Performance and Microbial Profiles of Broiler Chickens fed Phytogenic Feed Additives or Probiotics during Coccidiosis

By

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Abstract

The prophylactic use of antibiotics is rapidly being phased out of poultry operations. Diseases such as coccidiosis are extremely costly, and typically prevented with coccidiostats and antibiotics. New regulation banning antibiotics is mandating a search for new alternatives, such as probiotics and phytogenic feed additives (PFAs). Two separate studies were performed to evaluate the performance and microbial profiles of commercial broilers fed either PFAs or *Eimeria* challenged broilers fed probiotics. During the probiotics study, 1056 day-old male Ross broiler chicks were assigned to one of 6 treatments, 8 replicates per treatment, with 22 birds per floor pen in a 2x3 factorial arrangement, with DFM mix and coccidiosis challenge being the main factors. Treatments included a non-infected control (NC), a non-infected low level DFM-fed group (DL), a non-infected high level DFM-fed group (DH), an infected control (PC), an infected low level DFM-fed group (DL+), and an infected high level DFM-fed group (DH+). Performance measurements were taken on d0, d7, d14, d21, and d28. Mucosal scrapings from the ileum were obtained on d7, d14, d21, and d28 to determine microbial profiles. On d15, birds in the three infected groups were challenged with a mixed inoculum containing *Eimeria maxima, E. tenella,* and *E. acervulina.* Lesion scores were assessed on d21. Body composition was analyzed via Dual Energy X-Ray Absorptiometry (DXA) on d28. Results indicated a trend ($P < 0.1$) toward improved D28 body composition, lower *Clostridium* in DFM-fed challenged broilers compared to PC, and higher *Bacillus* in DH compared to DH+. Results were not significant for

BW, BWG, FI, or lesion scores. The second study evaluated the effects of PFA provided as a dietary supplement over a 6-week period on performance and immune responses in broilers. In total, 1056 day-old male chicks were randomly assigned to 8 replicate pens (22 birds/pen) with 6 treatments including a standard corn/wheat basal diet, a basal with antibiotics, and 4 various combinations of PFA (PHY 1, 2, 3, and 4). Performance was assessed on day 7 (D7), 14, 28, and 42. Starter diet was fed D0-14, grower D14-28, and finisher D28-42. Microbial profiling was assessed on D7, 14, and 42, and body composition measured on D42 using Dual Energy X-Ray Absorptiometry (DXA). Results varied by week, but cumulatively, body weight gain (BWG) per bird was higher in PHY1 and 4, statistically similar to AGP and CTRL. With feed conversion ratio (FCR), PHY2 was higher than any other treatment between D0-42, while all other treatments were similar. PCR results were not statistically significant; however, DXA results indicated a higher lean to fat ratio in birds from PHY1-4 when compared to AGP, indicating an overall leaner bird in PHY treatments. The results of these studies suggest that diets supplemented with DFMs or PFAs result in improved broiler performance.

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Table of Contents

Abstract i

Acknowledgments iii

Table of Contents v

List of Figures viii

List of Tables ix

CHAPTER I 1

Introduction 1

References 4

CHAPTER II 7

Literature Review 7

Avian Immune System 7

Innate Immunity 7

Physical and chemical barriers 8

Cellular defenses 10

Complement proteins 11

Major Histocompatibility Complex 11

Pattern Recognition Receptors and Microbe-Associated Molecular Patterns 12

Adaptive Immunity 12

Humoral Immunity 13

Humoral Immunity: The Bursa of Fabricius and B Cells 14

Humoral Immunity: Immunoglobulins 14

Cell-Mediated Immunity 15

Avian Cytokines and Chemokines 16

Gut-Associated Lymphoid Tissue (GALT) 18

Enteric Microbial Environment 19

Coccidiosis 20

Life Cycle of Eimeria 20

Treatment and Prevention 21

Response to Coccidiosis 22

Humoral Immune Response 23

Cell-Mediated Immune Response 23
Probiotics and Poultry 25
Modes of Action 25
Probiotics, Performance, and Intestinal Development 27
Probiotics and Innate Immunity 28
Probiotics and Adaptive Immunity 28
Probiotics and Host Defense against Pathogens 30
Phytogenic Feed Additives and Poultry 31
Modes of Action 32
Phytogenics, Performance, and Intestinal Effects 32
Phytogenics and Innate Immunity 35
Phytogenics and Adaptive Immunity 36
References 38
CHAPTER III 52
Performance and Response of DFM-fed broilers to a coccidiosis challenge 52
Abstract 52
Introduction 53
Materials and Methods 54
Birds and Experimental Treatments 54
Probiotics Mixture 55
Coccidiosis Challenge 55
Performance Measurements 55
Microbial Profiling 56
Statistical Analysis 58
Results 58
Performance Measurements 58
Coccidiosis Challenge 60
Microbial Profiling 60
Discussion 61
References 67
CHAPTER IV 81
Performance and response in broilers fed phytogenic feed additives 81

Abstract 81
Introduction 82
Materials and Methods 83
Birds and Experimental Treatments 83
Phytogenic Feed Additives Mixture 84
Performance Measurements 85
Microbial Profiling 85
Statistical Analysis 87
Results 88
Performance Measurements 88
Microbial Profiling 90
Discussion 90
References 96
CHAPTER V 111
Epilogue 111
Appendix 1 114
Non-significant data associated with Chapter III 114
Appendix 2 123
Non-Significant data associated with Chapter IV 123

List of Figures

Chapter III Figures

Figure 3.1. Effect of DFM diet (D0-14) or DFM diet*challenge interaction (D14-D28) on average body weight (BW) per bird per treatment of Ross male broiler chicks ……………72

Figure 3.2. Effect of DFM diet (D0-14) or DFM diet*challenge interaction (D14-D28) on average body weight gain (BWG) per bird per treatment of Ross male broiler chicks……..73

Figure 3.3. Effect of DFM diet (D0-14) or DFM diet*challenge interaction (D14-D28) on average feed intake (FI) per bird per treatment of Ross male broiler chicks………………..…74

Figure 3.4. Effect of DFM diet (D0-14) or DFM diet*challenge interaction (D14-D28) on feed conversion ratio (FCR) per treatment of Ross male broiler chicks…………………………76

Figure 3.5. Effect of DFM diet*challenge interactions on D28 body composition per bird (percent fat or percent lean) on male Ross broiler chicks……………………………………78

Figure 3.6. Effect of DFM diet*challenge interactions on microbial profiles of ileum of male Ross broiler chicks………………………………………………………………………79

Chapter IV Figures

Figure 4.1. Effect of PFAs on average body weight (BW) per bird of Ross male broiler chicks……………………………………………………………………………...…101

Figure 4.2. Effect of PFAs on weekly and cumulative body weight gain (BWG) per bird of Ross male broiler chicks……………………………………………………………………102

Figure 4.3. Effect of PFAs on weekly and cumulative feed intake (FI) per bird of Ross male broiler chicks…………………………………………………………..………..…105

Figure 4.4. Effect of PFAs on feed conversion ratio (FCR) of Ross male broiler chicks…………………………………………………………………………………....106

Figure 4.5. Effect of PFAs on percent mortality per treatment of male Ross broiler chicks …………………………..……………………………………………………….……....108

Figure 4.6. Effects of PFAs on D42 body composition per bird (percent fat or percent lean) on male Ross broiler chicks…………………………………………………………..…..…110

List of Tables

Chapter III Tables

Table 3.1. Description of treatments……………………………………………………69

Table 3.2. Composition of broiler diets during starter and grower phases……….…….70

Table 3.3. Primers used for qRT-PCR in microbial profiling………………….………71

Chapter IV Tables

Table 4.1. Description of treatments…………………………………………………...98

Table 4.2. Composition of broiler diets during starter, grower, and finisher phases…..99

Table 4.3. Primers used for qRT-PCR in microbial profiling…………….……………100

CHAPTER I

Introduction

Livestock production in the United States involves raising animals, including poultry, in close proximity to one another. For decades, the sub therapeutic application of antibiotics has been used in animals to improve growth rates and decrease incidence of disease that often occurs with large concentrations of animals. Antibiotics are now being phased for a number of reasons. Consumers are concerned over trace antibiotics in meat and antibiotic resistant strains of bacteria, such as *Salmonella,* which have been isolated from poultry slaughterhouses (Carmraminana, 2004; World Health Organization, 2000). The European Union has already banned antibiotic use, and the United States has implemented new rules governing the use of antibiotics that are important in human medicine, making routine antibiotic use more difficult in poultry production (Castanon, 2007; Veterinary Feed Directive, 2015). Improper antibiotic use has prompted research into natural alternatives, including probiotics and phytogenic feed additives, which may replace antibiotics previously used to promote growth and prevent diseases.

Among the most costly diseases in poultry is coccidiosis, with increased mortality, lower weight gains, and higher feed expenses costing the United States an estimated $3 billion annually (Williams, 1999; Dalloul and Lillehoj, 2006). Coccidiosis is caused by an intestinal parasite known as *Eimeria*, which is transmitted via the oral-fecal route. There are nine strains of *Eimeria* commonly found in chickens, each one infecting a different part of the intestinal tract and causing symptoms such as watery white diarrhea, increased mucus production, damaged intestinal villi, decreased ability to absorb nutrients, and stunted growth (Williams, 1999). This endemic disease can survive in poultry litter, which is often reused between flocks, resulting in

high levels of exposure to new birds (Stringfellow et al., 2011). Current prevention strategies include vaccination with live oocysts to induce immunity, improving litter management strategies, and coccidiostats that attempt to reduce parasite loads (Chapman, 1997; Bernhart et al., 2010; Peek and Landman, 2011). Other areas of research involve adding natural products to feed or water, such as probiotics and phytogenic feed additives.

Probiotics are live microorganisms that confer a health benefit when fed to a host (FAO/WHO, 2001). Common probiotics found in poultry additives include bacteria or yeast from the genera *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* (Kabir, 2009). They may improve bird health and defend against disease via numerous mechanisms. First, they act by competitive exclusion of pathogenic bacteria. They do this occupying space in the gut that pathogens could inhabit and producing antimicrobial metabolites, such as bacteriocins (Joerger et al., 2003; Yang et al., 2009). Second, they may directly interact with immune cells in the gut to improve immunity and prime the immune system for defense against disease (Edens, 2003). Probiotics in the diet may improve overall performance, including body weight gain and feed conversion ratios (Fritts et al., 2000; Lan et al., 2003; Huang et al., 2004). Early establishment of beneficial bacteria can result in a healthier gut, improved bird growth, and resistance to disease.

Another category of natural alternatives to antibiotics include phytogenic feed additives (PFAs). This is a broad category of plant-derived oils, spices, and herbs intended to improve poultry health (Yang et al., 2009). Common examples found in poultry feed supplements include compounds from cinnamon, oregano, thyme, pepper, citrus, and sage (Windisch and Kroismayr, 2006). Proposed mechanisms of action include antimicrobial activity, increased immune function, better feed palatability, and stimulation of digestion enzymes (Xue and Meng, 1996;

Cowen, 1999; Applegate et al., 2010). Numerous studies investigated the effects on performance in broilers, and found improved feed conversion ratios and average daily gain (Guo et al., 2004; Ertase et al., 2005; Garcia et al., 2007). Several studies (Allen et al., 1997; Youn et al., 2001; Christakia et al., 2004) showed anticoccidial activity when fed various types of PFAs. The effectiveness of PFAs depends on the type of plant used, time of harvest, method of extraction, interactions with other feed ingredients, and other physical properties (Wang et al., 1998). PFAs are a promising alternative that continue to be researched.

A healthy gut is especially essential in broiler production. Broilers grow rapidly and need to maintain a healthy gut for optimum nutrient absorption. Research is being done to reduce disease and improve gut health through the use of natural alternatives such as probiotics and PFAs. The goals of these experiments were to study the effects of probiotic or PFA supplementation on bird performance and microbial profiles, with or without a coccidiosis challenge.

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CHAPTER II

Literature Review

Information in this literature review covers the avian immune system, the prevalent parasitic disease known as coccidiosis, the benefits of feeding probiotics to challenged animals, and potential benefits of using antibiotic alternatives such as phytogenic feed additives in poultry in an attempt to improve performance. The studies yielded curious results pertaining to the immune system, broiler performance, and benefits of natural feed additives.

Avian Immune System

The avian immune system is similar to that of mammals, but different in a few key aspects. Both have a similar makeup, with the same main functions of recognizing and killing or neutralizing foreign microbes and pathogens, as well as recognizing and tolerating self antigens. The immune system is made up of the innate and adaptive components; both are unique from another but work together during a challenge (Juul-Madsen et al., 2014).

Innate Immunity

The avian innate immune system is a bird's first line of defense, acting at the earliest stages of invasion before the more specific adaptive immune response begins (Juul-Madsen et al., 2014). From time of hatch, the innate immune system is working to keep out unwanted pathogens and foreign material using physical and chemical barriers, cellular defenses, complement, and cytokines. Physical barriers include epithelial and mucosal layers, as well as a high body temperature (Butcher and Miles, 2001; Juul-Madsen et al., 2014). Chemical defenses include

antimicrobial secretions and pH changes. Cellular defenses involve a variety of innate cells that recognize and trap microbes, and present them to the adaptive immune system.

Physical and chemical barriers

The external environment is in endless contact with epithelial and mucosal layers covering the outside of the body, the gastrointestinal, and respiratory tracts. Foreign microbes and pathogens are kept out using multiple mechanisms. On the epithelium, skin acts as a natural barrier to invasion. The normal microbiota prevents the colonization of harmful pathogens. This is known as competitive exclusion, and is utilized both on the skin and in the gut (Van Immerseel et al., 2005). In the respiratory tract, mucus helps capture microbes and ciliary movement clears them from the body (Juul-Madsen et al., 2014). The peristaltic movements of the gastrointestinal tract carry foreign substances along the intestine, the site of nutrient and water absorption. There is a constant battle to keep out unwanted pathogens and foreign material. The intestines are lined with a single layer of columnar intestinal epithelial cells (IECs) that act as a barrier to foreign material in the gut. These cells remain close together, controlling the flow of water and nutrients, as well as allowing the secretion of antimicrobial proteins into the gut lumen to prevent dangerous bacteria from propagating (Pitman and Blumberg, 2000). Low pH in the gut is another barrier to microbial growth (Juul-Madsen et al., 2014). The avian gut maintains a complex microbiota that uses competitive exclusion as a means of deterring pathogens and toxins.

Inflammation quickly induces a chemical response changing the production of proteins involved in innate immunity. C-reactive protein (CRP), possessing both inflammatory and anti-inflammatory properties, increases in chickens infected with *Eimeria* spp. It aids in complement activation and opsonization by phagocytes (Murata et al., 2004). Mannan-binding lectin (MBL)

is produced by the liver and secreted into the blood during infection, aiding in complement activation (Murata et al., 2004). Fibrinogen (FB) aids in tissue repair by providing a substrate for fibrin formation (Murata et al., 2004). A collection of chemicals is produced by the epithelium, mucous membranes, and in the gastro-intestinal tract. They can destroy microbes, mark them for destruction, or aid in their removal.

Host defense peptides (HDPs) are proteins found in all avians and mammals. They directly kill microbes by creating holes in the cell membrane (Kagan et al., 1990). In chickens, there are two types of HDPs: cathelicidins and β-defensins. Cathelicidins are known to have cytotoxic activity as well as bind to lipopolysaccharides (LPS) on bacteria to block LPS-induced inflammatory response (van Dijk et al., 2005). Poultry β-defensins aid innate immune cells known as heterophils, the avian equivalent to mammalian neutrophils. The heterophils lack oxidative properties, and β-defensins assist in killing a number of microorganisms, including *Staphyloccocus aureas*, *Escherichia coli*, *Candida albicans*, *Salmonella* Enteritidis, and *Campylobacter jejuni* (Harmon, 1998; Higgs et al., 2005).

Macrophages are innate cells equipped with both respiratory burst and nitric oxide (NO) activity, chemical defenses against pathogens. Respiratory burst is performed when these cells reduce oxygen (O_2) to superoxide (O_2^-), which reacts with itself to form hydrogen peroxide (H_2O_2), eventually leading to hypochlorous acid (HOCl), a highly reactive oxidant (Golemboski et al., 1990; Lin et al., 1992; Babior, 1995, 2004). HOCl destroys bacteria through numerous mechanisms: disrupting glucose oxidation via disrupting cytochromes and iron-sulfur components in the electron transport chain; inhibiting DNA replication by decreasing affinity for necessary membrane proteins used in DNA replication; affecting post-translational modification

of bacterial proteins by aggregation and oxidizing cysteine and methionine (Venkobachar et al., 1975; Albrich et al., 1981; Rosen et al., 1998; Winter et al., 2008). In addition to respiratory burst, nitric oxide is also produced by activated macrophages in response to inflammatory signals (Kaspers et al., 2014b). Production of the antimicrobial reactive radical NO is derived from L-arginine and oxygen by the enzyme known as inducible nitric oxide synthase (iNOS) after stimulation with inflammatory cytokines (Fang, 2004). Certain bacterial antigens stimulate iNOS activity, commonly used as a measurement of avian macrophage activation (Kaspers et al., 2014b). Nitric oxide damages pathogens in a similar way to respiratory burst (Slauch, 2011).

Cellular defenses

If physical and chemical barriers are unable to keep out foreign material, innate immune cells are also ready to respond to the first sign of invasion. Cells include natural killer (NK) cells and phagocytes such as heterophils, dendritic cells (DC), and macrophages. Natural killer cells are large lymphocytes containing numerous granules (Göbel et al., 1994). Unlike B and T cells of the adaptive immune system, they lack MHC class II antigens, and do not need activation to release cytotoxic granules used in killing foreign microbes. (Göbel et al., 1994). Chicken heterophils are important phagocytic innate immune cells, but unlike their mammalian counterparts, the neutrophils, they lack myeloperoxidase to destroy microbes. They rely on the β-defensins found in granules that have antimicrobial abilities. Heterophils are stimulated by either pathogens or cytokines, and their activation leads to more cytokine production including interleukin (IL)-1, IL-6, and IL-8, further enhancing the inflammatory response (Kogut et al., 2005a, 2006). Phagocytic cells such as dendritic cells and macrophages are known as antigen-presenting cells (APCs). They take up foreign antigens and present its components to T and B

lymphocytes of the adaptive immune system to mount a more specific response. Macrophages will also release toxic agents when activated by antigens. One well-known enzyme is inducible nitric oxide synthase (iNOS) which leads to the production of nitric oxide. The nitric oxide and superoxide anions react to produce toxic substances that kill microbes by damaging the bacterial cytoplasm and essential molecules used in metabolism (Kaspers et al., 2014b).

Complement proteins

Another important part of innate immunity is the serum complement system. This group of 25 proteins is mostly produced by hepatocytes, but a few are produced by macrophages. They are in constant circulation until activation by inflammatory cytokines, promoting a cascade. Activation is achieved via three separate pathways: classical, lectin, or alternative pathway. These pathways are stimulated by antigen-antibody complexes or microbial surfaces. Once activated, effects of the complement cascade include phagocytosis, direct lysis of target cells, inflammation, and improved B and T cell response (Carroll, 2004).

Major Histocompatibility Complex

Major histocompatibility complex (MHC) molecules are found on host cells that function to bind foreign antigen and present it to T cells and B cells in the adaptive immune system. There are class I or class II MHC molecules. The class of MHC molecule expressing the antigen indicates the origin of the antigen. All host cells possess MHC class I molecules, and when they have been virally-infected or extremely damaged, they will present antigen to cytotoxic T cells to destroy the infected cell. Antigen presented on class II MHC molecules are from the surrounding

environment or cytoplasm. This alerts helper T cells and B cells that activate and begin producing antibodies against the antigen (Kaspers et al., 2014a).

Pattern Recognition Receptors and Microbe-Associated Molecular Patterns

In all animals, including poultry, innate cells have pattern recognition receptors (PRR) both extracellularly on their membranes and intracellularly in cytosol. Foreign microorganisms are coated in microbe-associated molecular patterns (MAMPs). These are conserved across groups of microbes, and innate cells recognize the MAMPs immediately upon contact, eliminating the need for a previous encounter to become active. The PRR on innate cells are created by conserved codes in the germline DNA, and have less specificity than adaptive immune cells (Juul-Madsen et al., 2014).

The innate immune system works to block foreign microbes from entering the body. It uses physical barriers, leukocytes, and various antimicrobial proteins to combat the initial stage of pathogenic invasion. Once an innate cell encounters a foreign pathogen, it takes immediate action to limit the infection and alert the adaptive immune system (Abbas et al., 2012a; Juul-Madsen et al., 2014).

Adaptive Immunity

The adaptive immune system is a second line of defense against pathogens. Cells of the adaptive immune system are known as lymphocytes, and they respond to antigens, which are any foreign substances that elicit a response of lymphocytes and antibodies. Adaptive immunity is categorized as either cell-mediated, involving T lymphocytes, or humoral, involving B lymphocytes. Cell-mediated immunity is mediated by T cells. They work to eliminate infection

by eliminating phagocytes or host cells that have been compromised by pathogens inside the cells. Humoral immunity uses antibodies produced by B cells to help neutralize and eliminate microbes and toxins (Abbas et al., 2012a).

Unlike innate immunity, which responds immediately to an infection in a similar way each time, adaptive immunity is acquired through exposure to an antigen. There are many fundamental features of the adaptive immune response. First, each adaptive immune cell, or lymphocyte, has specificity to a certain antigen. Lymphocytes are more diverse than leukocytes of the innate immune system. Exposure to antigen results in clonal expansion of the lymphocyte specific for the antigen, an immune response using cell-mediated and humoral immunity to eliminate antigens, return to homeostasis with the apoptosis of stimulated lymphocytes, and maintenance of memory for subsequent invasions (Abbas et al., 2012a). Each individual lymphocyte and its clone have a unique type of receptor created by the rearrangement of receptor genes in the DNA, allowing for an almost endless combination of receptors. This allows for an extremely diverse lymphocyte repertoire, capable of recognizing up to 10^7 to 10^9 different types of antigens. Each receptor is designed to recognize a specific part of an antigen, known as an epitope. When a lymphocyte is activated by a specific antigen, the cell undergoes clonal expansion to produce a strong, antigen-specific response. Once the antigen has been cleared, the immune system returns to homeostasis. Lymphocytes that are no longer needed will undergo apoptosis. Memory cells remain after the invasion has cleared, ensuring a faster, stronger response if the antigen is encountered again (Abbas et al., 2012a).

