University of Alberta

Nutritional mitigation strategies for antibiotic free broiler production

by

Kim Phuoc Ton

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Animal Science

Agricultural, Food and Nutritional Science

Fall, 2013
Edmonton, Alberta

ABSTRACT

Nutritional strategies to mitigate the loss of broiler performance due to the elimination of prophylactic antibiotics were investigated, consisting of two nutrient density starter diets (HIGH: 3,025 kcal/kg and 23.9% CP; LOW: 2,858 kcal/kg and 22.3% CP), 25-OH-D3 (0 or 69 µg/kg), and bacitracin methylene disalicylate (BMD®110; 0.5g/kg). All groups received the same basal diet for grower (3,150 kcal/kg, 21.7% CP) and finisher phases (3,200 kcal/kg, 20.0% CP). On d 14, subsets of broilers (n=128) were randomly selected, and either injected with *Salmonella typhimurium* lipopolysaccharide, or remained non-injected (n=64). The LOW diet decreased breast yield, body weight, and feed efficiency. BMD reduced mortality and interleukin-1β mRNA expression. 25-OH-D3 increased expression of LPS-induced tumor necrosis factor alpha and inducible nitric oxide synthase mRNA in the non-LPS group. A necrotic enteritis outbreak could have increased inflammatory response in the 25-OH-D3 birds, and may have led to decrease breast yield in this group.

ACKNOWLEDGMENTS

There are so many people that I have had reason to thank during my life. I wish I could list everyone's name and thank each individually. It was my privilege to meet so many wonderful people since I came to Edmonton. I could not have reached this stage without their love and care. I cannot fully express how grateful I am to friends and my extended family in Canada.

First and foremost, I am in debt to my supervisor, Dr. Martin Zuidhof, for teaching me so much. Martin is an academic supervisor and also a philosopher to me. Martin has given me encouragement in my research, an opportunity to be a scientist, and especially the strength to join my wife in marriage. His slogan is "there are a thousand ways to do something". This is true, but it was Martin who helped me choose the best way.

A huge thank you to Dr. Doug Korver and his research group, especially Dr. Jennifer Saunders-Blades for her work on the innate immune assays. I truly appreciate all of your patience with me. Dr. Korver gave me so much input and relentlessly trained me on academic writing. I will always remember the feeling whenever I received your email for paper corrections. I was nervous, but so excited.

I would like to express my sincere gratitude to Dr. Carolyn Fitzsimmons and Dr. Francois Paradis for teaching me how to perform an RT-PCR experiment. Dr. Paradis is very skilled in lab techniques. I greatly appreciate his instruction on how to perform lab work properly. His patience and support guided me through devastating moments when I made mistakes in the lab.

To achieve this thesis, I received a great deal of support and help from staff, technicians, and students at The Poultry Research Center. Many thanks to Dulal Paul and Paul Elaho for their hard work and care of the research animals. I greatly appreciate for the supports from Dr. Irene Wenger and Thania Moraes. Special thanks to Soledad Urrutia who I consider as my guru. Ms. Urrutia guided me through every obstacle presented to me when I first came to Canada. I hardly finished my writing without the assistance of Julia Sepkowski-Cook, a wonderful friend, for English tutor.

I would like to thank my girlfriend for agreeing to be my wife and giving birth to Kyle Ton. Special thanks to my newborn son who has kept me awake at night to write this thesis. My grandparents and my parents have always supported me in my study. My grandparents have expected to live healthy as long as they can just to see me with a degree on my return.

Last but not least, I would like to thank The Alberta Livestock Meat Agency for financial support for this project.

