysis used during the starter (0.08%), grower (0.25%), and finisher (0.23%) rations of the study period

NB-3000 Poultry Premix	Guaranteed Analysis
Manganese, (MIN)	4.0 %
Zinc, (MIN)	4.0 %
Iron, (MIN)	2.0 %
Copper, (MIN)	4,500 ppm
Iodine, (MIN)	600 ppm
Selenium, (MIN)	60 ppm
Vitamin A, (MIN)	1,400,000 IU/lb
Vitamin D3, (MIN)	500,000 ICU/lb
Vitamin E, (MIN)	3,000 IU/lb
Vitamin B12, (MIN)	2 mg/lb
Menadione, (MIN)	150 mg/lb
Riboflavin, (MIN)	1,200 mg/lb
Thiamine, (MIN)	200 mg/lb
D-Pantothenic Acid, (MIN)	1,200 mg/lb
Niacin, (MIN)	5,000 mg/lb
Vitamin B6, (MIN)	250 mg/lb
Folic Acid, (MIN)	125 mg/lb
Choline, (MIN)	70,000 mg/lb
Biotin, (MIN)	6 mg/lb

Table 6. Probiotic supplementation effect on performance of broilers raised from 0-6 wk of age during phase I of the trial[1]

Age (wk)	Treatment	CFI	CBWG	FCR	ADG
1	**C**	137 ± 1 [b]	112 ± 2 [b]	1.26 ± 0.02	2.74 ± 0.04 [b]
1	**P**	143 ± 1 [a]	120 ± 2 [a]	1.22 ± 0.02	2.93 ± 0.04 [a]
Probability		0.011	0.002	0.091	0.002
2	**C**	491 ± 4 [b]	366 ± 5 [b]	1.40 ± 0.02 [a]	8.94 ± 0.12 [b]
2	**P**	509 ± 4 [a]	398 ± 5 [a]	1.31 ± 0.02 [b]	9.71 ± 0.12 [a]
Probability		<0.001	<0.001	<0.001	<0.001
3	**C**	1,054 ± 9 [b]	835 ± 9	1.29 ± 0.02 [b]	20.37 ± 0.22
3	**P**	1,128 ± 9 [a]	848 ± 9	1.35 ± 0.02 [a]	20.69 ± 0.22
Probability		<0.001	0.312	0.023	0.312
4	**C**	2,172 ± 19 [a]	1,504 ± 18	1.46 ± 0.03 [a]	36.70 ± 0.44
4	**P**	2,036 ± 18 [b]	1,504 ± 19	1.37 ± 0.03 [b]	36.70 ± 0.46
Probability		<0.001	0.991	0.025	0.991
5	**C**	3,586 ± 29 [b]	2,279 ± 23	1.58 ± 0.03	55.59 ± 0.55
5	**P**	3,619 ± 29 [a]	2,259 ± 23	1.60 ± 0.03	55.10 ± 0.56
Probability		0.041	0.540	0.570	0.540
6	**C**	4,225 ± 24	2,945 ± 32	1.45 ± 0.02	71.83 ± 0.78
6	**P**	4,260 ± 24	2,909 ± 31	1.47 ± 0.02	70.96 ± 0.76
Probability		0.309	0.433	0.364	0.433

[1]Data=means ± SE; Means with different superscripts in the same column are different ($P<0.05$).
C = control; P = probiotic; CFI = cumulative feed intake; CBWG = cumulative body weight gain; FCR = feed conversion ratio; and ADG = average daily gain.

Table 7. Probiotic supplementation effect on body composition of broilers from 1-6 wk of age during phase I of the trial[1]