Humoral Immunity

Humoral immunity is mediated by B cells and works to eliminate extracellular pathogens. B cells, also referred to as B lymphocytes, are the only antibody-producing cells in the body. Antibodies are immunoglobulins (Ig), specialized immune proteins, with a more diverse range of antigen recognition than that of receptors found on T cells or MHCs (Abbas et al., 2012b). Antibodies can be membrane-bound to B cells or in circulation. When a membrane-bound receptor binds an antigen, it processes and presents the antigen-derived peptides on an MHC class II molecule on the B cell surface. Helper T cells in cell-mediated immunity interact with the B cell via a CD40L ligand and receptor to activate it. Activation renders the B cells responsive to inflammatory cytokines, and allows for proliferation and high levels of Ig secretion (Mills and Cambier, 2003).

Humoral Immunity: The Bursa of Fabricius and B Cells

In mammals, B cells mature in the bone marrow. Unlike mammals, avian B cells mature in the bursa of Fabricius, a structure dorsal to the cloaca (Glick et al., 1956). In early embryonic stages, circulating hemopoietic stem cells migrate to the bursa, where they soon begin lymphocyte proliferation and differentiation (Le Douarin et al., 1975). At hatch, gut-derived molecules are thought to aid in inducing proliferation and maturation of bursal B cells into naïve B cells (Ekino et al., 1980; Ekino, 1993). The naïve B cells then migrate into the periphery to reside in secondary lymphoid tissues such as the spleen and gut-associated lymphoid tissues (Oláh et al., 2008). Over the next 6 months of age, the chicken's bursa regresses. Many longer-lived (memory) B cells remain in circulation, and post-bursal B cells stem from the spleen (Paramithiotis and Ratcliffe, 1994a,b).

Humoral Immunity: Immunoglobulins

Immunoglobulins (Ig) are found in both mammals and birds. They are glycoproteins with antibody activity, circulating in the blood, lymph, and vascularized tissue (Marchalonis, 1977; Litman et al., 1993). Ig are made of polypeptide chains each having two heavy (H) and two light (L) chains. The Ig unit is shaped like a "Y" with both variable and constant regions. The variable (V) regions at the tips of the unit are known as V_H and V_L regions, while the constant regions known as C_H and C_L are found at the bottom of the unit. The variable regions are where previous somatic gene conversion generates a unique antigen-binding site in the immunoglobulin (McCormack et al., 1991; Davison et al., 2008).

In chickens, there are three different types of immunoglobulins, IgM, IgY (commonly called IgG), and IgA. Avian IgM has the same structure and function as mammalian IgM, and can be membrane-bound or in circulation. It is the primary B-cell antigen receptor and first isotype to be expressed during embryonic development. IgM is the major humoral response after an initial challenge, but expression decreases following infection. Avian IgY has similarities to mammalian IgG and IgE, and is often interchangeable with IgG in literature. It is found mostly in sera, and is the main secondary response once IgM production has decreased. Avian IgA is mostly found in bodily secretions and at mucosal sites (Davison et al., 2008). B cells and immunoglobulins work together with cell-mediated immunity to comprise the adaptive immune response.

Cell-Mediated Immunity

Cell-mediated immunity is mediated by T cells, functioning to eliminate intracellular pathogens, produce cytokines, and aid B cells in antibody production. The T cells are preceded by hematopoietic stem cells that migrate and mature in the thymus. In birds, the thymus lies on each

side of the neck, consisting of 7-8 lobes parallel to the vagus nerve and jugular veins (Hodges, 1974). After T cells mature, they leave the thymus to colonize peripheral lymphoid organs such as the spleen and gut-associated lymphoid tissues (Oláh et al., 2008). Just as B cells are covered in antigen receptors made of immunoglobulins, T cells express unique T cell receptors (TCR). These are randomly generated, containing specific ligands that work together with their corresponding major-histocompatibility complex. The TCR has a variable Ig-domain generated by somatic DNA recombination (Tonegawa, 1983). There is only one constant chain in a TCR, whereas there are four in Ig. The TCR is anchored in the plasma membrane of the T cell, where it waits to recognize and interact with antigens (Smith et al., 2014).

T cells are broadly divided into CD4+ or CD8+ types. CD4+ cells are coated in MHC class II molecules, and when an antigen is presented via an APC, the CD4+ cell is activated and proliferates into Type 1, Type 2, or Type 17 helper cells (T_H1, T_H2, or T_H17). T_H1 cells activate macrophages, T cells, and B cells. They release cytokines that inhibit T_H2 function, promoting inflammation and response to intracellular pathogens. On the other hand, T_H2 cells produce cytokines that inhibit T_H1 function while promoting B cell proliferation and antibody production to work against extracellular pathogens (Kaiser and Stäheli, 2014). The more recently identified T_H17 cell is less understood, but is so named for its primary cytokine IL-17, working to regulate inflammation (Chen et al., 2006; Harrington et al., 2006). The second class of T cells, CD8+ cells, is referred to as cytotoxic T cells (CTL). These are coated in MHC class I molecules, and lyse cells infected with pathogens (Dalloul and Lillehoj, 2006).

Avian Cytokines and Chemokines

Cytokines are small, short-lived, regulatory peptides produced by immune cells to send signals between cells (Cohen et al., 1974). Some of the major ones involved in immune regulation include interleukins, transforming growth factor (TGF)-β, interferons (IFN), colony-stimulating factors (CSF), tumor necrosis factor receptor superfamily (TNFRSF), and chemokines (Kaiser and Stäheli, 2014). Interleukins have a wide range of both pro- and anti-inflammatory effects. The T_H1 interleukins include interleukin (IL)-12, a driver of T_H1 inflammatory response. IL-23 drives the differentiation of T_H17 cells (Kaiser and Stäheli, 2014). T_H2 cytokines include IL-3, IL-4, IL-5, IL-13, granulocyte macrophage colony-stimulating factor (GM-CSF), and KK34. They generally work to respond against extracellular pathogens (Avery et al., 2004; Koskela et al., 2004). IL-10 is an anti-inflammatory cytokine, downregulating the inflammatory effects of IFN-γ (Rothwell et al., 2004). IL-17 produced by T_H17 cells is a key component in regulating the inflammatory response (Chen et al., 2006; Harrington et al., 2006). Interferons (IFN) are classified as either Type I or Type II. The Type I IFN subsets include IFN-α and IFN-β, which possess antiviral activity (Sekellick et al., 1994; Sick et al., 1996). The Type II IFN-γ is common to T_H1 responses, playing a role in controlling infections by intracellular pathogens (Kaiser and Stäheli, 2014). TGF-β is mostly an anti-inflammatory cytokine, preventing the damaging effects of an over active immune response (Elgert, 2009). TNFRSF molecules function more as costimulatory molecules rather than cytokines, but are still important to both innate and adaptive immunity, stimulating cell proliferation, the immune system, and apoptosis (Kaiser and Stäheli, 2014). As the name suggests, colony-stimulating factor (CSF) facilitates growth of myeloid cells from pluripotent hematopoietic stem cells (Kaiser and Stäheli, 2014). The chicken CSF1 stimulates macrophage growth from bone marrow (Garceau et al., 2010). CSF2 stimulates bone

marrow cells (Avery et al., 2004). Chicken CSF3 is responsible for the differentiation of myelomonocytic cells (Gibson et al., 2009).

Chemokines include a broad category of molecules that regulate how cells interact with one another (Kaiser and Stäheli, 2014). Chemokines are loosely divided into two overlapping categories. The first category is homeostatic, in which constitutively expressed chemokines are involved in trafficking of leukocytes. The second category, inflammatory chemokines, is inducible and recruits cells to sites of inflammation (Kaiser and Stäheli, 2014).

Gut-Associated Lymphoid Tissue (GALT)

One of the most important and vulnerable organs in avian immunity is the gut. Constant nutrient and water exchange across mucosal surfaces of the gut and contact with external substances in the lumen allows potential entry of pathogens into the body (Kaspers et al., 2014a). Tissues in the gut have more lymphocytes than other areas of the body, and are aptly named gut-associated lymphoid tissue (GALT), the largest part of the MALT, or mucosa-associated lymphoid tissues which make up nasal, lung, reproductive tract, and gut tissues (Kaspers et al., 2014a). GALT is a complex system that must work correctly to recognize pathogens versus commensal bacteria that naturally inhabit the gut, inhibiting pathogens. The gut regulates the environment to provide optimal growth of beneficial microbes that inhibit pathogens from entering the body or causing intestinal disease by overthrowing the natural balance found in the microbiota (Kaspers et al., 2014a).

The avian gut is a long tubular structure made of a single layer of epithelial cells laid upon a matrix known as the basement membrane. The epithelial cells of the gut are known as enterocytes, which absorb nutrients, water, and ions. They form a barrier between the intestinal contents and the host through structures known as “tight junctions” (Hermiston and Gordon, 1995). Some specialized enterocytes, known as goblet cells, secrete mucus to protect the surface of the epithelium (Smith et al., 2014).

Immune cells are located all along the gut and in strategic sites such as the lamina propria, Meckel’s diverticulum, Peyer’s patches, and cecal tonsils (CT). Many NK and T cells reside between epithelial cells, along the length of the gut. Below the epithelial surface is the lamina propria, where T cells, heterophils, macrophages, DCs, NK cells, and IgA-secreting B cells remain close to the gut lumen (Lebacq-Verheyden et al., 1972; Bienenstock et al., 1973; Mockett, 1986). Chickens lack lymph nodes, but along the gut are lymphoid aggregates, known as Peyer’s patches, with groups of microfold or M cells along the epithelium, that constantly sample the gut environment and present its content to macrophages and DCs that lie just below the surface. Nearby, there are lymphoid follicles within the lamina propria. These are found along the length of the gut, and maintain higher concentrations of the same types of immune cells found in the epithelium (Smith et al., 2014). A high concentration of immune cells so close to the gut ensures a quick response to invading pathogens.

Enteric Microbial Environment

Commensal organisms in the gut are responsible for keeping out pathogenic bacteria, as well as communicating with the immune system to both maintain homeostasis as well as respond to an

enteric invasion (Molloy et al., 2012). A healthy gut microbiota can resist infection to *E. coli, Salmonella, Campylobacter,* and other pathogens (Dalloul et al., 2003).

Coccidiosis

Coccidiosis is an enteric disease caused by the parasite *Eimeria* resulting in a wide range of diseases symptoms, depending on the age and health of the bird as well as the species of *Eimeria* (Smith and Beal, 2014). Avian coccidiosis is the most important disease economically, resulting in annual losses of $3 billion worldwide (Dalloul and Lillehoj, 2006). There are 9 species of *Eimeria* that infect the chicken: *E. tenella*, *E. maxima*, *E. acervulina*, *E. brunetti*, *E. mitis*, *E. necatrix*, *E. mivati, E. hagani,* and *E. praecox* with species *E. acervulina*, *E. tenella*, and *E. maxima* having higher diagnosis rates than others (Shirley et al., 2005; McDonald and Shirley, 2009). The species of *Eimeria* can have different symptoms in a bird, ranging from watery, white diarrhea (*E. acervulina* and *E. mivati),* malabsorption of nutrients (*E. mitis*), petechial hemorrhages and increased mucus production (*E. maxima* and *E. brunetti*), to hemorrhagic diarrhea and villar destruction, often leading to death (*E. tenella* and *E. necatrix)* (Dalloul and Lillehoj, 2006). Although the *Eimeria* spp. result in different degrees of infection, the results can have lasting impact on the recovered animal. Intestinal villi become damaged and blunt, reducing the ability to optimally absorb nutrients (Smith et al., 2014). Stunted growth may be recovered thanks to compensational growth, but economic loss is still a problem (Dalloul and Lillehoj, 2006).

Life Cycle of Eimeria

Eimeria is transmitted via an oral-fecal route in chickens (Shirley et al., 2005). An oocyst is an environmentally resistant, infectious stage of the *Eimeria* life cycle, persisting in feces and litter for long periods of time (Blake and Tomley, 2014). When a sporulated oocyst is ingested, the tough wall is disrupted in the gizzard, releasing four sporocysts from each oocyst. Enzymes in the stomach break down the sporocysts releasing the sporozoites. These sporozoites invade the intestinal epithelial layer in different parts of the intestine, depending on the species of *Eimeria*. The sporozoite transforms into a trophozoite and then undergoes asexual multiple fission, or schizogony. This asexual fission releases first generation merozoites, which escape the host cell and invade new cells. After more rounds of schizogony, final generation merozoites differentiate into macrogametes and microgametes for the sexual reproduction phase. Microgametes leave their host cell to fertilize macrogametes in neighboring host cells. This fertilization creates zygotes, which develop an environmentally resistant wall to become an oocyst. The oocyst ruptures the host cell, passes through the intestine, and is excreted into the environment, where warmth, oxygen, and moisture initiate meiotic and mitotic nuclear division in the oocyst. It is now considered an infective, sporulated oocyst (Blake and Tomley, 2014). The life cycle typically takes 4-6 days (McDougald, 1998; Allen and Fetterer, 2002).

Treatment and Prevention

Proper management and hygiene help reduce coccidiosis, such as removal of caked litter and application of a top dressing of fresh litter between flocks and controlling the house temperature and moisture, but only vaccination and use of prophylactic drugs can control it (Dalloul and Lillehoj, 2006; Blake and Tomley, 2014; Chapman and Jeffers, 2014). Coccidiostats are drugs currently administered at low doses in flocks to prevent disease occurrence. One major class of

coccidiostats is the ionophores, which are antimicrobials produced by fermentation that work to disturb the ion transport and osmotic balance of parasite metabolism (Dalloul, 2017). The other major class is anticoccidial drugs; synthesized chemicals antagonistic to parasite metabolism and distinct from ionophores (Blake and Tomley, 2014; Dalloul, 2017). All *Eimeria* spp. show drug resistance to anticoccidials currently in use; however, introducing drug sensitive strains into the vaccination programs while alternating use of anticoccidials may reduce the number of drug-resistant species found in a poultry house (Chapman and Jeffers, 2014). Using coccidiostats to combat the effects of disease are improved with coupled use of anticoccidial vaccines. Natural exposure to a specific *Eimeria* induces species-specific immunity, and a downfall of vaccines is that they must be specific for the type of *Eimeria* as well, or will not be effective (Dalloul, 2017). Current vaccines are either live wild type or attenuated, and generally consist of species prevalent in the region of application (Dalloul and Lillehoj, 2005). The live wild type vaccines contain small amounts of virulent mixed species that cause low level of infection before incurring immunity. The attenuated vaccines have undergone passage in embryos, to produce a vaccine with less pathogenicity (Blake and Tomley, 2014). Although these are effective at developing immunity, they must be administered at a young age to be effective, and can have temporary negative effects as minor disease affects the birds' health and growth for a short period of time. Uneven application or improper dosage can lead to disease outbreak, demonstrating a shortfall to vaccinations (Blake and Tomley, 2014).

Response to Coccidiosis

The host response to coccidiosis involves both innate and adaptive immunity. As previously stated, a single infection of *Eimeria* is enough to maintain immunity to a reinfection from the

same species (Chapman et al., 2013). Immunity to infection begins in the early asexual stages, when sporozoites have penetrated epithelial cells and are undergoing schizogony, thus working to prohibit further reproduction of sporozoites. Immunity also affects the stages of merozoites and sexual reproduction. The GALT responds by processing and presenting antigens, activating B cells to produce antibodies, and activating T cells in cell-mediated immunity (Dalloul, 2017). *Eimeria* antigens that provide protection to the host against reinfection seem to be presented to the immune system by the sporozoite-infected host cells (Shirley et al., 2005). Innate response to primary infection and adaptive response to primary and secondary infections are important; however, mechanisms of protection against reinfection are still not fully understood (Chapman et al., 2013).

Humoral Immune Response

Humoral immunity primarily involves the secretion of antibodies by B cells. The primary immunoglobulins found in intestinal secretions are IgA and IgM (Yun et al., 2000). During infection, chickens secrete parasite specific antibodies (Lillehoj and Lillehoj, 2000). When infected with *Eimeria,* the humoral response plays a relatively minor role in defending against *Eimeria* infection, as indicated by bursectomized chickens that were still resistant to infection (Lillehoj and Trout, 1996; Dalloul and Lillehoj, 2006; Chapman et al., 2013).

Cell-Mediated Immune Response

Since removal of the bursa had little effect on immunity against *Eimeria,* it was reported that the cell-mediated response involving T cells plays a critical role in host defense against intracellular parasites such as *Eimeria*. Suppressed T cells during infection led to impaired immunity (Dalloul

and Lillehoj, 2006). In studies involving adoptive transfer of T cells, peripheral blood leukocytes and spleen cells from infected chickens protected naïve recipients against *E. maxima* challenge (Rose, 1982). Proliferation of *Eimeria*-specific T lymphocytes in immune chickens was demonstrated in multiple studies (Rose et al., 1984; Lillehoj, 1986; Vervelde et al., 1996). Increased expression of CD8+ T cells were found in intestinal epithelial cells after infection with *E. acervulina* (Lillehoj and Bacon, 1991). Naïve chickens infected with *E. tenella* had higher numbers of CD4+ cells after 2 days, and immune chickens had more CD4+ and CD8+ cells in the lamina propria (Vervelde et al., 1996). The use of anti-CD8+ monoclonal antibodies prolonged oocyst shedding in birds infected with *E. tenella* or *E. acervulina* (Trout and Lillehoj, 1996). Studies depleting a certain subset of T cells indicate that CD4+ cells are more important in primary infection, and CD8+ cells are more important in reinfection (Chapman et al., 2013). Numerous studies demonstrate the effectiveness of both CD4+ and CD8+ T cells in providing immunity against *Eimeria* infection.

During *Eimeria* infection, T cell response is regulated by chemokines and cytokines (Lillehoj et al., 2003; Dalloul, 2017). One of the most important cytokines is IFN-γ, which is highly upregulated during infection. T_H1 response cytokines, which promote inflammation and respond to intracellular parasites, are upregulated during *E. maxima* infection and include IFN-γ, IL-1β, IL-6, IL-12, IL-15, IL-17, and IL-18, while upregulated T_H2 cytokines include IL-4, IL-10, IL-13, and GM-CSF (Hong et al., 2006b). Another study by Hong et al. (2006a) observed that infection with *E. acervulina* upregulated IFN-γ, IL-2, IL-12, IL-15, IL-16, IL-18, and GM-CSF while downregulating IL-4 and IL-13. In a separate study, infection with *E. acervulina* or *E. tenella* upregulated expression of IL-2, IL-18, and IFN-γ cytokines belonging to T_H1 response and an increase in T_H2 cytokines IL-4 and IL-10 and chemokine IL-8 (Cornelissen et al., 2009).

Contrastingly, a more recent study by Chow et al. (2011) found that infection with *E. tenella* was able to downregulate T_H1 cytokines IL-12 and IFN-γ, suggesting the parasites' ability to suppress certain immune defenses. The aforementioned studies demonstrate how both T_H1 and T_H2 responses work together as the main defense against *Eimeria* infection.

Probiotics and Poultry

For decades, it has been custom to use anticoccidials in poultry feed to control coccidiosis (Dalloul and Ritzi, 2016). Consumer concerns over drug residues in their food products and concerns about multidrug resistant parasites and bacteria have driven the modern research community to search for alternative methods. Vaccines have a positive outcome; however, they are expensive and must be carefully administered to be effective and not cause unwanted harm. To control coccidiosis and mitigate other health problems, immunomodulators such as probiotics have been at the forefront of worldwide research (Dalloul and Ritzi, 2016). Probiotics, also known as direct-fed microbials (DFMs), are classified by FAO/WHO (2001) as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." Probiotics specific to broilers include the genera *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* (Kabir, 2009). The beneficial effects of probiotics include improved performance, modulating the intestinal microbiota, inhibiting pathogens, improved intestinal integrity, immunomodulation, and improving microbiological and sensory characteristics of broiler meat (Kabir, 2009).

Modes of Action

Probiotics help maintain a healthy balance in the gut to promote proper health, performance, and defense against enteric diseases (Dalloul, 2017). Probiotics function through three main mechanisms: competitive exclusion, bacterial antagonism, and stimulation of the immune system (Ohimain and Ofongo, 2012). As soon as a chick hatches, gut microbiota begin to establish within hours, so early administration of probiotics has a better chance of improving gut microbial profiles (Timmerman et al., 2006; Torok et al., 2007). A chick would typically establish a gut microbiota via contact with adult chickens' feces, however, in a hatchery, probiotic supplementation is beneficial due to lack of contact with grown chickens (Kabir, 2009). Probiotics are able to competitively exclude pathogenic strains of bacteria by establishing in the gut, taking up the space and nutrients in the digestive tract that pathogens would otherwise use to colonize. Many probiotic species are antagonistic to bacterial pathogens through production of antimicrobial substances and secretions that lower pH in the gut, which inhibit pathogenic growth (Hume, 2011). Short-chain fatty acids (SCFA) produced by beneficial bacteria have been shown to increase host defense peptide gene expression (Sunkara et al., 2011, 2012), and in high concentrations, disrupt proton motive force and metabolic reactions in bacteria, when small, non-ionized acids cross bacterial membranes and then dissociate into protons and anions in the cytoplasm. The protons lead to acidification of intracellular compartments where proton motive force occurs, disturbing metabolic reactions, and anions disrupt osmotic balance (Sun and O'Riordan, 2013). Bacteriocins, small peptides or proteins secreted by beneficial bacteria, kill closely related strains of bacteria by forming pores in the cell membrane and disrupting enzyme function. Beneficial *Lactobacillus spp.* are known for secreting lactic acid, which lowers intestinal pH to inhibit pathogenic growth (Travers et al., 2011). The vital functions of probiotics to stimulate the immune system are discussed below.