TABLE OF CONTENTS

CHAPTER 1: Nutritional mitigation strategies for antibiotic free broilers..... 1

1.1 INTRODUCTION 1

1.2. LITERATURE REVIEW 3

1.2.1 A brief overview of poultry production in dominant countries and continents 3

1.2.2 A brief overview of the importance of broiler production in Canada ... 4

1.2.3 Prophylactic antibiotic use and its benefits to poultry industry 4

1.2.4 Chicken innate immunity 6

1.2.4.1 Overview of chicken innate immunity..... 6

1.2.4.2 Heterophils 7

1.2.4.3 Macrophages 10

1.2.4.4 Reactive nitrogen intermediates..... 12

1.2.5 Nutritional factors that potentially affect chicken immunity 13

1.2.5.1 Cholecalciferol 13

1.2.5.2 Early nutrition 15

1.2.5.3 Protein for broiler chicks during the starting phase 15

1.2.5.4 Energy for broiler chicks at starting phase..... 16

1.2.6 Impact of nutritional supplementations on gastrointestinal ecology... 17

1.3 Research application and research objectives..... 18

1.4 Approach..... 20

1.5 REFERENCES 22

CHAPTER 2: Effect of early nutrition, 25-hydroxy vitamin D_3 and bacitracin methylene disalicylate on broiler performance 33

2.1 INTRODUCTION 33

2.2 MATERIALS AND METHODS 35

2.2.2 Experimental design. 35

2.2.3 Stocks and management. 36

2.2.4 Data collection. 36

2.2.5 Economic analysis 37

2.2.6 Statistical analysis. 37

2.3 RESULTS AND DISCUSSION 38

2.3.1 Broiler Performance. 38

2.3.1.1 Body weight. 38

2.3.1.2 Feed conversion ratio. 40

2.3.1.3 Mortality. 41

2.3.2 Meat yield. 43

2.4 REFERENCES 50

CHAPTER 3: Effect of early nutrition, 25-hydroxy vitamin D_3 and bacitracin methylene disalicylate on small intestinal morphology of broilers. 54

3.1 INTRODUCTION 54

3.2 MATERIALS AND METHODS 55

3.2.1 Experimental design. 55

3.2.2 Stocks and management. 55

3.2.3 Tissue sampling and morphological examination. 56

3.2.4 Statistical analysis. 57
3.3 RESULTS AND DISCUSSION 57
3.3.1 Villus height. 57
3.3.3 Crypt depth. 60
3.3.4 Villus height to crypt depth ratio. 61
3.4 REFERENCES 67
CHAPTER 4: Effect of early nutrition, 25-hydroxy vitamin D_3 and bacitracin methylene disalicylate on broiler innate immunity 70
4.1 INTRODUCTION 70
4.2 MATERIALS AND METHODS 72
4.2.1 Experimental design. 72
4.2.2 Stocks and management. 73
4.2.3 Blood sampling. 73
4.2.4 Bacterial killing capacity. 74
4.2.5 Phagocytosis. 74
4.2.6 Plasma nitric oxide assay. 75
4.2.7 Statistical analysis. 76
4.3 RESULTS AND DISCUSSION 77
4.3.1 Bacterial killing capacity and phagocytosis. 77
4.3.2 Nitric oxide concentration. 79
4.4 REFERENCES 83

CHAPTER 5: Effect of early nutrition, 25-hydroxy vitamin D_3, and bacitracin methylene disalicylate on the expression of pro-inflammatory cytokines and inducible nitric oxide synthase mRNA 87

5.1 INTRODUCTION 87

5.2 MATERIALS AND METHODS 89

5.2.1 Experimental birds, diets and tissue sampling. 90

5.2.2 RNA extraction. 90

5.2.3 RNA integrity. 92

5.2.4 DNase treatment and cDNA transcription. 93

5.2.5 Primer design. 94

5.2.6 Selection of house-keeping genes. 95

5.2.7 Identification of the best combination of house-keeping genes. 95

5.2.8 Quantitative polymerase chain reaction of target genes. 96

5.2.9. Normalizing RT-PCR data 96

5.2.10 Statistical analysis. 97

5.3 RESULTS AND DISCUSSION 98

5.4 REFERENCES 107

CHAPTER 6: Research synthesis 111

6.1 SUMMARY 111

6.2 SYNTHESIS 113

6.3 REFERENCES 118

LIST OF TABLES

Table 2.1 Composition and analysis of experimental diets. Control diets contained similar amount of nutrients, but without supplementation of BMD and HyD premix at starter phase 456