Age (wk)	Treatment	Protein (g)	Fat (g)	Ash (g)	Water (g)
1	**C**	18.16 ± 0.31	21.13 ± 0.19 [a]	2.22 ± 0.05 [b]	96.93 ± 1.15 [b]
1	**P**	18.92 ± 0.31	17.35 ± 0.19 [b]	2.36 ± 0.5 [a]	103.59 ± 1.15 [a]
Probability		0.087	<0.001	0.041	<0.001
2	**C**	64.39 ± 0.86 [b]	38.03 ± 0.61 [b]	8.92 ± 0.13 [b]	275.43 ± 3.17 [b]
2	**P**	69.96 ± 0.86 [a]	40.71 ± 0.61 [a]	9.75 ± 0.13 [a]	296.89 ± 3.17 [a]
Probability		<0.001	0.002	<0.001	<0.001
3	**C**	147.81 ± 1.60	95.69 ± 1.29	21.31 ± 0.24	586.12 ± 5.84
3	**P**	150.28 ± 1.60	97.05 ± 1.29	21.68 ± 0.24	596.32 ± 5.84
Probability		0.281	0.461	0.286	0.224
4	**C**	264.61 ± 3.19	202.64 ± 2.94	39.01 ± 0.48	1,012.93 ± 11.58
4	**P**	264.77 ± 3.29	200.51 ± 3.03	39.03 ± 0.50	1,017.06 ± 11.91
Probability		0.973	0.620	0.981	0.807
5	**C**	397.10 ± 3.93	351.56 ± 4.03	59.49 ± 0.60	1,490.75 ± 14.17
5	**P**	393.71 ± 3.95	343.62 ± 4.05	58.96 ± 0.60	1,483.50 ± 14.25
Probability		0.551	0.174	0.537	0.724
6	**C**	507.68 ± 5.46	504.77 ± 6.21	77.07 ± 0.84	1,885.21 ± 19.54
6	**P**	501.59 ± 5.34	493.15 ± 6.08	76.07 ± 0.82	1,864.94 ± 19.12
Probability		0.432	0.190	0.406	0.467

[1]Data=means ± SE; Means with different superscripts in the same column are different ($P<0.05$).
C = control; P = probiotic.

Table 8. Probiotic supplementation effect on metabolic parameters of broilers raised from 1-6 wk of age during phase I of the trial[1]

Age (wk)	Treatment	MEC (kcal)	MER (kcal)	HP (kcal)	EMEU (kcal)
1	**C**	411 ± 4 [b]	299 ± 3 [a]	112 ± 3 [b]	0.74 ± 0.01 [a]
1	**P**	427 ± 4 [a]	268 ± 3 [b]	159 ± 3 [a]	0.63 ± 0.01 [b]
Probability		0.012	<0.001	<0.001	<0.001
2	**C**	1,467 ± 11 [b]	718 ± 10 [b]	754 ± 11	0.49 ± 0.01 [b]
2	**P**	1,521 ± 11 [a]	774 ± 10 [a]	747 ± 11	0.51 ± 0.01 [a]
Probability		<0.001	<0.001	0.670	0.013
3	**C**	3,249 ± 29 [b]	1,725 ± 21	1,534 ± 29 [b]	0.53 ± 0.01 [a]
3	**P**	3,476 ± 29 [a]	1,752 ± 21	1,724 ± 29 [a]	0.51 ± 0.01 [b]
Probability		<0.001	0.376	<0.001	0.023
4	**C**	6,693 ± 59 [a]	3,380 ± 45	3,319 ± 84 [b]	0.51 ± 0.01
4	**P**	6,276 ± 56 [b]	3,361 ± 47	2,905 ± 87 [a]	0.54 ± 0.01
Probability		<0.001	0.776	<0.001	0.084
5	**C**	11,393 ± 91	5,513 ± 60	5,880 ± 150	0.49 ± 0.01
5	**P**	11,498 ± 92	5,420 ± 60	6,078 ± 151	0.48 ± 0.01
Probability		0.4264	0.282	0.362	0.289
6	**C**	13,421 ± 77	7,563 ± 89	5,875 ± 122	0.56 ± 0.01
6	**P**	13,534 ± 77	7,420 ± 87	6,094 ± 119	0.55 ± 0.01
Probability		0.309	0.259	0.208	0.113

[1]Data=means ± SE; Means with different superscripts in the same column are different ($P<0.05$).
C = control; P = probiotic; MEC = metabolizable energy consumed; MER = metabolizable energy retained; HP = heat production; and EMEU: efficiency of metabolizable energy use.