Probiotics, Performance, and Intestinal Development

A healthy gut is linked with proper performance of poultry (Getachew, 2016). Probiotics can boost performance by helping to establish a healthy gut environment. In chickens, body weight gain (BWG) and feed conversion ratio (FCR) have been improved with probiotic supplementation (Kabir et al., 2004; Khaksefi di and Ghoorchi, 2006; Nayebpor et al., 2007; Talebi et al., 2008; Ignatova et al., 2009; Sen et al., 2012). A study by Mountzouris et al. (2007) even demonstrated performance results at the same level as the antibiotic growth promoter (AGP) avilamycin. Other studies have shown no improvement in performance due to probiotic supplementation (Rahimi et al., 2011; Wolfenden et al., 2011; Getachew, 2016). This could be explained by the strain of probiotic bacteria not having an effect, the viability being compromised during feed preparation, or improper dosage levels. Other factors that can affect probiotic efficacy is diet, overall health and age of birds, interactions with other additives in the feed, or stress factors such as temperature and stocking density (Patterson and Burkeholder, 2003; Mountzouris et al., 2007; Cox and Dalloul, 2015).

Probiotics have also shown the potential to improve gut development and makeup. They have resulted in increased length of intestinal villi and a decrease in crypt depth (Samanya and Yamauchi, 2002; Marković et al., 2009). Longer villi length correlates to a larger surface area for absorbing nutrients. Crypt sites are the location of enterocyte proliferation, and shallow crypts indicate less need for epithelial cell turnover, taking energy that would go towards cell turnover and placing it towards animal growth (Marković et al., 2009). Another example of probiotics benefiting gut health was demonstrated when the mycotoxin deoxynivalenol (DON) was added to feed (Awad et al., 2006). Typically, DON will damage the gut, decreasing villus height and

width in the duodenum and jejunum, but probiotic supplementation relieved the negative effects of decreased villus height (Awad et al., 2006).

Probiotics and Innate Immunity

Innate immune responses are non-specific defenses consisting of physical, chemical, and cellular barriers (Applegate et al., 2010). Physical barriers include epithelial cells (enterocytes), chemical barriers include secretions or mucus or antibacterial peptides (defensins, lysozymes), and cellular barriers include phagocytes and macrophages that engulf bacteria and present it to the adaptive immune system (Applegate et al., 2010). The avian heterophil is equivalent to the mammalian neutrophil. These cells play an important role in the first line of defense, phagocytosing pathogens, then using oxidative burst and degranulation to destroy them. Probiotics have been shown to enhance oxidative burst and degranulation, thus protecting against pathogens (Harmon, 1998). Heterophils isolated from probiotic-supplemented broilers displayed increased levels of oxidative burst and degranulation (Farnell et al., 2006; Stringfellow et al., 2011). Macrophages phagocytose pathogens, process them intracellularly, and present antigens to cells of the adaptive immune system, playing a role in lymphocyte differentiation. Broiler chicks fed a commercially available *Lactobacillus*-based product had increased levels of macrophages in the ileum and cecum, but after exposure to the pathogen *Salmonella* Enteritidis and supplementation with probiotics one hour after challenge, macrophage levels were reduced. The decrease in macrophages after challenge could be caused by competitive exclusion by the probiotics, reducing the pathogen load in the intestine (Higgins et al., 2007a). Probiotics play a role in enhancing innate immune response and the competitive exclusion of pathogens in the gut.

Probiotics and Adaptive Immunity

The adaptive immune system mounts a specific defense to foreign antigens, consisting of T cells and B cells that produce antigen-specific antibodies (Applegate et al., 2010). It is the second line of defense, and essential to protection against reinfection (Dalloul, 2017). Supplementation with probiotics increased antibody titers to sheep red bloods cells as well as Newcastle disease virus and infectious bursal disease, both important avian viral diseases (Kabir et al., 2004; Haghighi et al., 2005; Khaksefi di and Ghoorchi, 2006; Nayebpor et al., 2007; Apata, 2008; Karimi Torshizi et al., 2010). A study involving orally gavaging female broilers found that probiotic treated birds had increased natural antibody production, and increased levels of IgA responsive to tetanus toxoid, as well as increased IgG and IgM antibodies reactive to tetanus toxoid and alpha-toxin (Haghighi et al., 2006). Mountzouris et al. (2009) compared probiotic treatments to the AGP avilamycin and determined that both resulted in lower plasma IgA and IgG levels and decreased intestinal IgA against *Salmonella* Enteritidis compared to a challenged control. Reduced antibody levels may indicate enhance clearing and recovery of disease. *Eimeria* infected birds supplemented with probiotics produced more *Eimeria*-specific antibodies than controls (Lee et al., 2007a,b).

Probiotic supplementation has been shown to increase the number of intestinal intraepithelial lymphocytes expressing the cell surface markers CD3, CD4 and CD8 (Dalloul et al., 2003; Noujaim et al., 2008). A study by Karimi Torshizi et al. (2010) challenged birds with DNCB (1-chloro-2, 4-dinitrobenzene) and birds supplemented with a water application of probiotics displayed enhanced immune response. Birds challenged with a PHA-M (phytohemagglutinin-M) injection were supplemented with feed or water applied probiotics, and showed enhanced immune response as measured by increased skin thickness. Different strains of probiotics can have different effects on cytokine production, as indicated by studies using *Lactobacillus* and

Bacillus-based probiotics that modulated the levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-17a, IL-18), Th1 cytokines (IFN-γ, IL-2, IL-12), and Th2 cytokines (IL-4, IL-10, IL-13) (Dalloul et al., 2005; Brisbin et al., 2010; Lee et al., 2010a). The strain of probiotic used can have a profound effect on the immune response, as measured by lymphocyte and cytokine production.

Probiotics and Host Defense against Pathogens

Pathogens such as *Eimeria*, *Clostridium perfringens*, *Campylobacter jejuni* and *Salmonella* Enteritidis are making a comeback in modern poultry production due to the ban on prophylactic use of antibiotics (Dalloul and Ritzi, 2016). These diseases can have devastating effects on the intestines, leading to reduced performance, and possibly death. Probiotics can improve gut health and reduce the impacts of common enteric diseases. Reduced oocyst shedding has been observed in probiotic-fed chicks that were later infected with *E. acervulina* or *E. tenella* (Dalloul et al., 2003, 2005; Lee et al., 2007a,b). Severity of lesions from *E. maxima* infected broilers were reduced in probiotic supplemented treatments (Lee et al., 2010b). Ritzi et al. (2014) conducted studies on feed vs. water application of probiotics in assessing anticoccidial effects. It was found that a water-based probiotic led to lower duodenal and jejunal lesion scores, and those birds intermittently given water-based probiotic treatment shed fewer oocysts. The subsequent study concluded that probiotics in conjunction with vaccination lead to additional benefits, as seen by improved performance and reduced lesion scores when birds were challenged with *Eimeria* (Ritzi et al., 2016). Further, probiotics given to birds on embryonic day 18 had reduced disease severity after being challenged with *Eimeria* (Pender et al., 2016).

Other pathogens, such as *Salmonella*, *Campylobacter jejuni,* and *Clostridium perfringens*, were reduced by probiotic supplementation as well. A study by Revolledo et al. (2009) found that probiotic supplementation reduced colonization of *Salmonella* in the ceca, liver, and spleen of broiler chicks. Necrotic enteritis induced by *C. perfringens* was partially alleviated as indicated by reduced lesion scores, mortality, and levels of *C. perfringens* (McReynolds et al., 2009). Intracellular pathogens such as *Salmonella* Enteritidis and *C. jejuni* were reduced by probiotic supplementation as well (Higgins et al., 2007b; Ghareeb et al., 2012). Reducing pathogens in the gut can aid in protection against secondary infections that are often associated with a primary disease, for example, *Eimeria* infection often leads to necrotic enteritis by *C. perfringens* (Chapman et al., 2002).

Results from studies on probiotics are often varied and inconclusive; however, there is substantial evidence that probiotics are capable of modulating the gut and immune system. They have been shown to provide a benefit to the bird, thus offering a promising alternative to the diminishing use of antibiotic growth promoters.

Phytogenic Feed Additives and Poultry

Antibiotic-growth promoters (AGP) have been used to improve poultry performance for decades; however, recent bans in the European Union (EU) on these products and concerns over microbial resistance and health effects in humans has led to increased interest in natural alternatives (Alcicek et al., 2004). A broad category of feed additives commonly researched in both poultry and swine are phytogenic feed additives (PFAs), intended to improve gut health and function

(Alcicek et al., 2004). PFAs are plant-derived compounds such as essential oils, spices, and herbs intended to provide a health benefit when added to feed (Windisch et al., 2008). Some common examples are derivatives of rosemary, oregano, thyme, sage, cinnamon, citrus, pepper, and anise (Mountzouris, 2016). The efficacy of phytogenics can depend on numerous factors, including composition, inclusion level in the feed, bird genetics, and feed composition (Puvača et al., 2013). According to Mountzouris (2016), optimal gut function, animal health, and performance are linked and can be achieved when healthy gut microbiota, dietary factors, gut mucosa, and immune responses are balanced and work together to eliminate pathogens, improve nutrient absorption, and modulate inflammation (Koutsos and Arias, 2006; Choct, 2009; Applegate et al., 2010; Mountzouris, 2016).

Modes of Action

PFAs may promote a healthy gut and improve performance through various mechanisms: antioxidative and antimicrobial properties, improved palatability, improved digestion, growth promotion, and improved gut health (Alcicek et al., 2004; Windisch et al., 2008). Studies on palatability are inconclusive (Windisch et al., 2008), but the PFAs may improve feed quality due to antioxidative properties and ability to slow bacterial and fungal growth (Lambert et al., 2001; Soliman and Badeaa, 2002; Burt, 2004).

Phytogenics, Performance, and Intestinal Effects

Phytogenic feed additives have had varying effects on performance. Some studies demonstrated no differences in various performance parameters. One study used oregano essential oil at 50 or 100 mg/kg in a wheat-soybean meal diet with Cobb broilers, and there was a difference in body

weight (BW) or feed conversion ratio (FCR) from controls (Botsoglou et al., 2002). Another study using female Cobb broilers found that supplementation with thymol, cinnamaldehyde, and commercial preparation had no effects on feed intake (FI), body weight gain (BWG), or FCR (Lee et al., 2003).

Other studies conducted later demonstrated benefits of various types of PFAs in poultry feed given to different breeds of broiler chickens. Lee et al. conducted two studies involving different diets. The first diet included carboxylmethyl cellulose to corn in order to increase intestinal viscocity, and the negative effects on BWG were partially offset (Lee et al., 2004a). The second diet was rye-based to suppress weight gain, but the cinnamaldehyde partially offset the reduced weight gain over the first two weeks (Lee et al., 2004b). *Eimeria*-infected Cobb birds fed wheat-soybean meal with oregano supplementation at 300mg/kg had better BWG and FCR, although they were lower than the group fed a coccidiostat (Giannenas et al., 2003). In another 6 week study by Mountzouris et al. (2011), using different inclusion levels of PFA, no significant differences were seen during the starter and grower phases in BW, BWG, FI or FCR; however, during the finisher phase, BWG increased linearly, FI decreased linearly, and FCR improved linearly with the PFA inclusion level. Cumulatively, the addition of PFA improved BWG, FI, and FCR (Mountzouris et al., 2011). Similarly, another study demonstrated no significant difference in performance parameters, until the finisher phase, upon which BWG increased linearly, FI was reduced quadratically, and overall FCR was improved quadratically with increasing PFA inclusion level (Paraskeuas et al., 2017). A study by Soltan et al. (2008) demonstrated varying outcomes, depending on the PFA concentration, emphasizing the importance of composition in a feed additive. Anise seeds were included in a corn-soybean meal diet at 0.5-0.75 g/kg fed to Hubbard broilers for 6 weeks. Overall, these treatments resulted in

improved BWG with no effect on FI or FCR. The highest inclusion rate of anise seed was 1.5 g/kg, and actually reduced growth performance (Soltan et al., 2008). A proper balance of ingredients and concentrations of PFAs can lead to improved performance. The importance in concentration has been documented in several studies. Bolukbasi and Erhan (2007) added thyme to layer diets at 0, 0.1, 0.5, and 1% and observed improved FCR, egg production, and reduced fecal *E. coli* levels in the 0.1 and 0.5% concentrations. Another study evaluated three different levels of *Azadirachta indica* (Indian lilac) dried leaf meal at 0, 1.25, 2.5, and 5.0 g/kg of feed, resulting in better body weight, FCR, and dressing percent in the 2.5 g/kg diet, but no improvement in higher or lower amounts (Ansari et al., 2011). Jamroz and colleagues (2006) divided broilers into corn-fed or wheat and barley-fed diets, with or without carvacrol, cinnamaldehyde, and capsicum oleoresin plant extracts to investigate effect of diet composition on efficacy of PFAs. Significant effect of diet was observed by shorter jejunal villi height and shallower crypt depth in broilers fed corn and PFAs, while no difference was observed in the wheat and barley diet supplemented with PFAs. Both corn and wheat/barley diets that had PFAs had improved FCR. The range of efficacy varies depending on the type of plant used, type of basal diet, chicken breed, and age.

Phytogenic feed additives my improve digestibility by increasing enzyme action and mucus production in the gut. Broilers had greater trypsin, lipase, and amylase activity (Lee et al., 2003; Jamroz et al., 2005). Older broiler chickens began receiving PFAs at 41 days of age and exhibited a 38-46% increase in lipase activity (Jamroz et al., 2005). Another study reported increased thickness in the jejunum and stomach as well as increased mucus production, signifying a protective effect against pathogen colonization (Jamroz et al., 2006). The effects on gut microflora indicate PFAs have antimicrobial properties. Many phytogenic mixtures have

shown antimicrobial activity against foodborne pathogens including *Salmonella* Typhimurium, *S.* Enteritidis, *E. coli 0157:H7*, *Shigella dysenteria*, *Listeria monocytogenes*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Lambert et al., 2001; Burt, 2004; Chorianopoulos et al., 2004; Penalver et al., 2005; Si et al., 2006). Speculation as to the mechanism of these actions is credited towards PFAs phenolic structures (Burt, 2004; Penalver et al., 2005; Si et al., 2006). Phenolics are able to create disturbances in bacterial cytoplasmic membranes, disrupt the proton motive force, electron flow, active transport, and coagulate cell contents (Lambert et al., 2001; Burt, 2004). These aforementioned studies indicate that PFAs may be able to control the growth of pathogens in the gut; however, subsequent studies involving birds result in various outcomes. One commercial blend of essential oils was able to lower *E. coli* levels, and increase beneficial *Lactobacillus* levels (Jamroz et al., 2005). Another study using a blend of five herbs had no significant effect on the makeup of cecal or excreta microbial populations (Cross et al., 2007). Mountzouris et al. (2011) demonstrated no difference in microbial populations by day 14 or day 28, but variations by day 42 when linear increases in cecal aerobes, *Clostridium*, *Lactobacillus*, *Bifidobacterium,* and Gram +*ve* cocci were observed in direct relation to PFA inclusion levels.

Phytogenics and Innate Immunity

The innate immune system acts as a first line of defense against disease. In the gut, an important barrier to pathogens is mucus production by goblet cells in the epithelial layer. Mucus coats the epithelial cells lining the length of the gut, protecting them from pathogenic colonization (Mountzouris, 2016). Mucins are glycoproteins that can be produced, stored, and secreted by goblet cells. A study by Tsirtsikos et al. (2012) isolated mucin from day 14 broilers fed a control

diet, AGP (avilamycin) diet, or PFA treatments at increasing levels of 80, 125, and 150 mg/kg diet. In the mucin, increased mannose was found in the ileum and increased galactose was found in the duodenum of birds fed PFA compared to the negative control. Mannose and galactose are oligosaccharides that have been associated with increased response to enteric disease (Mountzouris, 2016). A separate trial used a different mixture of PFA and found a trend for increased ileal MUC2 gene expression in PFA treated birds at day 42. Those birds also exhibited a trend towards lower spleen iNOS levels, indicating an anti-inflammatory effect on the innate immune system (Paraskeuas et al., 2016). The same study substituted corn for wheat, and contrastingly, there were no observed differences in ileal MUC2 or spleen iNOS between treatments (Paraskeuas et al., 2016).

Phytogenics and Adaptive Immunity

The immune system strives to remain at homeostasis, between immunostimulation and immunosuppression (Applegate et al., 2010). In human literature, phytogenics have been shown to have immunostimulatory activity, as is the case with ginseng stimulating lymphocyte activity and increased cytokine production of IL-1, IL-6, IL-12, IL-6, TNF-α, and interferon-γ (Tan and Vanitha, 2004). Anti-inflammatory properties of ginko biloba have been noted in human studies, demonstrating how its flavonoids and terpenes mediate production of proinflammatory cytokines (Li, 2000). The study by Paraskeuas et al. (2016) was able to demonstrate increased levels of ileal IgA and a trend towards a decrease in spleen IL-18 of day 42 broilers fed a PFA mixture. The same study using wheat instead of corn observed no differences in ileal IgA or spleen IL-18.

As an alternative to antibiotic growth promoters, phytogenic feed additives have demonstrated improvements in performance, gut function, and gut health. The performance of many products

is dose dependent and the method of extracting the product is crucial in its function. PFA have varying effects depending on type of mixture used as well as the age and breed of the chicken. A more thorough understanding of these additives will play a pivotal role in improving poultry health, as commercial products continue to become available and research continues to provide information on phytogenic feed additives.

This literature review summarizes the avian immune system, and some basic defenses it utilizes against pathogens, particularly coccidiosis. Additionally, a background of probiotics and phytogenic feed additives, rather than antibiotics, is included to provide background for the subsequent studies performed with broilers and natural feed additives.

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CHAPTER III

Performance and Response of DFM-fed broilers to a coccidiosis challenge

Abstract

Coccidiosis is a costly parasitic disease to the poultry industry with multiple control methods being explored to reduce its impact. This study evaluated the effects of a direct-fed microbial (DFM) provided as a dietary supplement over a 4-week period on response of broilers to a coccidiosis challenge. In total, 1056 day-old male chicks were randomly assigned to 8 replicate pens (22 birds/pen) of 6 treatments in a 3x2 factorial arrangement including 3 DFM levels (zero, low, high) with or without a 10X coccidiosis vaccine as a challenge on D15. Performance parameters were measured weekly, microbial profiling was assessed weekly, coccidian pathology at D21, and body composition at D28 using Dual Energy X-Ray Absorptiometry (DXA). Data were analyzed using two-way ANOVA with LS Means to separate means with significance assigned at $P \leq 0.05$ with $0.05 \leq P \leq 0.10$ being a trend. Lesion scores were reduced in the duodenum of the DFM-low birds and jejunum of DFM-high birds compared to other groups, no lesion differences were observed in the ileum and ceca. As expected, the coccidiosis challenge main effect resulted in higher FCR and lower BWG and FI over the 28-day trial. Treatment had little effect on overall performance; however, birds on the DFM-low diet had lower fat and higher lean percentages than those of other groups without the challenge. Also, D28 DXA analysis revealed that body fat and lean tissue were reduced in the challenged groups, a potential indication of differential resource allocation to the defense response to an enteric infection rather to performance. The results of this study suggest that diets supplemented with DFM during an *Eimeria* infection may ameliorate the negative effects of coccidiosis and boost performance.

Introduction

Livestock producers have routinely applied sub therapeutic levels of antibiotics in feed for decades. The commercial poultry industry has moved away from using antibiotics for a number of reasons, including negative public perception of antibiotics used in food animals, development of antibiotic resistant bacteria, and the updated Veterinary Feed Directive (VFD), which makes it more difficult to routinely use medically important antibiotics in production and outlines stricter regulation for most drugs (Ratcliff, 2000; Veterinary Feed Directive, 2015). Research continues to focus on potential natural alternatives to antibiotics to maintain and improve the efficiency of commercial poultry. Probiotics are an important potential natural alternative to antibiotics, as demonstrated by their ability to improve overall bird performance through a variety of mechanisms. Probiotics can be defined as a feed or water supplement consisting of mono-strain or multi-strain mixtures of live microorganisms that provide a health benefit via the gut (Parker, 1974; Fuller, 1989). They can include fungi, bacteria, and yeast, most common are strains of *Lactobacillus, Streptococcus, Bacillus, Bifidobacterium, Enterococcus, Aspergillus, Candida,* and *Saccharomyces* (Kabir, 2009).

In a healthy gut, there is a balance of beneficial and harmful microbes, and in normal circumstances, the gut functions to absorb nutrients, regulate the immune system, serve as a protective barrier, and contribute to overall performance (Fuller, 2000; Ahmed, 2006). In stressful conditions, such as environmental changes or disease, the microbial balance may be disrupted and have negative consequences. Damage to the gut can stunt growth, increase cost of production, allow for secondary infections, or lead to death (Williams, 2005). The early application of probiotics aims at establishing beneficial bacteria in the gut to competitively exclude and inhibit pathogens that could also inhabit the gut, including coccidiosis (Dalloul,

2017). Coccidiosis is caused by the ubiquitous parasite *Eimeria*, and is among the most costly diseases to the poultry industry, resulting in annual losses of around US $3 billion (Dalloul and Lillehoj, 2006). The parasite is transmitted via the oral-fecal route, and reproduces within the epithelial cells of the gut (Chapman et al., 2013). Symptoms include gut lesions, tissue damage, and diarrhea, leading to impaired nutrient absorption and stunted growth performance. Although compensatory growth can usually correct the stunted growth, there is still an economic cost associated with the disease, even at subclinical levels (Peek and Landman, 2011). This study focused on whether the use of probiotics improved performance and reduced negative effects of coccidiosis in broiler chickens.