Table 2.2 Effect of starter nutrient density, 25-OH-D_3 and antibiotic on body weight of mix-sexed broiler chickens from 0 to 40 d 467

Table 2.3 Effect of starter nutrient density, 25-OH-D_3 and antibiotic on feed conversion ratio (FCR) 478

Table 2.4 Effect of starter nutrient density, 25-OH-D_3 and antibiotic on cumulative mortality 49

Table 2.5 Effect of starter nutrient density, 25-OH-D_3 and antibiotic treatments on broiler carcass yield at d 40 50

Table 3.1 Effect of starter nutrient density, 25-OH-D_3 and BMD on the ileal morphology of broiler chickens at d 7, d 21, d 35 64

Table 4.1 Effect of early nutrient density, 25-OH-D_3 and antibiotic on bacterial killing capacity and phagocytosis 812

Table 4.2 Effect of early nutrition, 25-OH-D_3 and antibiotic on nitric oxide concentration in plasma of chicks 823

Table 5.1 Primers used in real-time PCR plus accession numbers of the genomic sequence 10405

Table 5.2 PCR efficiencies of each amplified gene used for real-time 10506

Table 5.3 Effect of LPS, starter nutrient density, 25-OH-D_3 and antibiotic on IL-1β, ... 10607

LIST OF FIGURES

Figure 3.1 Villus height and crypt depth in the ileum of broiler chicks at d 7 of age, fed Low nutrient (22% CP and 2,858 kcal/kg) with 25-OH-D_3 (A), or Low nutrient without 25-OH-D_3 (B). (1) villus height, (2) crypt depth, (3) apical width, (4) basal width, magnification: X 10. 65

Figure 3.2 Dietary nutrient density x 25-OH-D_3 2-way interaction for ratio of villus height to crypt depth at d 7 ($P< 0.05$). Broiler chicks were fed either a HIGH (3,025 kcal/kg; 23.9% CP), or LOW (2,858 kcal/kg; 22.3% CP) nutrient density diet from 0 to 14 d, with 0 or 69 µg/kg of 25-OH-D_3. 66

Figure 3.3 Dietary nutrient density x 25-OH-D_3 x BMD 3-way interaction observed for ratio of villus height to crypt depth at d 21 ($p<0.05$). From 0 to 14 d of age, broiler chicks were fed either a HIGH (24% CP and 3,025 kcal/kg) or LOW (22% CP and 2,858 kcal/kg) nutrient density diet, with 0 or 69 µg/kg 25-OH-D_3, and 0 or 0.5g/kg Bacitracin Methylene Disalicylate (BMD® 110)..... 67

LIST OF ABBREVIATIONS

1, 25$(OH)_2D_3$ - 1, 25-dihydroxycholecalciferol

25-OH-D_3 - 25-dihydroxycholecalciferol

ACTB - Beta-actin

AGP - Antibiotic growth promoters

BMD - Bacitracin methylene disalicylate

BW – Body weight

CaBP - Calcium binding protein receptors

CP – Crude protein

D - day

E. coli - *Escherichia coli*

EEF1A2 - Eukaryotic translation elongation factor 1 alpha 2

FAO - United Nations Food and Agriculture Organization

FCR - Feed conversion ratio

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

H6PD - Hexose-6-phosphate dehydrogenase

HIGH – Early high nutrient diet (3,025 kcal/kg; 23.9% CP)

IL-1β – Interleukin 1 beta

IL-6 – Interleukin 6

iNOS – Inducible nitric oxide synthase

IU – International unit

L:D – Light:dark

LITAF - LPS-induced TNF-alpha factor

LOW- Early low nutrient diet (2,858 kcal/kg; 22.3% CP)