Table 9. Weekly performance data from broilers during stress periods raised from 4-6 wk in phase II of the trial[1]

Age (wk)	Treatment	Wkly BW	Wkly Gain	Wkly FI	Wkly FCR
4	**CTN**	1,457 ± 33	509 ± 21	797 ± 21 [a]	1.58 ± .04 [a]
4	**PTN**	1,445 ± 39	504 ± 25	757 ± 24 [a]	1.49 ± .05 [a]
4	**CHS**	1,410 ± 46	531 ± 30	623 ± 29 [b]	1.20 ± .06 [b]
4	**PHS**	1,442 ± 43	535 ± 28	790 ± 27 [a]	1.49 ± .05 [a]
Probability		0.875	0.814	<0.001	<0.001
5	**CTN**	2,142 ± 43	685 ± 23 [a]	1,116 ± 27 [a]	1.66 ± .07
5	**PTN**	2,044 ± 52	599 ± 28 [b]	1,043 ± 29 [a]	1.77 ± .08
5	**CHS**	1,927 ± 66	539 ± 35 [b]	900 ± 38 [b]	1.69 ± .10
5	**PHS**	2,051 ± 61	616 ± 33 [b]	997 ± 36 [b]	1.62 ± .09
Probability		0.066	0.009	<0.001	0.654
6	**CTN**	2,595 ± 49 [a]	492 ± 27 [a]	968 ± 42 [a]	2.03 ± .12
6	**PTN**	2,565 ± 59 [a]	521 ± 32 [a]	996 ± 49 [a]	1.97 ± .14
6	**CHS**	2,216 ± 98 [b]	344 ± 53 [b]	679 ± 69 [b]	2.32 ± .24
6	**PHS**	2,477 ± 80 [a]	464 ± 43 [ab]	784 ± 60 [b]	1.99 ± .19
Probability		0.012	0.0526	<0.001	0.650

[1]Data=means ± SE; Means with different superscripts in the same column are different ($P<0.05$). Wkly = weekly; BW = body weight; FI = feed intake; FCR = feed conversion ratio; CTN = control thermal neutral; PTN = probiotic thermal neutral; CHS = control heat stress; and PHS = probiotic heat stress.

Table 10. Probiotic supplementation effect on body composition of broilers raised from 4-6 wk of age during stress periods in phase II of the trial[1]

Age (wk)	Treatment	Wkly Protein (g)	Wkly Fat (g)	Wkly Ash (g)	Wkly Water (g)
4	**CTN**	91.39 ± 3.56	73.13 ± 3.45	13.50 ± 0.55	329.06 ± 13.28
4	**PTN**	90.85 ± 4.29	74.35 ± 4.16	13.44 ± 0.66	328.84 ± 16.02
4	**CHS**	95.23 ± 5.03	67.09 ± 4.87	14.12 ± 0.78	350.05 ± 18.78
4	**PHS**	89.01 ± 4.74	61.96 ± 4.60	13.94 ± 0.73	366.12 ± 17.71
Probability		0.837	0.167	0.877	0.319
5	**CTN**	118.86 ± 3.97 [a]	124.74 ± 4.57 [a]	18.12 ± 0.61 [a]	424.25 ± 14.34 [a]
5	**PTN**	103.32 ± 4.78 [b]	108.46 ± 5.51 [b]	15.84 ± 0.74 [b]	374.52 ± 17.29 [b]
5	**CHS**	93.82 ± 6.00 [b]	97.79 ± 6.90 [b]	14.30 ± 0.93 [b]	338.16 ± 21.68 [b]
5	**PHS**	108.89 ± 5.61[ab]	114.31 ± 6.46 [ab]	16.38 ± 0.87 [ab]	378.84 ± 20.28 [ab]
Probability		0.007	0.014	0.009	0.012
6	**CTN**	76.60 ± 6.23	101.63 ± 7.57	11.97 ± 0.98	264.83 ± 22.51
6	**PTN**	86.56 ± 7.51	111.24 ± 9.13	13.70 ± 1.18	311.89 ± 27.15
6	**CHS**	59.64 ± 12.46	87.32 ± 15.14	9.19 ± 1.95	202.43 ± 45.02
6	**PHS**	87.21 ± 10.17	121.94 ± 12.36	12.67 ± 1.59	256.49 ± 36.76
Probability		0.258	0.296	0.268	0.207

[1]Data=means ± SE; Means with different superscripts in the same column are different ($P<0.05$). Wkly = weekly; CTN = control thermal neutral; PTN = probiotic thermal neutral; CHS = control heat stress; and PHS = probiotic heat stress.