Materials and Methods

Birds and Experimental Treatments

A 28-day trial was conducted in floor pens (4 x 4 sq.ft.) in the Litton-Reaves animal research facilities at Virginia Tech. On day of hatch (DOH), 1056 day-old male Ross broiler chicks were randomly assigned to one of six treatments with 8 replicates per treatment and 22 birds per pen in a 3 x 2 factorial arrangement, 3 DFM doses (zero, low, high) with or without challenge. A negative control (NC) treatment consisted of a standard corn/soy broiler diet without a coccidiosis challenge. The next two treatments contained either low levels of direct-fed microbials (DL) or high levels of direct-fed microbials (DH) throughout the trial. A positive control (PC) treatment included a D15 coccidiosis challenge in addition to a standard broiler diet. The last two treatments were the DFM low-dose diet with a D15 coccidiosis challenge (DL+) and the DFM high-dose diet plus a D15 coccidiosis challenge (DH+). Feed and water were available ad libitum throughout the trial. A starter diet was fed from D0-D14, and a grower diet

was fed D14-D28. Animal protocols were approved and the experiment was conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee.

Probiotics Mixture

A poultry 3-strain DFM blend of *Bacillus subtilis, B. licheniformis,* and *B. pumilus* (BIO-CAT Microbials, Shakopee, Minnesota, US) was provided as a low-dose or high-dose treatment. The lose-dose (DL) treatment had a minimum total plate count of 350 million CFU/g and the high-dose (DH) treatment had a minimum total plate count of 9 billion CFU/g as measured by the manufacturer. Both were a powder added to starter and grower mash diets during feed preparation per manufacturer's instructed amount.

Coccidiosis Challenge

On D15, PC, DL+, and DH+ treatments were given a 10X Advent coccidiosis vaccine via oral gavage containing live oocysts of *E. tenella, E. acervulina,* and *E. maxima* (Huvepharma, Inc. Lincoln, NE, US). On D21, lesion scores (LS) were assessed in the afflicted areas of the small intestines (duodenum, jejunum, ileum, and ceca) on 3 birds per pen from challenged treatments using the Johnson and Reid scale (1970). A zero score indicates no lesions, with a four being severe lesions and symptoms.

Performance Measurements

On DOH, 7, 14, 21, and 28, birds and feeder weights (per pen) were recorded to keep track of performance parameters including body weight [BW], body weight gains [BWG], feed intake [FI] and feed conversion ratios [FCR]. On D28, Dual Energy X-Ray Absorptiometry (DXA, GE Healthcare, Lunar Prodigy Advance program) was used to measure body composition. Two birds per pen were humanely euthanized and tagged before going through a scalder and plucker to remove feathers. Birds were weighed, frozen, and thawed at time of measurements, which

included individual body mass, lean tissue mass, fat tissue mass, and bone mineral content. Results are presented as average bird weight (kg), fat percent in relation to body weight, and lean percent in relation to body weight for each treatment.

Microbial Profiling

Ileal Sample Preparation

Mucosal scrapings from the ileum were taken from 2 birds/pen on D7, 14, 21, and 28 to assess population counts of *Bacillus*, *Clostridium*, *Enterococcus*, and universal bacterial counts. Each bird was properly euthanized by cervical dislocation and the ileum was dissected out using scissors and tweezers cleaned with alcohol between birds. A two- to three-inch long section from the middle of the ileum was excised and washed with sterile phosphate buffered saline (PBS) before being placed in a sterile WhirlPak bag and immediately placed on dry ice before transfer to a -80˚C freezer. Bacterial DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following protocols for extraction of bacterial DNA. Approximately 45-mg tissue obtained from scraping the ileal mucosa was placed in a 2-mL microcentrifuge tube containing a stainless steel bead. To target Gram-negative bacteria, 180 µL Buffer ATL (Animal Tissue Lysis) were added to the tube, while 180 µL of enzymatic lysis buffer (a mixture of lysozyme, Tris-HCl, NaCl, and EDTA) were used to target Gram-positive bacteria; afterwards, all remaining steps were the same for each type of sample. Tubes were incubated in a 37°C water bath for 30 minutes. A stainless steel bead, 180 µL Buffer ATL and 20 µL proteinase K were added to each tube, which was placed in a TissueLyser at 30 Hz for 40 seconds to homogenize the contents. The bead was removed with a magnet and the 2 mL microcentrifuge tube and contents were incubated overnight at 56°C in a shaking incubator. Next, 200 µL Buffer AL and 200 µL 100% ethanol were added with vortexing after each addition. Approximately 700 µL of the mixture were

transferred into a new DNeasy Mini spin column resting in a collection tube. The DNeasy Mini spin column tubes were centrifuged at 3,578 x g for 1 minute. Supernatant from the collection tube was discarded and the spin column was transferred to a new collection tube. Next, 500 µL Buffer AW1 were added, and tubes centrifuged at 3,578 x g for 1 minute. Supernatant was discarded and spin columns were placed in a new collection tube where 500 µL Buffer AW2 was added. Tubes were spun at 10,956 x g for 3 minutes and the spin column was separated from the supernatant collection tube and placed in a new 1.5 mL microcentrifuge tube. Then, 200 µL Buffer AE were carefully placed on the DNeasy spin column membrane and the 1.5 mL tube containing the spin column was incubated at 25°C for 10 minutes, centrifuged at 3,578 x g for 1 minute, and the spin column was discarded, leaving the 1.5 mL microcentrifuge containing genomic DNA. The quality and concentration of genomic DNA were measured using NanoDrop before being stored at -20°C until DNA samples could be adjusted to concentrations of 50 ng/µL using DNase- and RNase-free water.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Genomic DNA from corresponding bacteria of interest had been previously isolated and concentrations were adjusted to 50 ng/µL. Primers (Table 3.3) for *Clostiridum*, *Enterococcus*, and universal bacterial counts were previously designed, purchased, and diluted to 2 µM in a previous study. Primers from *Bacillus* were purchased from Eurofin and diluted to 2 µM per manufacturer's instruction. A Master Mix was prepared containing 5 µL SYBER Green, 1 µL forward primer, 1 µL reverse primer, and 2 µL RNAse-free water for each sample.

A new 96-well plate was used for plating the samples. First, 1 µL RNase-free water was plated in three wells as a negative control. Second, 9-µL Master Mix was added to each well that would later contain a sample. Third, 1 µL genomic DNA was added to the three positive control wells.

Next, sample DNA was plated in triplicate. The samples were pooled by pen, using two birds from each pen, so 0.5 μL of sample DNA from each bird was combined to result in 1 μL of sample DNA in each well. Once all samples and Master Mix were plated, PCR plate film was placed over the 96-well plate before being centrifuged at 134 x g for 1 minute to spin down the mixture. Quantitative real-time PCR was completed using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, US) using manufacturer's instructions in an ABI 7500 FAST Real-Time PCR Machine (Life Technologies, Grand Island, NY). The PCR reaction consisted of denaturation at 95°C for 20 seconds (sec), then 40 cycles of denaturation at 95°C for 3 sec, and last, annealing and extension at 60°C for 30 sec. Results were graphed as Ct (cycle threshold) means.

Statistical Analysis

SAS JMP Pro 12 Fit Model was used to perform statistical analysis with results presented as least square means (LSMeans) with standard error means (SEM). Significance was set at $P \leq 0.05$ as being significant with $0.05 \leq P \leq 0.10$ being a trend.

Results

Performance Measurements

Body Weight (BW). There was a significant effect of diet ($P = 0.0181$) on D7 average BW per bird (Fig. 3.1-A) where PC had lower BW than DL, DH, DL+, and DH+. Results were similar on D14 ($P = 0.0221$) as shown in Figure 3.1-B.

Body Weight Gain (BWG). As shown in Figure 3.2-A, there was a significant effect of diet ($P = 0.0336$) on BWG per bird from D0-7, where PC had lower BWG than DL, DH, and DH+. From

D0-14 (Fig. 3.2-B), a significant effect of diet (*P* = 0.0259) was observed, with similar results to D0-07 in which PC had lower BWG per bird than DL, DH, and DH+.

Feed Intake (FI). Higher FI was observed in some of the DFM diets compared to controls during the beginning of the experiment. Figure 3.3-A shows a trend due to diet (*P* = 0.0880) from D0-7, in which DH and DH+ both had higher FI than PC. Similarly, Figure 3.3-B depicts D7-14 FI and Figure 3.3-C shows D0-14 in which a significant effect of treatment (*P* < .0001) was observed where DL, DH, and DH+ had higher FI than PC, and DH and DH+ had higher FI than NC and DL+. Although there was no significant interaction between diet and challenge from D21-28, there was a significant effect of diet (*P* = 0.0124) as shown by increased FI by DL, DH, DL+, and DH+ compared to PC in Figure 3.3-D. No significance was observed from D14-21, or cumulatively.

Feed Conversion Ratio (FCR). As presented in Figure 3.4-A, there was a significant effect of diet (*P* = 0.0344) on D0-7 FCR, where DL had lower FCR than all other diets. There was also a significant effect of diet (*P* < 0.0001) on D7-14 FCR (Fig. 3.4-B), where DH had a higher FCR than all other treatments, and DL had lower FCR than DH and DH+. From D21-28, there was no significant effect of treatment due to interaction of diet and challenge, but there was a significant effect of diet alone (*P* = 0.0002) shown in Figure 3.4-C, where the challenged DFM-supplemented diets had higher FCRs than NC, DL, and PC. Cumulative D0-14 FCR (Fig. 3.4-D) had a significant effect of treatment (*P* = 0.0008), where NC and DL had lower FCRs than DH. There was a trend (*P* = 0.0555) from D0-28 (Fig. 3.4-E), in which NC and DL+ had higher FCRs than the DL treatment.

Some non-significant effects of treatment were observed in average BWs per bird (Appendix 1, Table S3.1), BWGs per bird (Appendix 1, Table S3.2), FI per bird (Appendix 1, Table S3.3), and FCR (Appendix 1, Table S3.4).

Based on Dual X-ray Absorptiometry (DXA) measurements, there was no significant effect of treatment found in the average D28 body weight of birds used for DXA (Table S3.5). There was a trend ($P = 0.08$) in D28 percent fat per bird (Fig. 3.5-A) where DL+ and DH+ had lower fat percent than DL. The DL+ treatment had a lower fat percent than NC, DL, DH, and PC.

Coccidiosis Challenge

Lesion Scores. There was no significant effect of diet on lesion scores measured on D21. Data are reported in Appendix 1, Figure S3.1 and Table S3.7.

Microbial Profiling

No significant effects of dietary treatments were observed prior to the coccidiosis challenge, i.e. on D7 and D14, and those results are reported in Appendix 1, Table S3.8.

Universal Bacterial Counts. There was a significant effect of treatment ($P = 0.00059$) on D21 (Fig. 3.6-B), in which DH+ had higher bacterial counts than all other treatments.

Enterococcus Counts. In Figure 3.6-A, results from D21 microbial profiling indicate a significant effect of treatment ($P = 0.028$), indicating higher levels in DH+ when compared to all other treatments, as shown by a lower Ct mean number.

Bacillus Counts. On D28 microbial profiling of the ileum (Fig. 3.6-C), there was a significant effect of treatment ($P = 0.03$) in which a higher level was observed in DH than NC and DH+, as indicated by a lower Ct number.

Clostridium Counts. Figure 3.6-D shows a significant effect of treatment ($P = 0.0027$) on D28, where PC had lower Ct Means and higher bacterial levels than all other treatments.

Discussion

The objective of this study was to evaluate the effects of a low dose and high dose probiotic during a coccidiosis challenge. Overall, there was little significant effect of treatment when looking at the interaction between diet and challenge. Performance parameters were improved with supplementation of DFM during the beginning of the trial, as shown by increased BW, BWG, and FI in DL, DH, DL+, and DH+ compared to controls during the first two weeks.

For average BW and BWG per bird, there was a significant effect of diet before the challenge, where DFM-supplemented birds had improved BW and BWG over the controls; as expected however, the challenged birds tended to have lower BW towards the end of the experiment, especially by D28, compared to the non-challenged birds. Lower BW in challenged birds compared to non-challenged birds gives partial indication of a somewhat effective coccidiosis challenge. The low-dose and high-dose of DFM tended to have similar BW and BWG during the first two weeks. There was no significant effect of treatment on BW and BWG at the end of this relatively short experiment, despite significance at the beginning. Giannenas and colleagues (2012) reported no difference in BW between probiotic and control groups until D28 of their trial. A study by Li and colleagues (2008) tested probiotic and antibiotic supplementation on broilers for 42 days, and also did not find significant differences in average daily gain with probiotic supplementation. Mohan and colleagues (1996) suggest that there is a lag phase of about 4 weeks before beneficial effects of probiotics on BWG are exhibited, as they did not observe effects until later based on reviews of previous studies. A short study (22 days) fed broilers probiotics and also observed no difference in BWG (Kim et al., 2009). One study fed birds a probiotic from DOH and observed no difference in BWG during the starter phase, but reported improvements during the grower phase due to probiotic supplementation, also

suggesting a possible lag phase in effects of probiotics on BWG (Mountzouris et al., 2015). Probiotics can take time to properly establish in the gut, and do not have an effect until then. This colonization may be sped up by the addition of prebiotics to the probiotic supplement, which promotes probiotic growth and health in the intestines by feeding the bacteria and creating an environment tailored to the specific needs of the probiotic strains. Perhaps additional effects of probiotics may be further exhibited beyond the four weeks used in this trial.

Although there was no significant effect of treatment on feed intake (FI) per bird throughout the trial, the effects of diet were initially significant. A trend was observed from D0-7 and a significant effect was seen from D7-14 and across D0-14, showing improvements in FI by DFM-supplemented diets. The DH and DH+ diets tended to perform better than DL or DL+, but both diets improved FI over controls during the first two weeks. There were no significant effects of treatment on FI during the last two weeks, once the broilers were challenged. Similar studies in broilers fed probiotics found no significant effect on cumulative feed intake (Mohan et al., 1996; Li et al., 2007). Contrastingly, Mountzouris and colleagues (2015) found no significant effect on FI from DFM supplementation during the starter phase, but observed improvement by probiotics during the grower phase.

With feed conversion ratio (FCR) there was a significant effect of diet from D0-7, D7-14, and D21-28, as well as cumulatively from D0-14 and an overall trend from D0-28. While the only change in the first week was a lower FCR in DL, there was higher FCR in DH during the second week. Although there was no interaction between diet and challenge from D21-28, FCR was worse in DFM supplemented diets. From D0-28, there was a trend ($P = 0.0555$) where DL had a lower FCR than DL+, and all other diets performed similarly. During the coccidiosis challenge, the low-dose DFM diet performed differently than it did without a challenge, in which it had

lower FCR. Overall, FCR was not significantly affected by diet. A study using probiotics without challenge observed no difference in FCR over the 42 day trial (Mohan et al., 1996). Another trial by Mountzouris and colleagues (2015) using probiotic supplementation reported a significant effect of probiotics on FCR during the grower phase only, but no difference in the overall results. The varying effects of DFMs on FI and FCR have been reported by numerous researchers and could be attributed to multiple factors. DFMs may have little effect on birds living in clean environments, which are not exposed to much stress or disease, since DFMs can inhibit and exclude pathogens (Gunal et al., 2006). Type of feed ingredients or feed preparation techniques can affect the bacterial strain in the DFM, since some bacterial strains thrive better with specific feed types (Pan and Yu, 2013). For example, fermented feeds can improve the colonization of beneficial bacteria often found in DFMs, including *Bifidobacterium* and *Lactobacillus* (Xu et al., 2003). Hammons and colleages (2010) found that feeding a standard corn/soy diet to broilers increased *Lactobacillus agilis* type R5, while a diet containing more wheat middlings favored *L. agilis* type R1. Substituting soybean meal with a fermented cottonseed meal increased *Lactobacillus* population and decreased the number of coliforms in broiler ceca (Sun et al., 2013). Animal fat, such as tallow and lard, in substitution for a soy oil in a broiler diet increased ileal levels of *Clostridium perfringens* (Knarrebord et al., 2002).

Dual X-ray absorptiometry (DXA) results showed biological trends ($P = 0.08$) in percent lean and fat between the treatments. Of the birds that were sampled, there was no difference in their average weight per treatment. There was no significant difference between the non-challenged treatments. DL+ diet had higher lean and lower fat compared to the three non-challenged diets and PC diet. Feeding a probiotic during a coccidiosis challenge led to better carcass composition, as less fat would lead to a higher yield. A study by Schobert (2015) saw better body composition

in probiotic-fed broilers during the starter phase of a trial, but did not see further effects in subsequent phases.

To observe the effects of the D15 coccidiosis challenge, lesion scores were measured in the duodenum, jejunum, ileum, and ceca of PC, DL+, and DH+ diets. Birds exposed to coccidiosis at this age are most likely to display symptoms, including intestinal lesions, approximately 4 to 5 days after inoculation, after adequate time is given for the intracellular *Eimeria* parasite to reproduce and cause symptoms before the bird gains immunity and heals (Allen and Fetterer, 2002). The vaccine used contained live oocysts from *E. tenella*, *E. acervulina*, and *E. maxima* (Huvepharma, Inc. Lincoln, NE, US). *E. tenella* mainly infects the ceca, where it can cause lesions, thickening of the walls, and blood in the contents. The *E. acervulina* main site of infection is the duodenum, where it can form plaque-like lesions, cause watery contents, discoloration of the mucosal wall, and thickened intestinal walls filled with mucus in severe cases. *E. maxima* is more difficult to score, because the visual symptoms of lesions are briefer than in the other species. Damage occurs mostly in the jejunum and upper ileum around day 6 to 7 post inoculation. Lesion scoring ranges from 0, indicating no lesions, to 4, indicating the highest level of intestinal damage. In this experiment, lesion scores were minimal, with averages ranging from 0 to 1.93. There was no significant effect between the treatments, yet there appeared to be lower duodenal lesions in DL+ and DH+, lower jejunal and ileal lesions in DH+, and lower cecal lesions in DL+. The DFM products may boost the immune system and improve host defense against coccidiosis (Yang et al., 2009). Probiotic supplementation has been shown to increase levels of chicken IFN-γ, which stimulates acquired immunity and boosts protection to coccidiosis (Dalloul et al., 2005). The bacteria found in these products may also contribute to competitive exclusion, occupying intestinal space and attachment sites that the parasites need to

infiltrate (Edens, 2003), although little progress has been made in discovering receptors needed for sporozoite penetration into epithelial cells. Mortality was measured to observe effects of DFM products on a severe coccidiosis challenge. Before the challenge, there was no significant difference between the treatments, as expected. After the challenge, there was also no significant difference. Collectively examining mortality, performance, and lesion scores, the coccidiosis challenge may not have been severe enough to have large disease related effects between treatments, perhaps an outcome of more controlled environment and shorter growing period relative to commercial settings.

Microbial profiling via qRT-PCR was performed to analyze differences in the bacterial populations of the ileum each week. Differences in *Bacillus, Clostridium, Enterococcus,* and universal bacteria counts were measured using Ct (cycle threshold) means, where a lower Ct mean indicates a higher number of corresponding bacteria. There was no significant difference in diet on D7 and D14. On D21, there was a significant effect of treatment in *Enterococcus* and total (universal) bacterial counts. In both, the DH+ treatment had a lower Ct mean than the other treatments, indicating higher numbers of bacteria. The other treatments were similar, perhaps because the DFM bacteria did not establish well in the gut. By D28, there was a significant difference in the *Bacillus* Ct means between treatments. The DH diet had higher *Bacillus* numbers than the DH+ diet, most likely due to the effect of the challenge lowering overall microbial integrity in the challenged birds. All other treatments had similar results. Unlike this current study, Zarei and colleagues (2017) fed broilers a control, probiotic, or whey powder diet for 42 day. They measured ileal microbial populations of *Bacillus* and found higher levels in the probiotic treatment on D42. The *Clostridium* levels were also significantly different. All treatments had similar Ct means, except an increase in PC. This treatment had been challenged,

and had higher numbers of *Clostridium* than the DL+ or DH+ diets. This may indicate a benefit of feeding the DFM product to challenged birds, and reducing harmful *Clostridium* loads. Murray and colleagues (2006) also found lower *Clostridium* levels in *Lactobacilli*-fed birds, even though it did not improve performance parameters such as BWG and mortality. Overall, there did not appear to be much of a difference between the DFM diets and controls when responding to a challenge.

There were no negative aspects associated with feeding a DFM product to broilers. The non-significant results may have been due to a mild coccidiosis challenge that was not strong enough to elicit response. A DFM product had beneficial aspects to it when added to broiler feed, even though not all effects were significant towards the end of the trial. For example, there tended to be higher BW, BWG, and FI in birds fed a low-dose or high-dose DFM. There was a trend toward improved body composition of birds fed a DFM product. The microbial profiles indicated more species of beneficial *Bacillus* in a DH+ group, and higher levels of *Enterococcus* and universal numbers as well in later weeks of the experiment. Lower numbers of *Clostridium* in the DL+ and DH+ compared to PC indicate a beneficial effect of DFM product during challenge. Due to the nature of probiotics, they may perform differently during a disease challenge than without, and the nature of bacteria used, preparation methods, and other environmental factors all affect the function of probiotics (Edens, 2003; Huang et al., 2004). Although much of the diet and challenge interaction results were not significant, there were still important conclusions to be drawn about the benefits of feeding DFM products.

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Table 3.1. Description of treatments.

Treatment	Description	Challenge
1. NC	Control = basal (standard corn/soy) diets	-
2. DL	Control + DFM Low	-
3. DH	Control + DFM High	-
4. PC	Control + Challenge	+
5. DL+	Control + DFM Low + Challenge	+
6. DH+	Control + DFM High + Challenge	+

Table 3.2. Composition of broiler diets during starter and grower phases.

Item	Starter (DOH to D15)	Grower (D15 to D28)
Ingredient, %		
Corn	60.12	65.20
Soybean meal	22.42	16.43
Distiller's grain	7.00	8.00
Poultry by-product meal	5.00	5.00
Grease (yellow)	1.91	2.12
Dicalcium phosphate	1.15	0.90
L-Lysine	0.63	0.60
Big Spring Mills vitamin and mineral premix*	0.63	0.63
Limestone	0.58	0.54
DL-Methionine	0.18	0.30
Salt	0.27	0.17
L-Threonine	0.10	0.10
Optiphos**	0.01	0.01
Total	100.00	100.00
Calculated nutrient Level		
ME, kcal/kg	3,036.00	3,102.00
CP, %	21.00	19.00
Ca, %	0.90	0.80
Available P, %	0.45	0.40
Total P, %	0.71	0.64
Digestible Lys, %	1.50	1.33
Digestible Meth, %	0.50	0.60
Digestible Thr, %	0.89	0.81
Digestible Trp, %	0.22	0.19

* Cobalt (Min) 34 ppm; Copper (Min) 540 ppm; Iodine (Min) 134 ppm; Iron (Min) 6,750 ppm; Manganese (Min) 8,580 ppm; Zinc (Min) 6,500 ppm; Vitamin A (Min) 400,000 IU/lb; Vitamin D3 (Min) 134,000 ICU/lb; Vitamin E (Min) 100 IU/lb; Vitamin B12 (Min) 0.4 mg/lb; Menadione (Min) 70 mg/lb; Riboflavin (Min) 250 mg/lb; D-Pantothenic acid (Min) 368 mg/lb; Niacin (Min) 1,200 mg/lb; Choline (Min) 23,147 mg/lb.

**Phytase source; Huvepharma

Table 3.3. Primers used for qRT-PCR in microbial profiling.

Gene	Primer ID	Primer Sequence
Bacillus	005F	5' – TGGAGAGTTTGATCCTGGCTCAG – 3'
	531R	5' – TACCGCGGCTCGTGGCAC – 3'
C. perfringens	Clost_7_F	5' – ATGCAAGTCGAGCGA(G/T)G – 3'
	Clost_7_R	5' – TATGCGGTATTAATCT(C/T)CCTTT – 3'
Enterococcus	RT-chEntero2_F	5' – GATGCATAGCCGACCTCAGAA – 3'
	RT-chEntero2_R	5' – AGTTTGGGCCGTGTCTCAGT – 3'
Universal Bacteria	RT-chUni_F	5' – TCCTACGGGAGGCAGCAGT – 3'
	RT-chUni_R	5 – GGACTACCAGGGTATCTAATCCTGTT – 3'

Figure 3.1. Effect of DFM diet (D0-14) or DFM diet*challenge interaction (D14-D28) on average body weight (BW) per bird per treatment of Ross male broiler chicks. Data are presented as ratios using Least Square Means ± Standard Error Means. Significant difference represented by bars lacking the same letter. There was a significant effect of diet (P = 0.0181) on D7 (Fig. 3.1-A) and a significant effect of diet (P = 0.0221) on D14 (Fig. 3.1-B). NC = control; DL = DFM Low; DH = DFM High; PC = control plus challenge; DL+ = DFM Low plus challenge; DH+ = DFM High plus challenge.

Figure 3.1-A

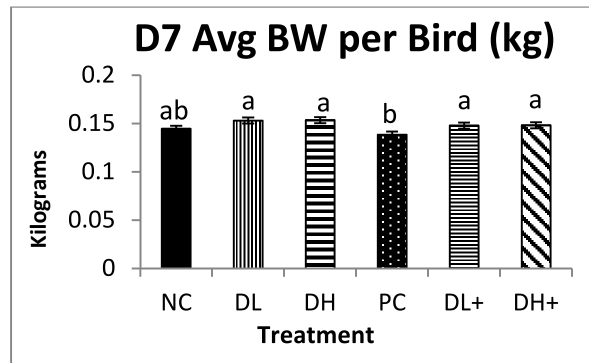


Figure 3.1-B

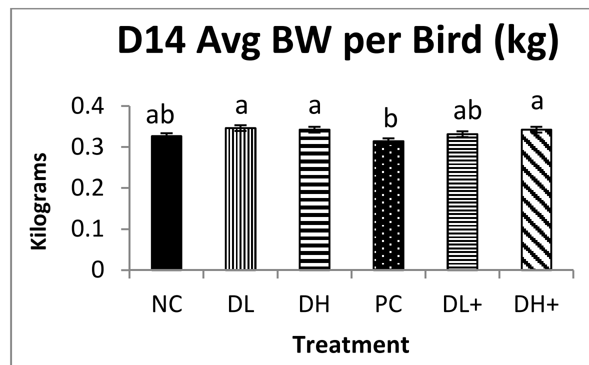


Figure 3.2. Effect of DFM diet (D0-14) or DFM diet*challenge interaction (D14-D28) on average body weight gain (BWG) per bird per treatment of Ross male broiler chicks. Data are presented as ratios using Least Square Means ± Standard Error Means. Significant difference represented by bars lacking the same letter. There was a significant effect of diet (P = 0.0336) from D0-7 (Fig. 3.2-A) and a significant effect of diet (P = 0.0259) from cumulative D0-14 (Fig. 3.2-B). NC = control; DL = DFM Low; DH = DFM High; PC = control plus challenge; DL+ = DFM Low plus challenge; DH+ = DFM High plus challenge.

Figure 3.2-A

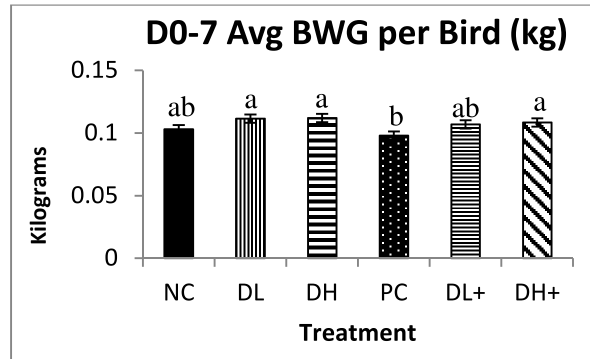


Figure 3.2-B

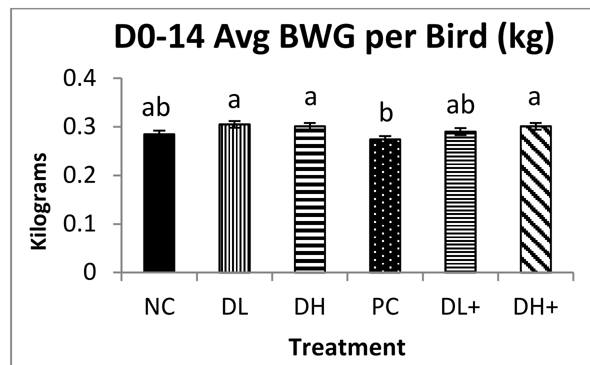


Figure 3.3. Effect of DFM diet (D0-14) or DFM diet*challenge interaction (D14-D28) on average feed intake (FI) per bird per treatment of Ross male broiler chicks. Data are presented as ratios using Least Square Means ± Standard Error Means. Significant difference represented by bars lacking the same letter. There was a trend due to diet (P = 0.0880) from D0-7 (Fig. 3.3-A), a significant effect of diet (P < .0001) from D7-14 (Fig. 3.3-B), a cumulative significant effect of diet (P < 0.0001) from D0-14 (Fig. 3.3-C), and a significant effect of diet (P = 0.0124) from D21-28 (Fig. 3.3-D). NC = control; DL = DFM Low; DH = DFM High; PC = control plus challenge; DL+ = DFM Low plus challenge; DH+ = DFM High plus challenge.

Figure 3.3-A

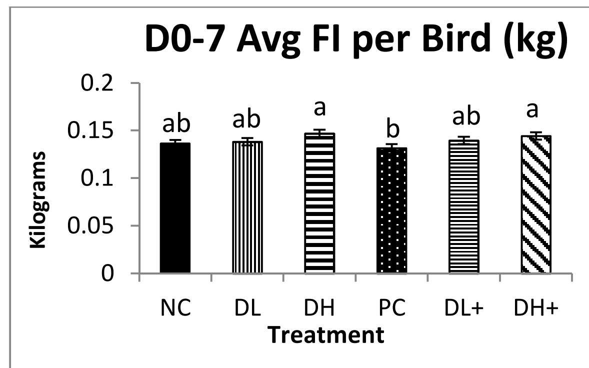


Figure 3.3-B

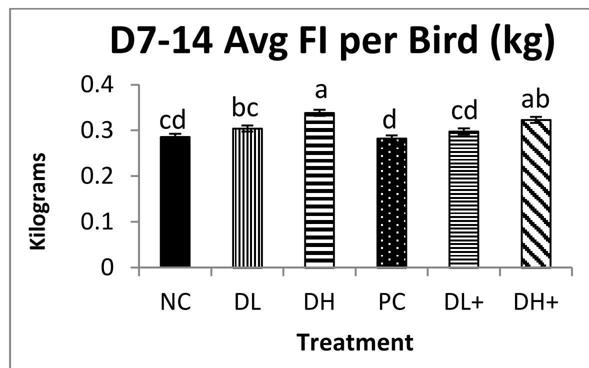


Figure 3.3-C

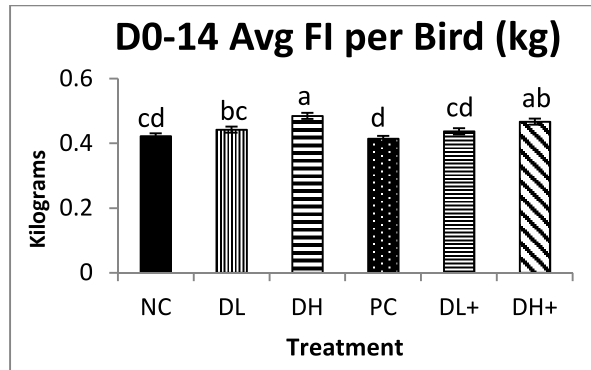


Figure 3.3-D

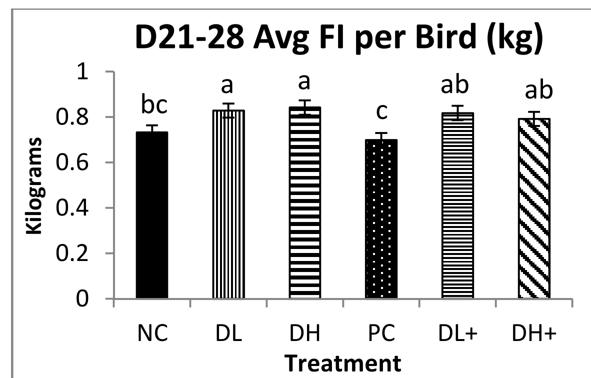


Figure 3.4. Effect of DFM diet (D0-14) or DFM diet*challenge interaction (D14-D28) on feed conversion ratio (FCR) per treatment of Ross male broiler chicks. Data are presented as ratios using Least Square Means ± Standard Error Means. Significant difference represented by bars lacking the same letter. There was a significant effect due to diet ($P = 0.0344$) from D0-7 (Fig. 3.4-A), a significant effect of diet ($P < .0001$) from D7-14 (Fig. 3.4-B), a significant effect of diet ($P = 0.0002$) from D21-28 (Fig. 3.4-C), a cumulative significant effect of diet ($P = 0.0008$) from D0-14 (Fig. 3.4-D), and a cumulative trend due to treatment ($P = 0.0555$) from D0-28 (Fig. 3.4-E). NC = control; DL = DFM Low; DH = DFM High; PC = control plus challenge; DL+ = DFM Low plus challenge; DH+ = DFM High plus challenge.

Figure 3.4-A

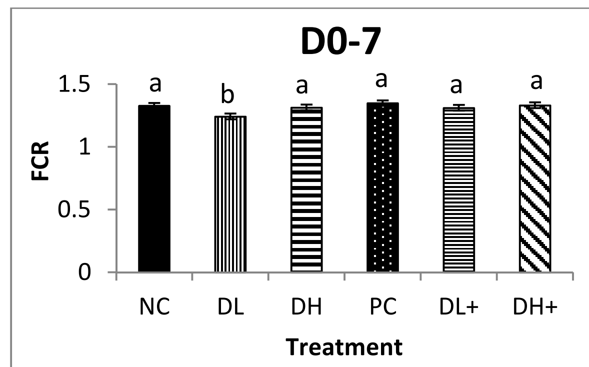


Figure 3.4-B

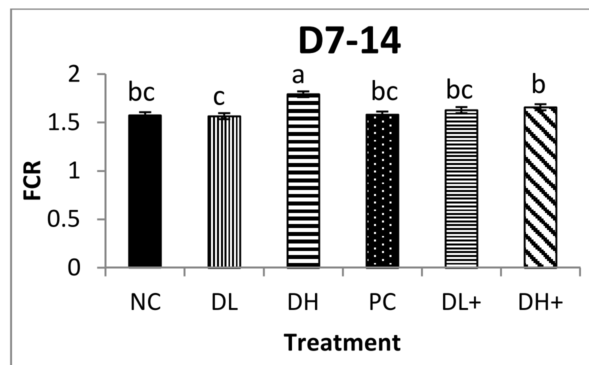


Figure 3.4-C

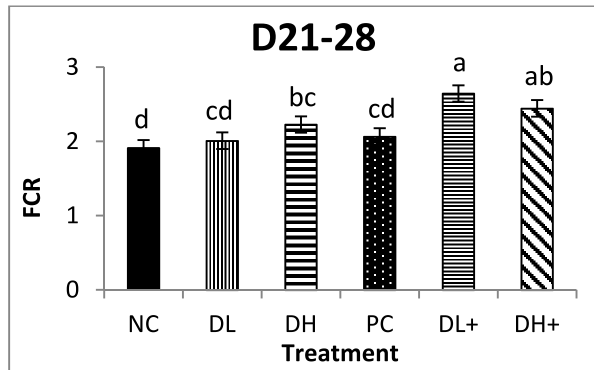


Figure 3.4-D

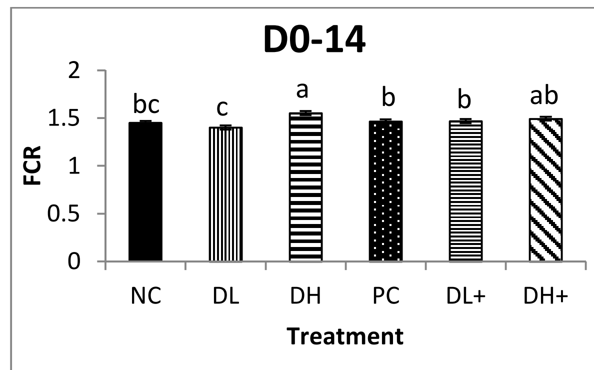


Figure 3.4-E

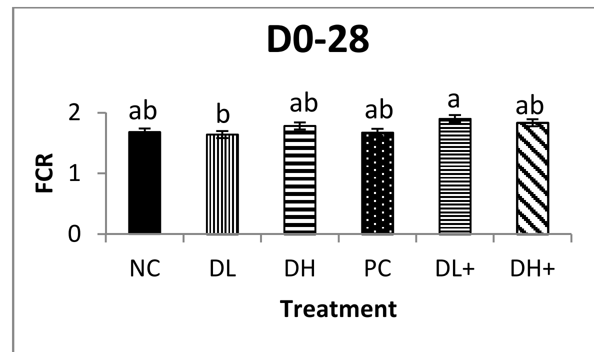


Figure 3.5. Effect of DFM diet*challenge interactions on D28 body composition per bird (percent fat or percent lean) on male Ross broiler chicks. Data are presented as percent using Least Square Means ± Standard Error Means. Significant difference represented by bars lacking the same letter. There was a trend ($P = 0.08$) on D28 average percent fat per bird (Fig. 3.5-A) and a trend ($P = 0.08$) on D28 average percent lean per bird (Fig. 3.5-B). NC = control; DL = DFM Low; DH = DFM High; PC = control plus challenge; DL+ = DFM Low plus challenge; DH+ = DFM High plus challenge.

Figure 3.5-A

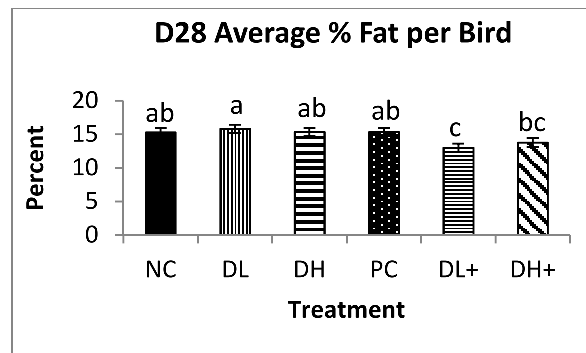


Figure 3.5-B

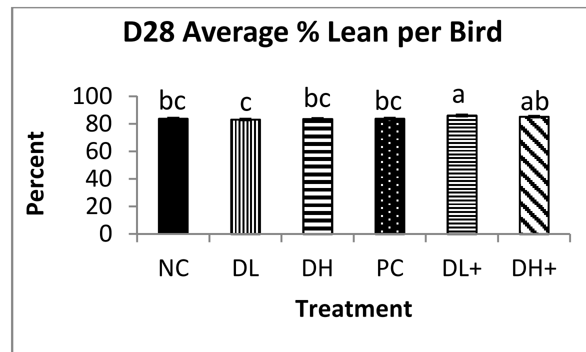


Figure 3.6. Effect of DFM diet*challenge interactions on microbial profiles of ileum of male Ross broiler chicks. Data are presented as Ct Means using Least Square Means ± Standard Error Means. Significant difference represented by bars lacking the same letter. There was a significant difference (*P* = 0.0028) on D21 Enterococcus counts (Fig. 3.6-A), a significant difference (*P* = 0.00059) on D21 Universal bacterial counts (Fig. 3.6-B), a significant difference (*P* = 0.03) on D28 Bacillus counts (Fig. 3.6-C), and a significant difference (*P* = 0.0027) on D28 Clostridium counts (Fig. 3.6-D). NC = control; DL = DFM Low; DH = DFM High; PC = control plus challenge; DL+ = DFM Low plus challenge; DH+ = DFM High plus challenge.

Figure 3.6-A

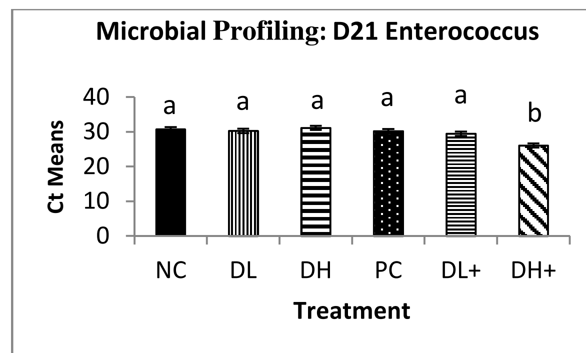


Figure 3.6-B

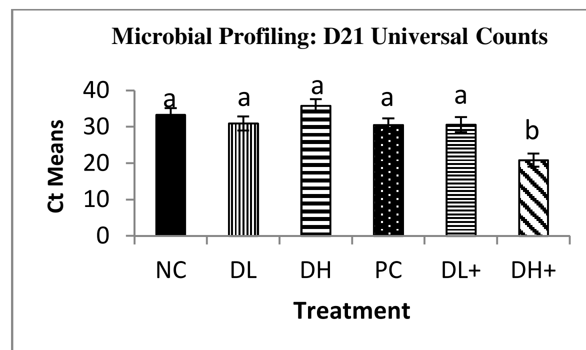


Figure 3.6-C

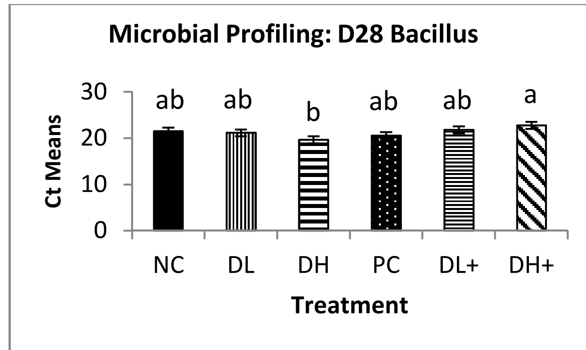
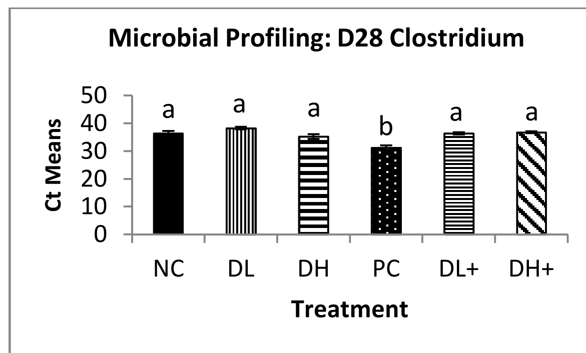


Figure 3.6-D



CHAPTER IV

Performance and response in broilers fed phytogenic feed additives

Abstract

Alternatives to antibiotics are a subject of heavy research. One group of natural alternatives are phytogenic feed additives (PFA). This study evaluated the effects of PFA provided as a dietary supplement over a 6-week period on performance and immune responses in broilers. In total, 1056 day-old male chicks were randomly assigned to 8 replicate pens (22 birds/pen) with 6 treatments including a standard corn/wheat basal diet, a basal with antibiotics, and 4 various combinations of PFA (PHY 1, 2, 3, and 4). Performance was assessed on day 7 (D7), 14, 28, and 42. Starter diet was fed D0-14, grower D14-28, and finisher D28-42. Microbial profiling was assessed on D7, 14, and 42, and body composition measured on D42 using Dual Energy X-Ray Absorptiometry (DXA). Data were analyzed using one-way ANOVA with LS Means to separate means with significance assigned at $P \leq 0.05$ and trends set at $0.05 \leq P \leq 0.10$. Overall, the antibiotic growth promoter (AGP) treatment performed similarly or lower than the control (CTRL), although not significantly. The PFA treatment results varied depending on the week and the measurement. For mortality, differences towards the beginning of the trial were observed with PHY 2, PHY3, CTRL, and AGP having lower mortality than PHY1 and PHY4. Observable differences towards the beginning of the trial in average bird weight and feed intake per bird suggested that PHY2 and CTRL had higher weights than AGP at D14. PHY1, 2, 4, and AGP had similar D28 weights while PHY1 and 2 were also similar to CTRL. During the first week, only one PHY treatment consumed more than the other treatments, while FI was reduced in the second week for two PHY treatments and increased in one PHY diet. Results varied by week, but

cumulatively, body weight gain per bird (BWG/Bird) was higher in PHY1 and 4, statistically similar to AGP and CTRL. With feed conversion ratio (FCR), PHY2 was higher than any other treatment between D0-42, while all other treatments were similar. PCR results were not statistically significant; however, DXA results showed variation. There was a higher lean to fat ratio in birds from PHY1-4 when compared to AGP, indicating an overall leaner bird in PHY treatments. The results of this study suggest that diets supplemented with PFA suggest some improved performance results in broilers.