Table 11. Probiotic supplementation effect on metabolic parameters of broilers raised from 4-6 wk of age under stress periods during phase II of the trial[1]

Age (wk)	Treatment	Wkly MEC (kcal)	Wkly MER (kcal)	Wkly HP (kcal)	Wkly EMEU (kcal)
4	**CTN**	2,455 ± 63 [a]	1,196 ± 52	1,259 ± 41 [a]	0.49 ± 0.02 [c]
4	**PTN**	2,332 ± 73 [a]	1,205 ± 63	1,088 ± 49 [b]	0.52 ± 0.02 [b]
4	**CHS**	1,920 ± 90 [b]	1,162 ± 73	758 ± 58 [c]	0.61 ± 0.02 [a]
4	**PHS**	2,434 ± 85 [a]	1,079 ± 69	1,355 ± 55 [a]	0.44 ± 0.02 [c]
Probability		<0.001	0.520	<0.001	<0.001
5	**CTN**	3,547 ± 85 [a]	1,832 ± 65 [a]	1,693 ± 94	0.52 ± 0.02
5	**PTN**	3,313 ± 92 [a]	1,592 ± 78 [b]	1,723 ± 107	0.49 ± 0.02
5	**CHS**	2,860 ± 121 [b]	1,440 ± 98 [b]	1,420 ± 134	0.50 ± 0.03
5	**PHS**	3,168 ± 113 [b]	1,678 ± 91 [a]	1,490 ± 125	0.53 ± 0.03
Probability		<0.001	0.012	0.204	0.529
6	**CTN**	3074 ± 135 [a]	1,487 ± 79	1,580 ± 116	0.48 ± 0.03
6	**PTN**	3165 ± 156 [a]	1,524 ± 92	1,628 ± 136	0.51 ± 0.03
6	**CHS**	2157 ± 220 [b]	1,149 ± 153	1,282 ± 225	0.47 ± 0.06
6	**PHS**	2490 ± 191 [b]	1,627 ± 125	1,224 ± 184	0.58 ± 0.05
Probability		<0.001	0.120	0.227	0.364