Introduction

Using sub-therapeutic levels of antibiotics has been common practice to reduce disease and improve weight gains for decades, but using antibiotics in feed animals is becoming increasingly difficult, as demonstrated by the Veterinary Feed Directive in the United States and bans in the European Union (Castanon, 2007; Veterinary Feed Directive, 2015). Concerns over antibiotic resistant bacteria and antibiotic residue in meat products are driving the search for natural alternatives to antibiotics to maintain animal health and productivity (Silbergeld et al., 2008).

One group of natural alternatives is known as phytogenic feed additives (PFAs). This is a broad category of naturally derived oils, herbs, and spices that can be added to the feed or water of poultry. There are hundreds of types of PFAs, but common examples include derivatives of cinnamon, oregano, thyme, citrus, capsaicin, and rosemary (Greathead, 2003). There are numerous types of PFAs and a number of mechanisms of action that improve poultry health and performance. The addition of PFAs may result in improved performance, such as body weight gains and feed conversion ratios (Alcicek et al., 2003; Lewis et al., 2003). Digestibility may also improve due to increased enzyme and mucus production in the gut. Many in vitro studies

demonstrate the antimicrobial properties of various essential oils (Moleyar and Narasimham, 1992; Nychas, 1995; Rota et al., 2004). Some PFAs may improve the gut microbiota, which improves nutrient absorption, disease resistance, and overall health. Immune response is stronger, and some PFAs demonstrate anti-inflammatory properties (Brenes and Roura, 2010).

The diet composition, age, breed, and health status of the bird, in addition to the type of PFA in a diet are all important factors when researching natural alternatives to antibiotics. Research continues to find optimal combinations of PFAs and discover the mechanisms of action that lead to improved poultry performance. The following study was performed to assess broiler performance and gut microbial profiles when fed differing PFAs over a six-week period.

Materials and Methods

Birds and Experimental Treatments

A 42-day trial was conducted using individual 4 feet by 8 feet floor pens using clean pine shavings at the Virginia Tech Turkey Research Center. On day of hatch (DOH), 1056 Ross 708 male chicks were randomly assigned to one of 6 treatments. Each treatment had 8 replicates with 22 birds per replicate. The first treatment was a control (CTRL), with no feed additive. The second was referred to as the antibiotic-growth promoter (AGP), using the recommended dose of the coccidiostat Amprolium at 113.4 g/US ton and the poultry anti-infective Stafac at 453.6 g/US ton for the entire trial. Treatments PHY1, PHY3, and PHY4 were varying mixtures of PFAs plus Amprolium, while treatment PHY2 contained solely a PFA without Amprolium. Each PHY product was added at 907.4 g/US ton.

Birds were fed a standard corn/wheat diet, using formulations for starter in crumble form (D0-14), pelleted grower (D14-28) and finisher (D28-42). Feed and water were provided ad libitum throughout the trial. All protocols and experimental guidelines were approved and conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee.

Phytogenic Feed Additives Mixture

A feed additive intended to improve feed palatability and performance was tested by splitting up a commercial product into three different combinations, referred to as Products 3, 4, and 5. Table 4.2 in the Results section below contains a visual description of the diets. Although the exact composition of each of the three test products was not provided for proprietary reasons, they contain blends of mainly origanum essential oil, clove bud essential oil, cinnemaldehyde, and other non-active ingredients (Oleobiotec, Probiotech International Inc.). During the starter phase (D0-14), PHY1, PHY3, and PHY4 contained Amprolium and Product 3. PHY2 during starter was solely Product 4. In the grower phase (D14-28), PHY1 and PHY 4 contained the same mixture of Amprolium and Product 3 as before and PHY 2 was also the same, containing Product 4. PHY3 changed, adding Product 5 to the mixture in addition to Product 3 and Amprolium. In the finisher phase (D28-42), PHY1 remained the same as in the starter and grower phase, using Amprolium and Product 3. PHY3 contained Amprolium and Product 3, as it did in the starter, but not the grower phase. PHY2 continued to solely use Product 4, and PHY4 changed to using only Product 4 during finisher as well, in contrast to using Amprolium and Product 3 throughout the starter and grower phase. Again, each PHY product was added to each basal diet at 907.4 g/US ton before being crumbled (starter) or pelleted (grower, finisher).

Performance Measurements

Bird and feeder weights were taken on a per pen basis on DOH, D7, D14, D28, and D42 to measure body weight [BW] and feed intake [FI], and calculate body weight gains [BWG] and feed conversion ratios [FCR]. At the conclusion of the experiment, D42 body composition was analyzed using Dual Energy X-Ray Absorptiometry (DXA) to calculate lean and fat percentages of body weight. Three birds per pen were euthanized via cervical dislocation, their feathers plucked, and frozen until time of measurement. The DXA unit (GE Healthcare, Lunar Prodigy Advance program) measures individual body mass, lean tissue mass, fat tissue mass, bone mineral content, and bone mineral density on 10 birds at a time. Results are presented as average bird weight (kg), percent fat, and percent lean in relation to body weight on a treatment basis. Mortality was measured daily and reported during corresponding times as FCR, BW, BWG, and FI.

Microbial Profiling

Ileal Sample Preparation

Mucosal scrapings from the ileum were taken from 2 birds/pen on D7, 14, 21, and 28 to assess population counts of *Bacillus*, *Clostridium*, *Enterococcus*, and universal bacterial counts. Each bird was properly euthanized by cervical dislocation and the ileum was dissected out using scissors and tweezers cleaned with alcohol between birds. A two- to three-inch long section from the middle of the ileum was excised and washed with sterile phosphate buffered saline (PBS) before being placed in a sterile WhirlPak bag and immediately placed on dry ice before transfer to a -80˚C freezer. Bacterial DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following protocols for extraction of bacterial DNA. Approximately 45-mg tissue obtained from scraping the ileal mucosa was placed in a 2-mL microcentrifuge tube containing a stainless steel

bead. To target Gram-negative bacteria, 180 µL Buffer ATL (Animal Tissue Lysis) were added to the tube, while 180 µL of enzymatic lysis buffer (a mixture of lysozyme, Tris-HCl, NaCl, and EDTA) were used to target Gram-positive bacteria; afterwards, all remaining steps were the same for each type of sample. Tubes were incubated in a 37°C water bath for 30 minutes. A stainless steel bead, 180 µL Buffer ATL and 20 µL proteinase K were added to each tube, which was placed in a TissueLyser at 30 Hz for 40 seconds to homogenize the contents. The bead was removed with a magnet and the 2 mL microcentrifuge tube and contents were incubated overnight at 56°C in a shaking incubator. Next, 200 µL Buffer AL and 200 µL 100% ethanol were added with vortexing after each addition. Approximately 700 µL of the mixture were transferred into a new DNeasy Mini spin column resting in a collection tube. The DNeasy Mini spin column tubes were centrifuged at 3,578 x g for 1 minute. Supernatant from the collection tube was discarded and the spin column was transferred to a new collection tube. Next, 500 µL Buffer AW1 were added, and tubes centrifuged at 3,578 x g for 1 minute. Supernatant was discarded and spin columns were placed in a new collection tube where 500 µL Buffer AW2 was added. Tubes were spun at 10,956 x g for 3 minutes and the spin column was separated from the supernatant collection tube and placed in a new 1.5 mL microcentrifuge tube. Then, 200 µL Buffer AE were carefully placed on the DNeasy spin column membrane and the 1.5 mL tube containing the spin column was incubated at 25°C for 10 minutes, centrifuged at 3,578 x g for 1 minute, and the spin column was discarded, leaving the 1.5 mL microcentrifuge containing genomic DNA. The quality and concentration of genomic DNA was measured using NanoDrop before being stored at -20°C until DNA samples could be adjusted to concentrations of 50 ng/µL using DNase- and RNase-free water.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Genomic DNA from corresponding bacteria of interest had been previously isolated and concentrations were adjusted to 50 ng/µL. Primers (Table 4.2) for *Clostiridum*, *Enterococcus*, and universal bacterial counts were previously designed, purchased, and diluted to 2 µM in a previous study. Primers from *Bacillus* were purchased from Eurofin and diluted to 2 µM per manufacturer's instruction. A Master Mix was prepared containing 5 µL SYBER Green, 1 µL forward primer, 1 µL reverse primer, and 2 µL RNAse-free water for each sample.

A new 96-well plate was used for plating the samples. First, 1 µL RNase-free water was plated in three wells as a negative control. Second, 9-µL Master Mix was added to each well that would later contain a sample. Third, 1 µL genomic DNA was added to the three positive control wells. Next, sample DNA was plated in triplicate. The samples were pooled by pen, using two birds from each pen, so 0.5 µL of sample DNA from each bird was combined to result in 1 µL of sample DNA in each well. Once all samples and Master Mix were plated, PCR plate film was placed over the 96-well plate before being centrifuged at 134 x g for 1 minute to spin down the mixture. Quantitative real-time PCR was completed using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, US) using manufacturer's instructions in an ABI 7500 FAST Real-Time PCR Machine (Life Technologies, Grand Island, NY). The PCR reaction consisted of denaturation at 95°C for 20 seconds (sec), then 40 cycles of denaturation at 95°C for 3 sec, and last, annealing and extension at 60°C for 30 sec. Results were graphed as Ct (cycle threshold) means.

Statistical Analysis

SAS JMP Pro 12 Fit Model was used to perform one-way ANOVA with results presented as least square means (LSMeans) with standard error means (SEM). Significance was set at $P \leq 0.05$ as being significant and trends set at $0.05 \leq P \leq 0.10$.

Results

Performance Measurements

Body Weight (BW). There was a significant effect of treatment (*P* = 0.0013) in D14 average BW per bird (Fig. 4.1-A). The CTRL, PHY1, and PHY2 diets had higher average BW than the AGP or PHY4 treatments. In Figure 4.1-B, there was a significant effect of treatment (*P* = 0.0129) on D28 average BW per bird, with PHY3 having a lower average BW than CTRL, AGP, or PHY1 treatments.

Body Weight Gain (BWG). As seen in Figure 4.2-A, there was a significant effect of treatment (*P* = 0.0052) in D7-14 BWG per bird in which CTRL had higher BWG than AGP, PHY1, PHY3, and PHY4. In addition, PHY4 had lower BWG than CTRL or PHY2. There was also a significant effect of treatment (*P* = 0.0277) from D14-28 (Fig. 4.2-B), where PHY3 had lower BWG than CTRL and AGP, and PHY4 had lower BWG than CTRL. In Figure 4.2-C, from cumulative D0-14 BWG per bird, there was a significant effect of treatment (*P* = 0.0086) where AGP, PHY1, PHY3, and PHY4 had lower BWG than CTRL. PHY2 also had a higher BWG than the PHY4 treatment. Looking again at cumulative results, there was a significant effect of treatment (*P* = 0.0136) from D0-28 (Fig. 4.2-D), in which PHY1, PHY2, and PHY4 had similar BWG as the AGP diet, yet PHY1, PHY3, and PHY4 were lower than CTRL. Figure 4.2-E shows the significant effect of treatment (*P* = 0.0156) of cumulative D0-42 BWG per bird, with PHY1 and PHY4 having the same levels as CTRL and AGP, and PHY2 and PHY3 being lower than the CTRL and AGP treatments.

Feed Intake (FI). A trend due to treatment effect (*P* = 0.0738) was observed from D0-7 feed intake (Fig. 4.3-A), where PHY1, PHY3, and PHY4 had similar FI to both the CTRL and AGP diets. Within the PFA diets, PHY2 had highest FI. In Figure 4.3-B, a significant effect of

treatment ($P = 0.0009$) was observed during D7-14, where lower FI was observed in PHY3 and PHY4 in comparison to CTRL, AGP, and PHY2. PHY2 had higher FI than the other PFA treatments, similar to levels of CTRL and AGP. From cumulative D0-14 (Fig. 4.3-C), a trend ($P = 0.0696$) was observed, in which PHY3 and PHY4 shared similar, lower FI with CTRL and AGP treatments, while PHY2 had a higher FI than the CTRL or AGP.

Feed Conversion Ratio (FCR). Significant effect of treatment ($P = 0.039$) was observed in D0-7 FCR (Fig. 4.4-A), in which PHY2 had a higher FCR than other PFA treatments and the CTRL. PHY1, PHY3, and PHY4 had similar FCR to the CTRL. A similar effect of treatment ($P = 0.0052$) was seen in the cumulative results from D0-14 FCR (Fig. 4.4-B), where PHY3 and PHY4 had lower FCR than AGP and PHY2. PHY2 still had higher FCR than the other PFA treatments. Across the first four weeks, D0-28 FCR (Fig. 4.4-C) showed significance ($P = 0.0125$) with higher FCR in PHY2 when compared to all other treatments, and overall results, D0-42 FCR (Fig. 4.4-D), showed a significant effect of treatment ($P = 0.0023$) with PHY2 maintaining higher FCR than the other five treatments.

Mortality. Between D0-7 (Fig. 4.5-A), a significant effect of treatment ($P = 0.0475$) was observed, where PHY2 and PHY3 had lowest mortality, similar to the CTRL treatment, and PHY4 had higher mortality, similar to the AGP treatment. PHY1 had mortality levels similar to all treatments. In Figure 4.5-B, a significant effect of treatment ($P = 0.0349$) was observed from D7-14, with PHY4, CTRL, and AGP having a lower mortality than PHY1. Significant effect of treatment ($P = 0.0481$) from cumulative D0-28 (Fig. 4.5-C) demonstrated percent mortality was lower in PHY2, PHY3, CTRL, and AGP compared to PHY1.

Dual X-ray Absorptiometry (DXA). There was no significant effect of treatment observed in the average D42 body weight of birds using in DXA measurements, but there was a significant effect

of treatment ($P < 0.0001$) observed in D42 percent fat (Fig. 4.6-A), where all PFA treatments had lower percent fat than the AGP diet, and PHY2 and PHY3 had lower percent fat than the control. D42 percent lean (Fig. 4.6-B) also had a significant effect of treatment ($P < 0.0001$) in which AGP had lower percent lean than PHY1, PHY2, PHY3, and PHY4. PHY2 and PHY3 had higher percent lean than the CTRL. Within the PFA treatments, PHY3 had higher lean than PHY1 and PHY4.

Microbial Profiling

Results from D7, D14, and D42 microbial profiling of *Bacillus, Clostridium, Enterococcus,* and total bacterial counts using qRT-PCR were not significant and are reported in Appendix 2, Figure S4.7

Discussion

This study tested four different PFA treatments, of varying compositions, against a standard control diet and a diet using Stafac and Amprolium. A goal of this study was to determine if the PFAs used in a wheat diet could improve performance and gut health. Adding wheat to a diet can lead to more mucus production in the gut, and increase susceptibility to diseases like necrotic enteritis (Annett et al., 2002; Jia et al., 2009). There was variation in weekly performance and between the PFA diets. PFA additions to the diet improved different aspects of performance without significant impact on microbial profiles. Adding PFAs to the diet often led to similar results as adding Amprolium and Stafac. It is important to note that PHY1, PHY3, and PHY4 included Amprolium and a PFA mixture, but PHY2 consisted of a PFA mixture only.

During the starter and grower phases, the dietary supplements significantly influenced the birds' growth expressed as body weights, which were comparable by the end of the study. Interestingly

at 2 weeks of age, those birds fed the PHY1 and PHY2 mixtures had similar BW as the control birds, while those on the PHY3 and PHY4 diets weighed as much as the AGP birds. D14 BW was lower in AGP compared to the CTRL and some of the PHY diets. This may be due to the effect of Amprolium and Stafac inhibiting beneficial microbes that are needed for a healthy gut. These birds took longer to establish the normal microbiota, but were able to catch up in BW by the end of the trial. Antibiotics are more beneficial to a bird during a challenge, when pathogens would be inhibited. All the diets had similar BWs by D42, demonstrating that PFA had the same BW as both the CTRL and AGP, and although they were not higher than these treatments, they showed no sign of negative effect.

More effect of treatment was seen in results from average body weight gain (BWG) per bird. From D7-14, CTRL had higher BWG than the other treatments, while PHY4 was lower than CTRL and PHY2. During the grower phase, D14-28, PHY3 had significantly lower BWG than CTRL and AGP, while PHY4 was lower than CTRL, similar to the previous week's result. Although there was no significant difference in the finisher phase, cumulative results had an effect of treatment on BWG. From D0-14, CTRL had higher BWG, and at this point PHY2 had higher BWG than PHY4. In results from D0-28, diets PHY1, PHY2, and PHY4 had similar BWG to AGP, even though PHY1, PHY3, and PHY4 were lower than CTRL. Cumulatively, from D0-42, PHY1 and PHY4 evened out and had similar BWG to CTRL and AGP. PHY2 and PHY3 were lower than CTRL and AGP, but all the PFA treatments were significantly similar to each other, even if they varied from the CTRL and AGP.

Most significant effect of treatment on feed intake (FI) was seen towards the beginning of the trial. In the first week, D0-7, PHY2 had a trend towards higher FI than all other diets. This treatment also had lower FCR compared to treatments yet no improvement in BWG over other

treatments, suggesting better results from PHY1, PHY3, and PHY4 which contained Amprolium in addition to the PFA product in the diet. This difference may not be due to Amprolium however, since all PFA diets had similar FI to CTRL, which did not contain Amprolium. During week 2, from D7-14, the results were somewhat similar to those from the first week. Here, PHY1 and PHY2 had similar FI to CTRL and AGP, while PHY3 and PHY4 had lower FI compared to CTRL, AGP, and PHY2. By the end of the trial, and cumulatively from D0-42, there was no significant difference in FI. The PFAs did not have a negative impact on FI, but seemed to increase FI in one treatment during the first and second week, while reducing FI in other treatments during the second week.

FCR was impacted towards the beginning of the experiment, as well as cumulatively. From D0-7, PHY1, PHY3, and PHY4 had similar FCRs to the CTRL and AGP. PHY2 was worse than the CTRL, but similar to AGP. When analyzing D7-14, D14-28, and D28-42, there was no difference between treatments, however, a significant effect of treatment remained during cumulative analyses of data. From D0-14, PHY3 and PHY4 had significantly lower FCRs than AGP and PHY2. PHY2 remained high, but was not significantly different from CTRL or AGP. Cumulatively, in D0-28 and D0-42, the only difference was a higher FCR in PHY2 than any of the other five treatments. When considering the entire experiment, there was little difference in FCR between treatments, except for a higher ratio in PHY2. This treatment was the only PFA treatment that excluded Amprolium, so perhaps feeding the PFA with Amprolium in the other treatments kept FCR at levels closer to control and AGP. Feeding a PFA has been shown to lower, raise, or have no effect on FCR in other studies, perhaps due to the broad category of PFA compositions and feeding methods. Mountzouris and colleagues (2011) saw improved FCR in

PFA treatments compared to a negative control, and Brenes and Roura (2010) reported improved FCR due to lower FI but no change in bird weight gains.

Other studies have shown mixed results on broiler performance. Some studies tested essential oils on broiler performance and found no effects on growth parameters (Jang et al., 2007; Zhang et al., 2005; Botsoglou et al., 2002). Garcia and colleagues (2007) tested plant extracts and found the average daily gain (ADG) was similar to the NC and better than an antibiotic treatment, but FCR was lower compared to NC. In 2005, Jamroz and colleagues also saw no change in ADG or FCR in broilers fed phytogenic extracts, while Ertas and colleagues (2005) saw improvement in both ADG and FCR.

The results for D42 body composition using DXA showed significant effect of treatment in both percent lean and percent fat, but no effect in body weight between the treatments. For percent fat, all PFA treatments had lower amounts than the AGP diet. Additionally, PHY2 and PHY3 had lower percentages than CTRL. Corresponding to the percent fat results were the percent lean results. All PFA treatments had higher lean percentages than the AGP diet, and PHY2 and PHY3 had more lean than CTRL. There was no difference in percent fat within the four PFA treatments, but PHY3 had higher lean than either PHY1 or PHY4. Overall, there was improved body composition due to PFA supplementation, resulting in higher lean to fat ratios.

There were significant differences in mortality in the first and second weeks of the trials, with higher mortality in the AGP, PHY1, and PHY4 treatments during week one. Mortality percent did not exceed 3.9 percent for any given treatment, which is not unusual for broilers during the first seven days. During week two, PHY1 was higher than the other treatments, at 4.5%

mortality. The second half of the trial saw no significant differences across any treatments. Cumulatively, there were no significant differences in percent mortality, as expected.

Microbial profiles of the ileum were analyzed on D7, D14, and D42 of the experiment to observe potential differences in *Bacillus*, *Clostridium*, *Enterococcus*, and total bacteria counts. Results were reported using Ct (cycle threshold) means, and no significant effect of treatment was seen during any of the weeks. The PFA treatments did not seem to alter the observed microbial populations, in either a negative or positive way. Only a few microbial populations were chosen for analysis. The chicken microbiome consists of thousands of bacterial species (Pan and Yu, 2013). The major genera found in chickens are *Clostridium*, *Ruminococcus*, *Lactobacillus*, and *Bacteroides* (Wei et al., 2013). In the current study, there may have been differences in other populations that were not observed, or there may have been differences within species of the broader genus of *Bacillus*, *Clostridium*, or *Enterococcus*.

Adding a PFA supplement to broiler diets showed some significant differences in performance measures, but no difference in the observed microbial populations. Most of the PFA treatments had similar cumulative FCR to the CTRL and AGP diets. Body weight at D14 was better in PHY1 and PHY2 than AGP. Diets PHY1 and PHY4 had similar cumulative BWG results as both CTRL and AGP treatments. Feed intake was higher in the PFA treatments during the first week, and they had better body composition on D42, where the PFA treatments had higher percent lean to fat ratios. As a whole, the PFA supplementation did not have negative effects on performance or microbial profiles, and the treatments often improved some measurements during this study. Further research might include expanding the number of microbial populations measured. Sampling from different parts of the intestine, besides just the ileum, may also give more insight

into the enteric microbial population. Adding a disease challenge to this experiment would also be another way to test the effects of PFA supplementation on broiler performance.

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Table 4.1. Description of treatments.

Treatment	Description
1. NC	Control=basal (corn/wheat/SBM) diets [Starter, Grower, Finisher]
2. AGP	NC + Amprolium + AGP (Stafac) [Starter, Grower, Finisher]
3. PHY1	NC + Amprolium + Product 3 [Starter, Grower, Finisher]
4. PHY2	NC + Product 4 [Starter, Grower, Finisher]
5. PHY3	NC + Amprolium + Product 3 [Starter, Finisher] NC + Amprolium + Product 3 + Product 5 [Grower]
6. PHY4	NC + Amprolium + Product 3 [Starter, Grower] NC + Product 4 [Finisher]

Table 4.2. Composition of broiler diets during starter, grower, and finisher phases.

Item	Starter (DOH to D15)	Grower (D15 to D28)	Finisher (D28 to D42)
Ingredient, %			
Corn	31.28	33.24	36.60
Wheat	25.00	25.00	25.00
Soybean meal	22.56	18.74	13.44
Distiller's grain	8.00	9.00	10.00
Poultry by-product meal	7.00	7.00	7.00
Grease (yellow)	2.13	3.40	4.53
Limestone	1.43	1.20	1.04
Big Spring Mills vitamin and mineral premix*	0.63	0.63	0.63
Di-calcium Phosphate	0.50	0.50	0.50
L-Lysine	0.41	0.35	0.37
Salt	0.40	0.40	0.40
DL-Methionine	0.35	0.30	0.27
L-Threonine	0.15	0.11	0.09
Choline	0.14	0.11	0.11
Quantum Blue**	0.02	0.02	0.02
Total	100.00	100.00	100.00

*Cobalt (Min) 34 ppm; Copper (Min) 540 ppm; Iodine (Min) 134 ppm; Iron (Min) 6,750 ppm; Manganese (Min) 8,580 ppm; Zinc (Min) 6,500 ppm; Vitamin A (Min) 400,000 IU/lb; Vitamin D3 (Min) 134,000 ICU/lb; Vitamin E (Min) 100 IU/lb; Vitamin B12 (Min) 0.4 mg/lb; Menadione (Min) 70 mg/lb; Riboflavin (Min) 250 mg/lb; D-Pantothenic acid (Min) 368 mg/lb; Niacin (Min) 1,200 mg/lb; Choline (Min) 23,147 mg/lb.
** Phosphatase; AB Vista

Table 4.3. Primers used for qRT-PCR in microbial profiling.

Gene	Primer ID	Primer Sequence
Bacillus	005F	5' – TGGAGAGTTTGATCCTGGCTCAG – 3'
	531R	5' – TACCGCGGCTCGTGGCAC – 3'
C. perfringens	Clost_7_F	5' – ATGCAAGTCGAGCGA(G/T)G – 3'
	Clost_7_R	5' – TATGCGGTATTAATCT(C/T)CCTTT – 3'
Enterococcus	RT-chEntero2_F	5' – GATGCATAGCCGACCTCAGAA – 3'
	RT-chEntero2_R	5' – AGTTTGGGCCGTGTCTCAGT – 3'
Universal Bacteria	RT-chUni_F	5' – TCCTACGGGAGGCAGCAGT – 3'
	RT-chUni_R	5 – GGACTACCAGGGTATCTAATCCTGTT – 3'

Figure 4.1. Effect of PFAs on average body weight (BW) per bird of Ross male broiler chicks. Data are presented in kilograms using Least Square Means ± Standard Error Means. Significant difference is represented by bars lacking the same letter. There was a significant effect of treatment (P = 0.0013) on D14 (Fig. 4.1-A) and a significant difference (P = 0.0129) on D28 (Fig. 4.1-B). CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

Figure 4.1-A

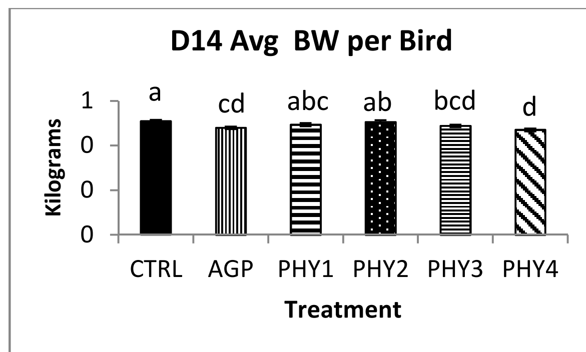


Figure 4.1-B

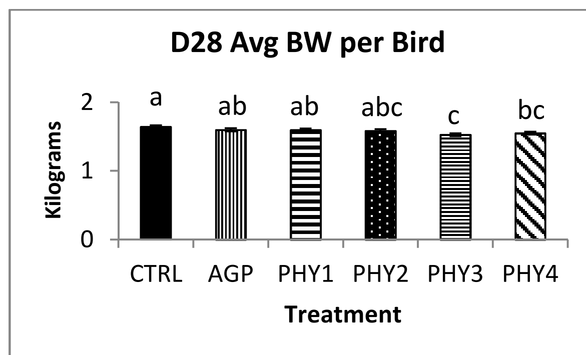


Figure 4.2. Effect of PFAs on weekly and cumulative body weight gain (BWG) per bird of Ross male broiler chicks. Data are presented in kilograms using Least Square Means ± Standard Error Means. Significant difference is represented by bars lacking the same letter. There was a significant effect of treatment (*P* = 0.0052) from D7-14 (Fig. 4.2-A), a significant effect (*P* = 0.0277) from D14-28 (Fig. 4.2-B), a significant effect (*P* = 0.0086) from D0-14 (Fig. 4.2-C), a significant effect (*P* = 0.0136) from D0-28 (Fig. 4.2-D), and a significant effect (*P* = 0.0156) from D0-42 (Fig. 4.2-E). CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

Figure 4.2-A

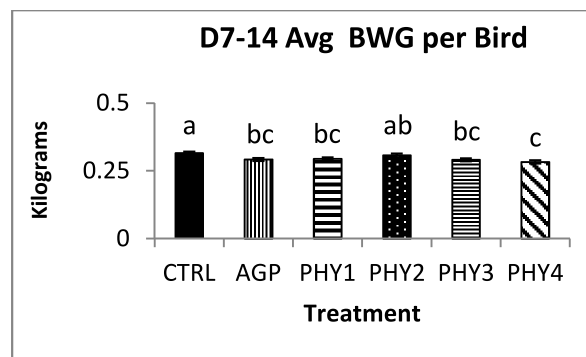


Figure 4.2-B

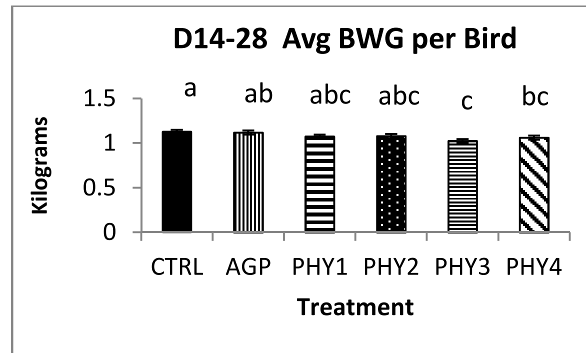


Figure 4.2-C

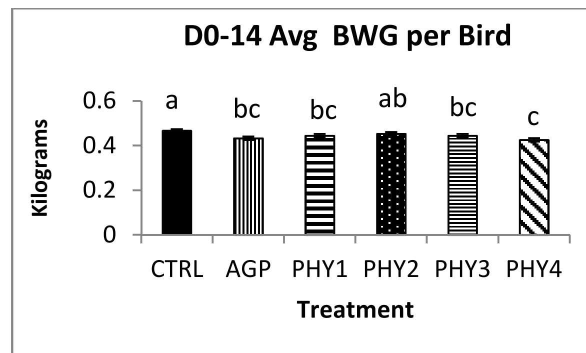


Figure 4.2-D

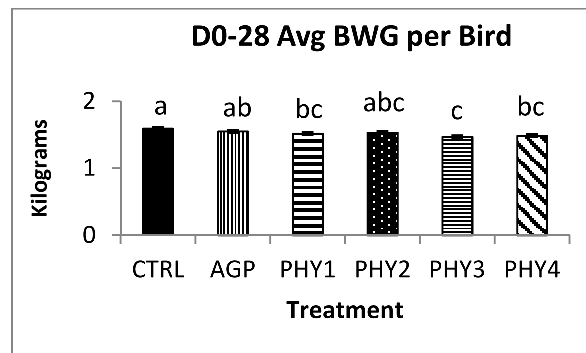


Figure 4.2-E

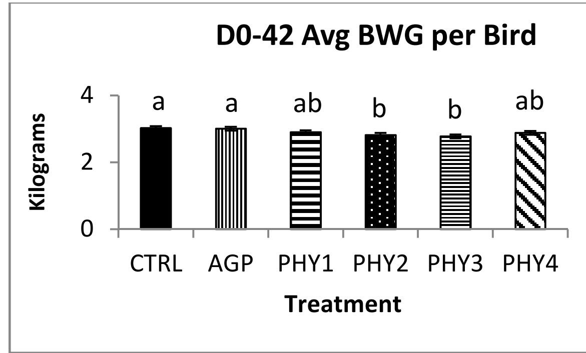


Figure 4.3. Effect of PFAs on weekly and cumulative feed intake (FI) per bird of Ross male broiler chicks. Data are presented in kilograms using Least Square Means ± Standard Error Means. Significant difference is represented by bars lacking the same letter. There was trend due to diet (P = 0.0738) from D0-7 (Fig. 4.3-A), and a significant effect of diet (P = 0.0009) from D7-14 (Fig. 4.3-B). CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

Figure 4.3-A

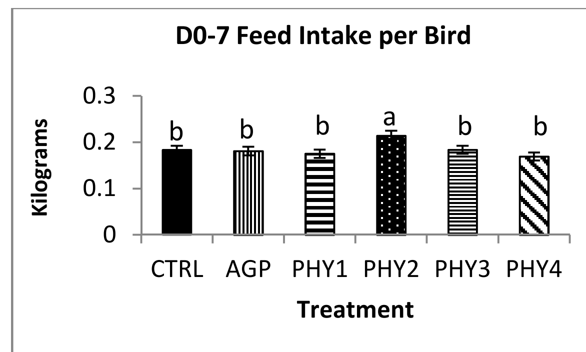


Figure 4.3-B

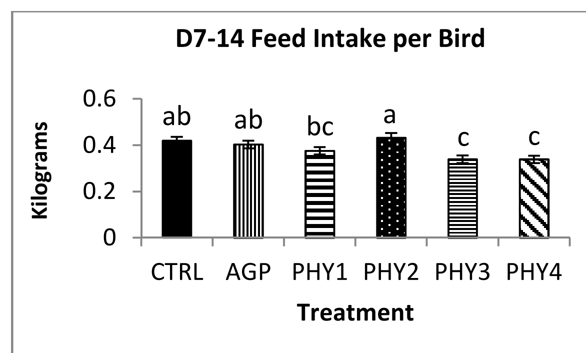


Figure 4.4. Effect of PFAs on feed conversion ratio (FCR) of Ross male broiler chicks. Data are presented as ratios using Least Square Means ± Standard Error Means. Significant difference is represented by bars lacking the same letter. There was a significant effect of treatment (*P* = 0.039) from D0-7 (Fig. 4.4-A), and from cumulative results, such as a significant difference (*P* = 0.0052) from D0-14 (Fig. 4.4-B), a significant difference (*P* = 0.0125) from D0-28 (Fig. 4.4-C), and a significant difference (*P* = 0.0023) from D0-42 (Fig. 4.4-D). CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

Figure 4.4-A

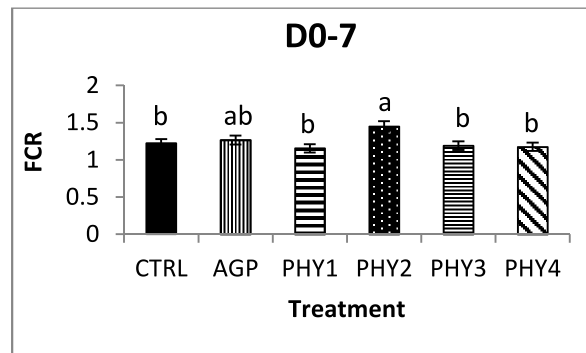


Figure 4.4-B

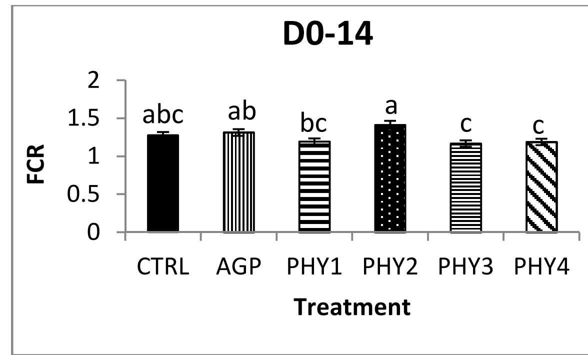


Figure 4.4-C

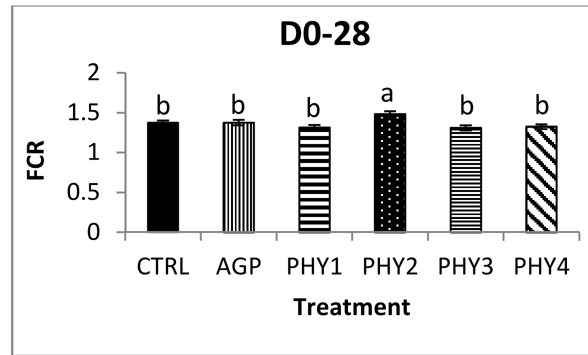


Figure 4.4-D

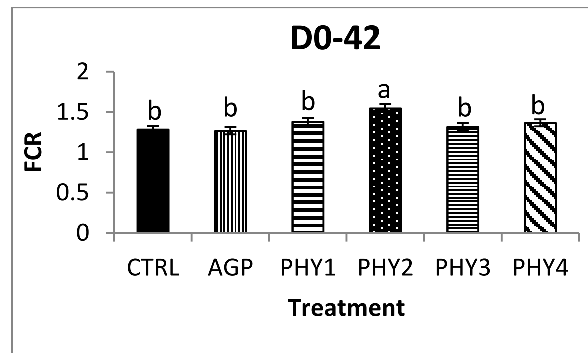


Figure 4.5. Effect of PFAs on percent mortality per treatment of male Ross broiler chicks. Data are presented as percent using Least Square Means ± Standard Error Means. Significant difference represented by bars lacking the same letter. There was a significant effect of treatment (*P* = 0.0475) on D0-7 (Fig. 4.5-A), a significant effect of treatment (*P* = 0.0349) on D7-14 (Fig. 4.5-B), and a significant effect (*P* = 0.0481) on cumulative mortality percentage from D0-28 (Fig. 4.5-C) . CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

Figure 4.5-A

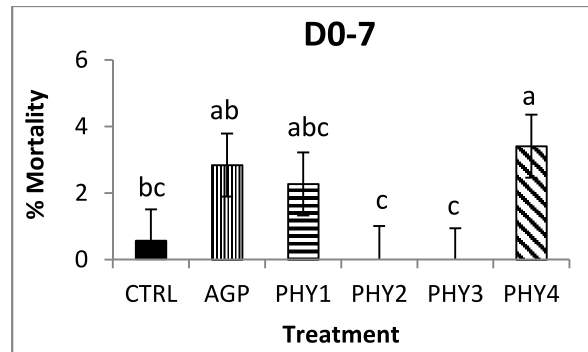


Figure 4.5-B

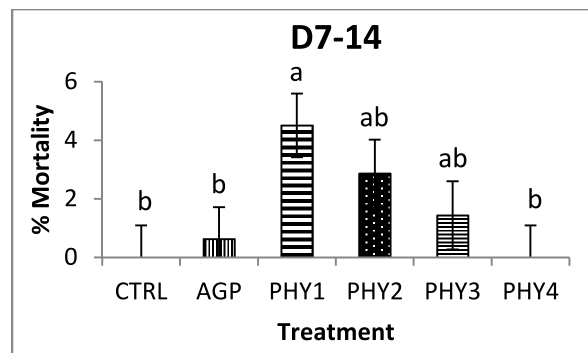


Figure 4.5-C

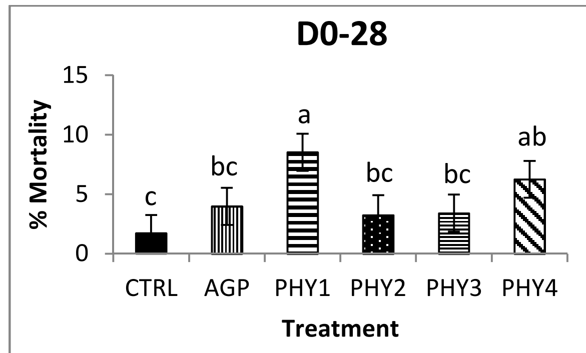


Figure 4.6. Effect of PFAs on D42 body composition per bird (percent fat or percent lean) on male Ross broiler chicks. Data are presented as percent using Least Square Means ± Standard Error Means. Significant difference represented by bars lacking the same letter. There was a significant effect of treatment ($P < 0.0001$) on D42 average percent fat per bird (Fig. 4.6-A) and a significant effect of treatment ($P < 0.0001$) on D42 average percent lean per bird (Fig. 4.6-B). CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

Figure 4.6-A

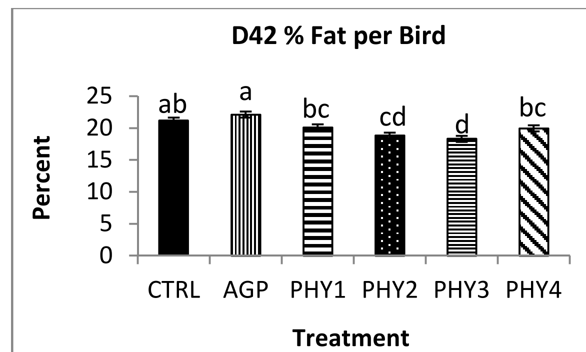
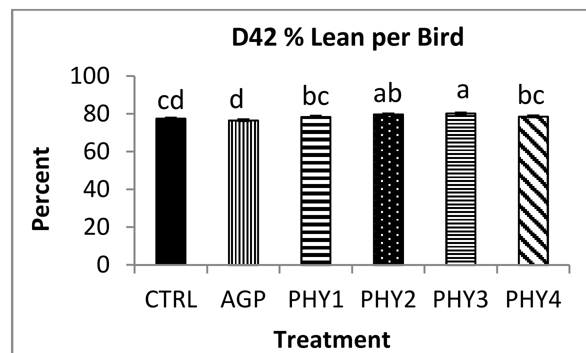


Figure 4.6-B



CHAPTER V

Epilogue

The importance of natural alternatives stems from the ban on prophylactic use of antibiotics in the European Union and increasing difficulty of use in the United States. There is much research on both probiotics and phytogenic feed additives, yet more is needed to continue studying the mechanisms of action, optimal blends of each product, and results from various studies that may or may not involve disease. This research indicates that feeding PFAs may improve poultry performance and that adding probiotics before and during a coccidiosis challenge had various benefits from supplementation in the diet from an early age.

Coccidiosis is among the most costly diseases to the poultry industry worldwide. This endemic parasite is difficult to control, and although mortality is less of an issue with this disease, the economic cost associated with extra feed consumption and slower growth are major aspects for continued research. Coccidiosis can be managed or treated through improved flock and litter management, use of coccidiostats, live vaccination, and addition of natural alternatives to feed and water.

Probiotics are intended to support healthy guts in broilers. Early establishment of these live microorganisms can create a healthy microbiome, preventing pathogens from colonizing through competitive exclusion. The probiotics may stimulate the immune system and prime it for response against disease. Results from this study showed that the low-dose and high-dose DFM products used in the first experiment provided some performance benefits to birds before and during a coccidiosis challenge. Although statistically not significant, there were trends toward improved body composition through higher lean to fat ratios in DFM-fed broilers, and perhaps

such effects could be better accentuated with more replication and extended growth period. The challenged birds fed a high-dose DFM diet had higher levels of beneficial *Bacillus* and *Enterococcus*, and lower levels of *Clostridium* in both high-dose and low-dose DFM fed chickens. It would be useful to carry out the trial to 42 days instead of 28 days, and add prebiotics to the mixture with the probiotics. Other analysis could be done with this type of study, such as short-chain fatty acid production and populations of immune cells. Many additional aspects could be explored using probiotics, and future studies will continue to study their positive effects in broilers.