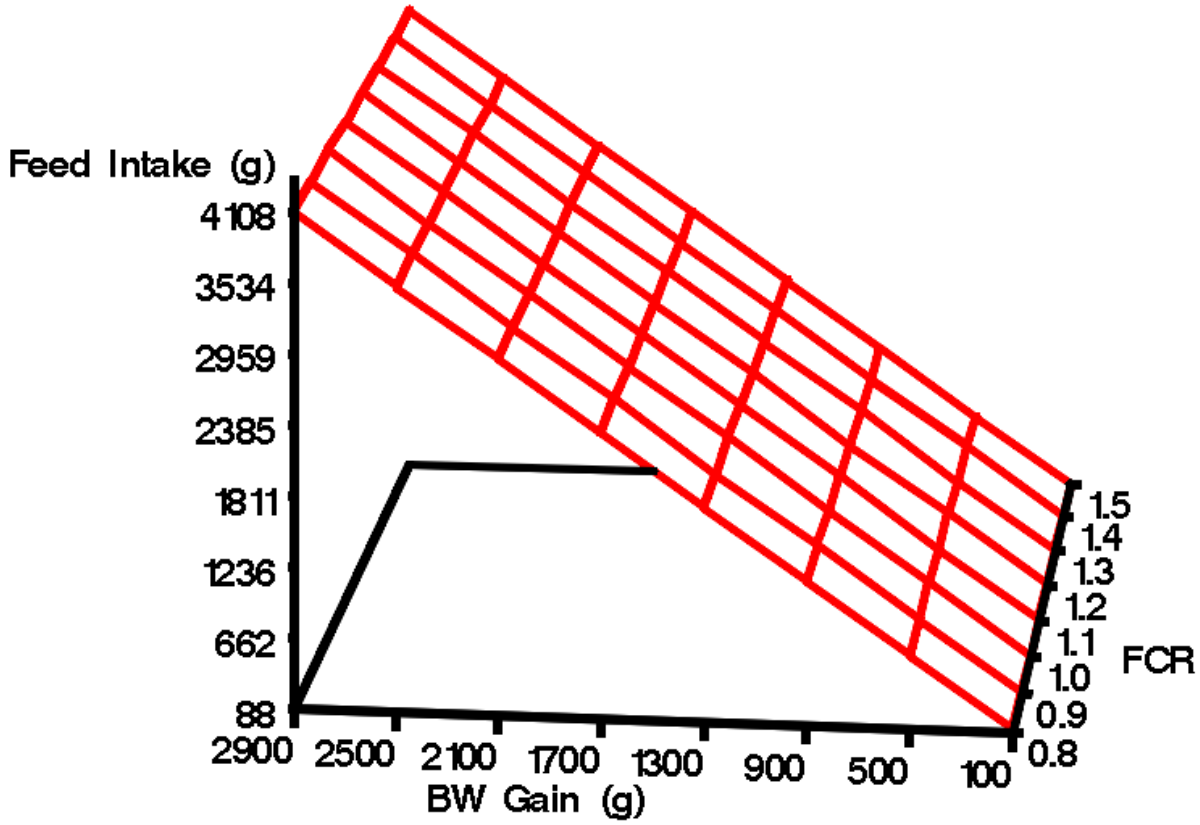
[1]Data=means ± SE; Means with different superscripts in the same column are different (P<0.05).
Wkly = weekly; CTN = control thermal neutral; PTN = probiotic thermal neutral; CHS = control heat stress; PHS = probiotic heat stress; MEC = metabolizable energy consumed; MER = metabolizable energy retained; HP = heat production; and EMEU: efficiency of metabolizable energy use.

Table 12. Respiration per minute of broilers raised from 4-6 wk of age during stress periods during phase II of the trial[1]

	4 (wk)	5 (wk)	6 (wk)	4 -6 ARW (wk)
Treatment				
PTN	52.00 ± 5.65 [b]	62.89 ± 3.80 [c]	81.60 ± 4.44 [b]	63.85 ± 2.96 [c]
CHS	140.95 ± 5.65 [a]	135.11 ± 3.80 [a]	133.87 ± 4.44 [a]	137.04 ± 2.96 [a]
PHS	126.10 ± 5.65 [a]	115.78 ± 3.80 [b]	122.40 ± 4.44 [a]	121.63 ± 2.96 [b]
Probability	<.0001	<0.001	<0.001	<0.001