The use of PFAs in feed may stimulate bird health and improve performance in a variety of ways. This broad category can include essential oils, herbs, and spices from sources such as oregano, cinnamon, thyme, sage, and citrus. They possess antioxidant and antimicrobial properties, may improve digestion and gut health, and improve immune function. In this study, the beneficial effects of PFA supplementation could be seen in some of the performance results. The birds eating diets containing PFAs had similar FCRs to the control and antibiotic growth promoter diets. Body weight and body weight gains were better in some of the PFA diets. Feed intake contrasted between the treatments in the beginning of the experiment, where one diet had higher FI, while other diets had lower FI, making it hard to draw conclusions about palatability based off of this study. The broilers fed PFAs had higher lean to fat ratios at the end of the experiment as well. Overall, supplementation had some beneficial effects, and no negative effects on broilers. In future studies, it would be useful to try these products in a disease setting. Another measurement to investigate would be nutritional aspects, such as short-chain fatty acid measurements, enzyme production and activation, and expression of nutrient transporters to further devise the mechanisms of action during PFA supplementation in the feed.

As a whole, the probiotics with a coccidiosis challenge provided useful results, and indicated some benefit in DFM supplementation. The addition of PFAs to the diet also showed improvements in performance. Further research will add to the information already known about these natural alternatives.

Appendix 1

Non-significant data associated with Chapter III

Table S3.1. Results of DFM diet and coccidiosis challenge interaction on average body weight per treatment in male Ross broiler chickens on a weekly basis. NC = control; DL = DFM low dose; DH = DFM high dose; PC = control plus challenge; DL+ = DFM low dose plus challenge; DH+ = DFM high dose plus challenge.

Average Body Weight (kg); Diet*Challenge Interactions		
	D21	**D28**
	P = 0.86	*P* = 0.84
	SEM = 0.016	SEM = 0.029
Diet	**LSMeans**	**LSMeans**
NC	0.65	1.04
DL	0.68	1.10
DH	0.68	1.07
PC	0.63	0.96
DL+	0.67	0.99
DH+	0.66	0.97

Table S3.2. Results of DFM diet and coccidiosis challenge interaction on average body weight gain (BWG) per bird per treatment of male Ross broiler chickens on a weekly and cumulative basis. NC = control; DL = DFM low dose; DH = DFM high dose; PC = control plus challenge; DL+ = DFM low dose plus challenge; DH+ = DFM high dose plus challenge.

	Average BWG per Bird (kg); Diet Interactions (D0-14) Diet*Challenge Interactions (D14-28)					
	D7-14	**D14-21**	**D21-28**	**D0-14**	**D14-28**	**D0-28**
	P = 0.07	P = 0.70	P = 0.28	P = 0.55	P = 0.49	P = 0.75
	SEM = 0.005	SEM = 0.014	SEM = 0.018	SEM = 0.007	SEM = 0.013	SEM = 0.016
Diet	**LSMeans**	**LSMeans**	**LSMeans**	**LSMeans**	**LSMeans**	**LSMeans**
NC	0.18	0.33	0.39	0.28	0.32	0.60
DL	0.19	0.34	0.42	0.30	0.34	0.64
DH	0.19	0.33	0.39	0.30	0.34	0.64
PC	0.18	0.32	0.34	0.27	0.31	0.58
DL+	0.18	0.34	0.31	0.29	0.34	0.63
DH+	0.19	0.31	0.32	0.30	0.31	0.61

Table S3.3. Results of DFM diet and coccidiosis challenge interaction on average feed intake (FI) per bird per treatment of male Ross broiler chickens on a weekly and cumulative basis. NC = control; DL = DFM low dose; DH = DFM high dose; PC = control plus challenge; DL+ = DFM low dose plus challenge; DH+ = DFM high dose plus challenge.

	Average FI per Bird (kg); Diet Interactions (D0-14) Diet*Challenge Interactions (D14-28)				
	D14-21 *P* = 0.14 SEM = 0.045	**D21-28** *P* = 0.82 SEM = 0.031	**D0-14** *P* = 0.76 SEM = 0.009	**D14-28** *P* = 0.31 SEM = 0.066	**D0-28** *P* = 0.42 SEM = 0.067
Diet	**LSMeans**	**LSMeans**	**LSMeans**	**LSMeans**	**LSMeans**
NC	0.63	0.73	0.42	1.37	1.79
DL	0.58	0.83	0.44	1.41	1.85
DH	0.60	0.84	0.48	1.44	1.92
PC	0.54	0.70	0.41	1.24	1.65
DL+	0.67	0.82	0.44	1.49	1.92
DH+	0.60	0.79	0.47	1.39	1.86

Table S3.4. Results of DFM diet and coccidiosis challenge interaction on feed conversion ratio (FCR) per treatment of male Ross broiler chickens on a weekly and cumulative basis. NC = control; DL = DFM low dose; DH = DFM high dose; PC = control plus challenge; DL+ = DFM low dose plus challenge; DH+ = DFM high dose plus challenge.

	Average FCR per Bird; Diet Interactions (D0-14) Diet*Challenge Interactions (D14-28)			
	D14-21 *P* = 0.32 SEM = 0.16	**D21-28** *P* = 0.08 SEM = 0.11	**D14-28** *P* = 0.11 SEM = 0.12	**D0-28** *P* = 0.0555 SEM = 0.057
Diet	**LSMeans**	**LSMeans**	**LSMeans**	**LSMeans**
NC	1.9	1.9	1.9	1.68
DL	1.7	2.0	1.9	1.64
DH	1.8	2.2	2.0	1.78
PC	1.7	2.1	1.9	1.68
DL+	2.0	2.6	2.3	1.90
DH+	1.9	2.4	2.2	1.84

Table S3.5. Results of DFM diet and coccidiosis challenge interaction on actual body weight (BW) per treatment of selected male Ross broiler chickens used for D28 Dual X-ray Absorptiometry (DXA). NC = control; DL = DFM low dose; DH = DFM high dose; PC = control plus challenge; DL+ = DFM low dose plus challenge; DH+ = DFM high dose plus challenge.

Avg. D28 DXA BW (kg) ; Diet*Challenge Interactions	
	D28
	P = 0.35
	SEM = 0.040
Diet	**LSMeans**
NC	0.99
DL	1.05
DH	1.02
PC	0.93
DL+	0.89
DH+	0.94

Table S3.6. Results of DFM diet and coccidiosis challenge interaction on mortality percentage per treatment of male Ross broiler chickens on a weekly and cumulative basis. NC = control; DL = DFM low dose; DH = DFM high dose; PC = control plus challenge; DL+ = DFM low dose plus challenge; DH+ = DFM high dose plus challenge.

Average %Mortality; Diet Interactions (D0-14) Diet*Challenge Interactions (D14-28)							
	D0-7	**D7-14**	**D14-21**	**D21-28**	**D0-14**	**D14-28**	**D0-28**
	P = 0.32	P = 0.73	P = 0.69	P = 0.14	P = 0.89	P = 0.11	P = 0.33
	SEM = 0.17	SEM = 0.32	SEM = 0.30	SEM = 0.22	SEM = 0.40	SEM = 0.33	SEM = 0.54
Diet	**LSMeans**	**LSMeans**	**LSMeans**	**LSMeans**	**LSMeans**	**LSMeans**	**LSMeans**
NC	0.5	0.5	0.1	0.1	1.0	0.3	0.9
DL	0.3	0.8	0.3	0.0	1.0	0.3	1.3
DH	0.5	0.4	0.6	0.0	0.9	0.6	2.5
PC	0.1	0.5	0.0	0.3	0.6	0.3	1.3
DL+	0.4	0.5	0.6	1.0	0.9	1.6	1.9
DH+	0.3	0.6	0.6	0.4	0.9	1.0	1.5

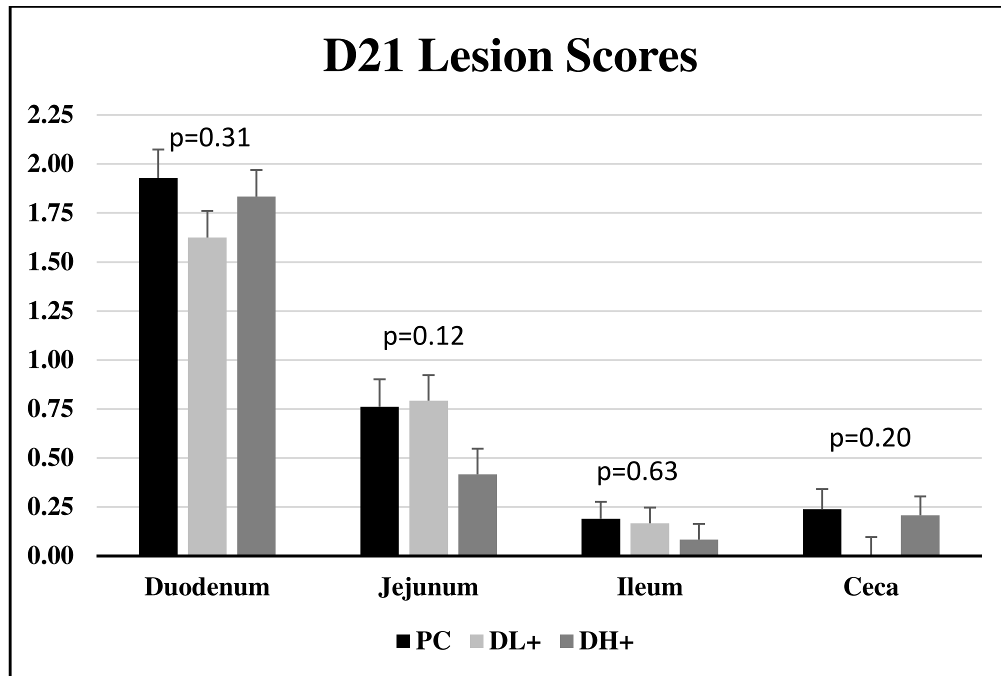
Table S3.7. Results of DFM diet and coccidiosis challenge interaction on Day 21 lesion scores in the duodenum, jejunum, ileum, and ceca of each challenged treatment of sampled male Ross broiler chickens. Results presented as LSMeans ± SEM. PC = control plus challenge; DL+ = DFM low dose plus challenge; DH+ = DFM high dose plus challenge.

Treatment	Duodenum	Jejunum	Ileum	Ceca
PC	1.9 ± 0.15	0.8 ± 0.14	0.19 ± 0.086	0.24 ± 0.103
DL+	1.6 ± 0.14	0.8 ± 0.13	0.17 ± 0.080	0.00 ± 0.096
DH+	1.8 ± 0.14	0.4 ± 0.13	0.08 ± 0.080	0.21 ± 0.096

Table S3.8. Results of DFM diet and coccidiosis challenge interaction on *Bacillus, Clostridium, Enterococcus,* and Universal microbial profiles in ileums of sampled male Ross broiler chickens. Results presented as LSMeans of Ct Means with Standard Error Means (SEM). NC = control; DL = DFM low dose; DH = DFM high dose; PC = control plus challenge; DL+ = DFM low dose plus challenge; DH+ = DFM high dose plus challenge.

	Treatment						
	NC	**DL**	**DH**	**PC**	**DL+**	**DH+**	***P* value**
D7 *Bacillus*	20.6±0.66	21.2±0.66	20.8±0.66	20.8±0.66	19.7±0.66	21.7±0.66	0.20
D7 *Clostridium*	35.2±5.33	34.1±3.07	36.2±2.01	36.1±1.88	31.7±1.88	34.3±1.88	0.88
D7 *Enterococcus*	31.2±0.87	30.1±0.93	29.1±0.87	27.1±0.93	25.0±0.87	27.1±0.87	0.21
D7 Universal	25.4±1.09	25.5±1.17	22.5±1.09	22.0±1.09	19.8±1.17	21.5±1.09	0.13
D14 *Bacillus*	22.7±0.88	23.2±0.94	22.7±0.88	23.2±0.88	22.2±0.88	23.3±0.88	0.60
D14 *Clostridium*	37.7±1.19	36.8±0.97	36.8±0.75	37.0±0.64	35.2±0.64	35.7±0.64	0.88
D14 *Enterococcus*	30.4±0.83	29.6±0.83	29.9±0.83	29.4±0.83	26.8±0.83	27.3±0.83	0.48
D14 Universal	32.6±1.41	30.2±1.41	29.8±1.41	30.0±1.41	22.3±1.41	24.1±1.41	0.17
D21 *Bacillus*	20.9±0.78	21.8±0.78	21.4±0.78	20.8±0.78	20.7±0.78	19.6±0.78	0.55
D21 *Clostridium*	33.4±3.38	30.6±4.14	36.7±3.38	28.9±4.14	37.7±4.14	34.9±2.62	0.35
D28 *Enterococcus*	32.3±0.80	32.0±0.80	31.0±0.80	29.3±0.80	28.2±0.80	28.8±0.80	0.62
D28 Universal	32.4±1.55	29.3±1.55	27.8±1.55	23.9±1.55	25.6±1.55	24.0±1.55	0.22

Figure S3.1. Results of DFM diet and coccidiosis challenge interaction on Day 21 lesion scores in the duodenum, jejunum, ileum, and ceca of each challenged treatment of sampled male Ross broiler chickens. PC = control plus challenge; DL+ = DFM low dose plus challenge; DH+ = DFM high dose plus challenge.



Appendix 2

Non-Significant data associated with Chapter IV

Table S4.1. Effect of PFAs on feed conversion ratio (FCR) of Ross male broiler chicks. Data are represented as Least Squares Means ± Standard Error Means. CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

	Average FCR; Trt Interactions		
	D7-14 $P = 0.066$	**D14-28** $P = 0.087$	**D28-42** $P = 0.159$
Diet	**LSMeans ± SEM**	**LSMeans ± SEM**	**LSMeans ± SEM**
CTRL	1.33 ± 0.055	1.56 ± 0.028	1.58 ± 0.048
AGP	1.36 ± 0.059	1.56 ± 0.030	1.46 ± 0.045
PHY1	1.23 ± 0.055	1.61 ± 0.030	1.48 ± 0.048
PHY2	1.37 ± 0.070	1.63 ± 0.030	1.58 ± 0.048
PHY3	1.16 ± 0.059	1.64 ± 0.030	1.43 ± 0.048
PHY4	1.20 ± 0.055	1.54 ± 0.030	1.48 ± 0.045

Table S4.2. Effect of PFAs on average body weight (BW) per bird of Ross male broiler chicks. Data are represented in grams as Least Squares Means ± Standard Error Means. CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

	Average BW per Bird (g); Trt Interactions		
	D0 *P* = 0.22	**D7** *P* = 0.26	**D42** *P* = 0.20
Diet	**LSMeans ± SEM**	**LSMeans ± SEM**	**LSMeans ± SEM**
CTRL	43.1±.0.50	194±2.7	3130±39
AGP	43.8±0.43	186±2.7	3094±39
PHY1	43.0±0.43	194±2.7	3046±39
PHY2	43.4±0.46	191±2.9	3023±42
PHY3	42.7±0.43	193±2.9	3003±39
PHY4	42.2±0.46	189±3.1	3017±39

Table S4.3. Effect of PFAs on average body weight gain (BWG) per bird of Ross male broiler chicks. Data are represented in grams as Least Squares Means ± Standard Error Means. CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

	Average BWG per Bird (g); Trt Interactions	
	D0-7	**D28-42**
	$P = 0.11$	$P = 0.27$
Diet	**LSMeans ± SEM**	**LSMeans ± SEM**
CTRL	150 ± 3.1	1429.35 ± 0.045
AGP	141 ± 3.1	1456.92 ± 0.045
PHY1	150 ± 3.1	1379.88 ± 0.045
PHY2	148 ± 3.1	1353.73 ± 0.052
PHY3	151 ± 3.1	1308.38 ± 0.045
PHY4	142 ± 3.1	1392.02 ± 0.045

Table S4.4. Effect of PFAs on average feed intake (FI) per bird of Ross male broiler chicks on a weekly or cumulative basis. Data are represented in kilograms as Least Squares Means ± Standard Error Means. CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

	Average FI per Bird (kg); Trt Interactions				
	D14-28 *P* = 0.321	**D28-42** *P* = 0.337	**D0-14** *P* = 0.330	**D0-28** *P* = 0.505	**D0-42** *P* = 0.202
Diet	**LSMeans ± SEM**	**LSMeans ± SEM**	**LSMeans ± SEM**	**LSMeans ± SEM**	**LSMeans ± SEM**
CTRL	1.740 ± 0.027	2.325 ± 0.068	0.604±0.047	2.374±0.069	4.698±0.099
AGP	1.738 ± 0.027	2.188 ± 0.068	0.584±0.050	2.275±0.069	4.463±0.099
PHY1	1.761 ± 0.025	2.155 ± 0.068	0.551±0.047	2.312±0.069	4.466±0.099
PHY2	1.767 ± 0.027	2.270 ± 0.072	0.461±0.050	2.228±0.073	4.498±0.105
PHY3	1.719 ± 0.025	2.140 ± 0.068	0.515±0.047	2.234±0.069	4.374±0.099
PHY4	1.690 ± 0.025	2.161 ± 0.068	0.508±0.047	2.197±0.069	4.358±0.099

Table S4.5. Results of PFA treatment on average body weight (BW) per treatment of selected male Ross broiler chickens used for D42 Dual X-ray Absorptiometry (DXA). Results presented in kilograms using LSMeans. CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

Avg. D42 DXA BW (kg); Trt*Challenge Interactions	
	D42 $P = 0.275$ SEM = 0.056
Diet	**LSMeans**
CTRL	2.985
AGP	3.016
PHY1	3.026
PHY2	2.910
PHY3	2.867
PHY4	2.926

Table S4.6. Effect of PFAs on percent mortality of Ross male broiler chicks on a weekly or cumulative basis. Data are represented in percent as Least Squares Means ± Standard Error Means. CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

	Average %Mortality per Treatment; Trt Interactions			
	D14-28 *P* = 0.500	**D28-42** *P* = 0.151	**D0-14** *P* = 0.121	**D0-42** *P* = 0.141
Diet	**LSMeans ± SEM**	**LSMeans ± SEM**	**LSMeans ± SEM**	**LSMeans ± SEM**
CTRL	1.389 ± 1.402	3.484 ± 2.213	0.568 ± 1.342	3.977 ± 2.245
AGP	0.735 ± 1.402	2.083 ± 2.213	3.409 ± 1.342	5.682 ± 2.245
PHY1	3.682 ± 1.402	3.768 ± 2.213	5.682 ± 1.342	11.364 ± 2.245
PHY2	0.794 ± 1.499	8.413 ± 2.366	2.597 ± 1.342	9.740 ± 2.400
PHY3	2.741 ± 1.402	9.441 ± 2.213	1.136 ± 1.342	10.795 ± 2.245
PHY4	3.773 ± 1.402	4.556 ± 2.213	3.409 ± 1.342	9.659 ± 2.245

Table S4.7. Results of PFA treatment on *Bacillus, Clostridium, Enterococcus,* and Universal microbial profiles in ileums of sampled male Ross broiler chickens. Results presented as LSMeans of Ct Means with Standard Error Means (SEM). CTRL = control, basal diet; AGP = basal diet with Amprolium and Stafac; PHY1 = basal diet, Amprolium, and PFA Product #3; PHY2 = basal diet with PFA Product #4; PHY3 = basal diet, Amprolium, and PFA Product #3 during all three feeding periods with the addition of Product #5 during the grower phase; PHY4 = basal diet, Amprolium and PFA Product #3 in starter and grower phases, and only basal diet plus PFA Product #5 in the finisher phase.

	Treatment						
	CTRL	**AGP**	**PHY1**	**PHY2**	**PHY3**	**PHY4**	***P* value**
D7 *Bacillus*	22.48±0.62	23.39±0.62	22.32±0.62	22.01±0.62	22.22±0.62	21.75±0.62	0.54
D7 *Clostridium*	32.57±3.35	34.74±2.90	33.24±3.35	32.05±2.90	33.88±2.90	36.13±2.90	0.93
D7 *Enterococcus*	32.90±0.31	32.37±0.31	32.59±0.31	32.50±0.31	33.00±0.31	32.84±0.31	0.65
D7 Universal	24.16±0.66	24.97±0.66	23.99±0.66	23.70±0.66	24.09±0.66	23.37±0.66	0.66
D14 *Bacillus*	24.26±0.81	24.33±0.81	26.43±0.81	24.16±0.81	24.34±0.81	23.32±0.81	0.17
D14 *Clostridium*	34.57±3.27	32.10±3.06	35.39±3.27	27.85±3.06	30.75±3.06	30.87±3.06	0.58
D14 *Enterococcus*	33.32±0.27	33.22±0.27	32.83±0.27	32.59±0.27	33.28±0.27	32.52±0.27	0.14
D14 Universal	23.76±0.78	25.35±0.78	25.12±0.78	22.98±0.78	24.11±0.78	23.35±0.78	0.21
D42 *Bacillus*	24.59±0.79	22.42±0.79	21.83±0.79	22.43±0.79	21.77±0.79	22.52±0.79	0.15
D42 *Clostridium*	35.63±1.28	34.42±1.28	35.17±1.28	34.30±1.28	35.23±1.28	35.12±1.28	0.98
D42 *Enterococcus*	32.07±0.32	31.60±0.32	31.28±0.32	31.97±0.32	31.75±0.32	32.13±0.32	0.41
D42 Universal	26.44±0.56	25.45±0.56	25.00±0.56	25.53±0.56	24.97±0.56	25.87±0.56	0.44