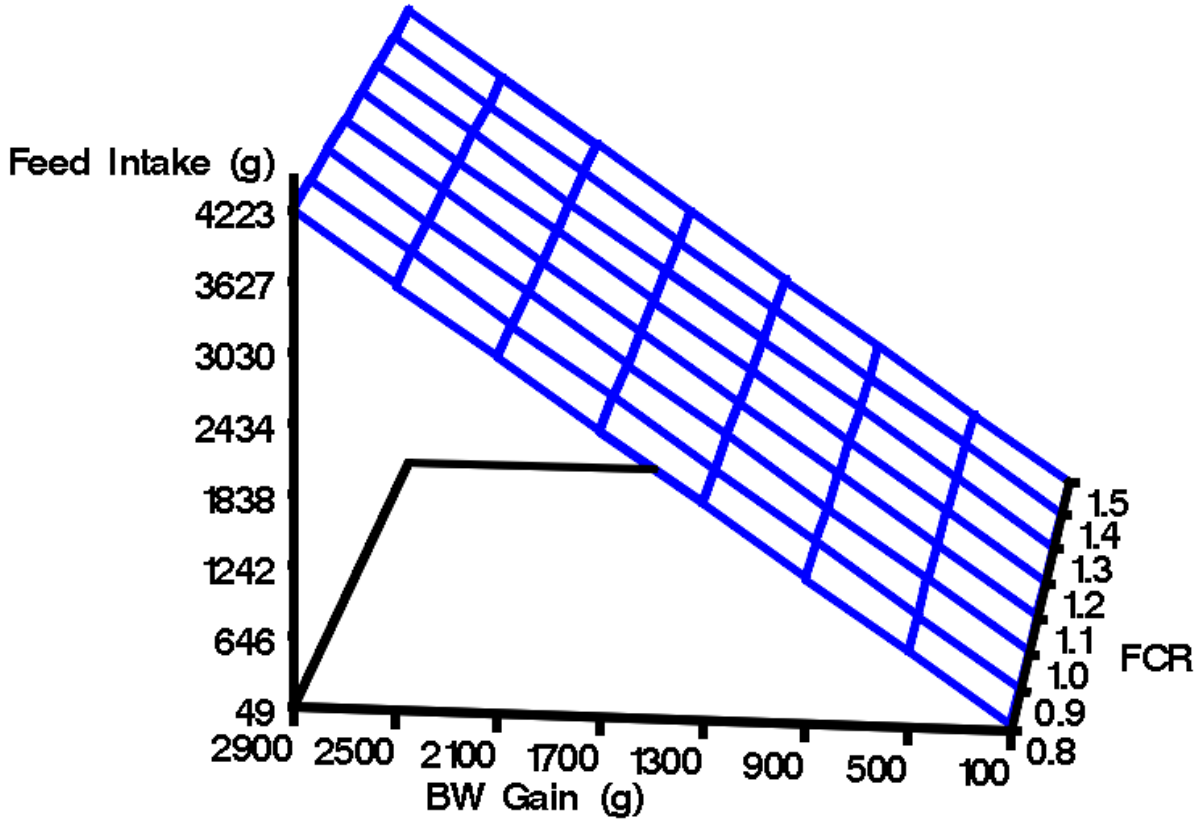
[1]Data=means ± SE; Means with different superscripts in the same column are different (P<0.05).
ARW = average respiration per week; PTN = probiotic thermal neutral; CHS = control heat stress; and PHS = probiotic heat stress.

Figure 1. Relationships of feed intake, body weight gain, and feed conversion ratio (FCR) of control broilers raised to 6 wk of age



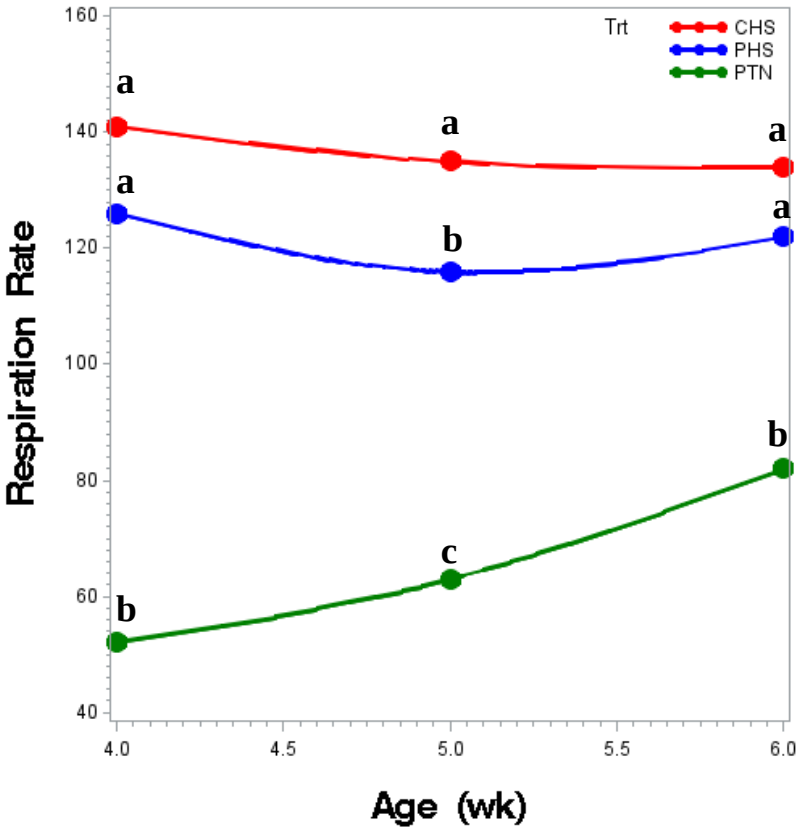
FeedI**C**=1.42664*BWTgain**C** - 36.61285*FCR**C**

Figure 2. Relationships of feed intake, body weight gain, and feed conversion ratio (FCR) of probiotic-supplemented broilers raised to 6 wk of age



FeedI**P**=1.47416*BWTgain**P** - 65.3574*FCR**P**

Figure 3. Respiration rate of broilers raised under either TN or HS



Different superscripts within a treatment are different ($P<0.05$).
TN = thermo-neutral; HS = heat stress; CHS = control heat stress; PHS = probiotic heat stress; PTN = Probiotic thermo-neutral.

APPENDICES

Appendix 1.

Oklahoma State University
Institutional Animal Care and Use Committee (IACUC)

Protocol Expires: 10/8/2017

Date : Monday, February 29, 2016 Animal Care and Use Protocol (ACUP) AG1412

Proposal Title: Assessment of growth efficiency in poultry with a probiotic diet supplement

Principal Investigator:

Patricia Rayas-Duarte
Biochemistry & Molecular Biology
123 FAPC
Campus

Reviewed and Processed as: Special Review **Modification**

Approval Status Recommended by Reviewer(s): Approved

The modification request for change in animal numbers and change in procedure is approved. You are approved to use 500 additional chicks for a heat stress study as described. The total number of chickens now approved for this protocol is 810 (250+60+500).

Signatures :

Karen McBee

Dr. Karen McBee, IACUC Chair — Monday, February 29, 2016 Date

cc: Department Head, Food & Agricultural Products Research & Tech Ctr.
LAR

Approvals are valid for three calendar years, after which time a request for renewal must be submitted. Any modifications to the research project, course, or testing procedures must be submitted for review and approval by the IACUC, prior to initiating any changes. Modifications do not affect the original approval period. Modification approvals are valid for the duration of the protocol approval (see protocol expiration date). Approved projects are subject to monitoring by the IACUC. OSU is a USDA registered research facility and maintains an Animal Welfare Assurance document with the Public Health Service Office of Laboratory Animal Welfare, Assurance number AA3722-01.

VITA

Sarah Elsa Marie Schobert

Candidate for the Degree of

Master of Science

Thesis: PROBIOTIC SUPPLEMENTATION EFFECTS ON PERFORMANCE, BODY COMPOSITION, AND ENERGETIC EFFICIENCY OF BROILERS UNDER THERMO-NEUTRAL AND HEAT STRESS CONDITIONS

Major Field: Animal Science

Biographical:

Education:

Completed the requirements for the Master of Science in Animal Science at Oklahoma State University, Stillwater, Oklahoma, in May 2017.

Completed the requirements for the Bachelor of Science in Animal Science at Oklahoma State University, Stillwater, Oklahoma, in May 2015.

Completed the requirements for the Associate of Science at Black Hawk College East Campus, Galva, Illinois, in May 2013.

Experience: Stable Hand, Mount Regis Stables, Salem, Virginia; Sales Associate, The Men's Warehouse, Roanoke, Virginia; Student Intern, Sport Horse Incorporated, Westfield, Indiana; Resident Assistant, Prairie Pointe Apartments, Galva, Illinois; Assistant Horse Judging Coach, Black Hawk College East Campus, Galva, Illinois; Junior Consultant Intern, Denali Executives Inc., Indianapolis, Indiana; Sales and Marketing Intern, Alltech Inc., Springfield, Missouri; Graduate Research and Teaching Assistant, Oklahoma State University, Stillwater, Oklahoma.

Professional Memberships: Alpha Zeta, American Paint Horse Association, American Quarter Horse Association, American Society of Animal Science, Phi Theta Kappa, Pony of Americas, Inc. – Carded Judge, Poultry Science.