LPS- Lipopolysaccharide

ME – Metabolize energy

NE - Necrotic enteritis

NO – Nitric oxide

OECD - Organization for Economic Co-operation and Development

PAMP - Pathogen-associated molecular patterns

PCR – Polymerase chain reaction

PRR - Pattern recognition regions

qRT-PCR – Quantitative real time polymerase chain reaction

RNI - Reactive nitrogen intermediates

ROS - Reactive oxygen species

RPL19 - Ribosomal protein L19

SEM – Standard error of the mean

SRBC - Sheep red blood cells

TLR - Toll like receptors

TNF-α - Tumor necrosis-like factor alpha

UBB - Ubiquitin B

VDR - vitamin D receptors

Vitamin D_3 – Cholecalciferol

wk- week

CHAPTER 1: Nutritional mitigation strategies for antibiotic free broilers

1.1 INTRODUCTION

Poultry consumption is increasing due to high protein value of poultry meat, reasonable price, and convenience for further processed products. Scanes (2007) reported that world chicken production increased by 53% between 1995 and 2005. To grow broilers intensively, which often involves a high stocking density, prophylactic antibiotics have been used in the broiler industry to prevent gastro-intestinal diseases (Torok et al., 2011), increase feed efficiency and promote growth of broilers (Stutz and Lawton, 1984; Miles et al., 2006). However, prophylactic antibiotic use has been banned by the European Union, and the pressure from activists and consumers will likely lead to a decrease in non-therapeutic use in other countries (Casewell et al., 2003). According to the World Health Organization (2002), prophylactic antibiotic use in the long run could increase bacteria with resistant genes to the therapeutic antibiotics, and could pose a potential threat to human if there was a bacteria cross-contamination between human and livestock. Necrotic enteritis (NE) could cause a substantial negative economic impact in broiler flocks when antibiotic feed additives are not used (Hofacre et al., 2003). NE is caused by *Clostridium perfringens*, and causes approximately a $2 billion annual loss to the poultry industry internationally due to high mortality and poor feed efficiency (Skinner et al., 2010).

Nutrition, genetic composition and flock management are considered as the three major determinants of disease susceptibility in broilers (Kogut, 2009). Among

those three components, nutritional regimes can be used to modulate the resistance of broilers to infectious diseases (Korver et al., 1997; 1998). Hofacre et al. (2003) reported that broilers fed competitive exclusion cultures and mannan-oligosaccharide could mitigate mortality when broilers were challenged with *Clostridium perfringens*. Cholecalciferol (vitamin D_3) was found to increase calcium metabolism, reproduction, bone quality and immune function in chickens (Chou et al., 2009). The active form of vitamin D_3 is 1, 25-dihydroxycholecalciferol (1, 25$(OH)_2D_3$), a hormone that is essential for several internal processes, including calcium metabolism and cellular differentiation (Norman, 1990). This hormone is produced in the kidney using 25-hydroxycholecalciferol (25-OH-D_3), which is a metabolite of vitamin D_3 produced in the liver (Holick et al., 1976). Recently, 25-OH-D_3 has been identified as a component in cellular immunity (Cantorna et al., 2004; Chou et al., 2009). Several studies have shown that 25-OH-D_3 can modulate the immune response, via vitamin D receptors on the surface of immune cells (Bhalla et al., 1983; Praslickova et al., 2008).

In the next few pages, I will attempt to summarize the current understanding of chicken immunity and its relationship with early dietary nutrient densities, 25-OH-D_3, or prophylactic bacitracin methylene disalicylate. The effects of nutrition and those supplementations on broiler health and productivity will be explored using broiler performance, cellular immunity, gut morphology and pro-inflammatory cytokine gene expressions as indicators.

1.2. LITERATURE REVIEW

1.2.1 A brief overview of poultry production in dominant countries and continents

World poultry production increased dramatically (158%) between 1985 and 2005, attributable to greater demand in Western countries for high protein, low fat, low carbohydrate food products and increased consumption in emerging markets such as Brazil and Thailand (Agriculture and Agri-Food Canada, 2006). Asia and North America have the highest poultry production in the world, at 32% and 26.9% of global production, respectively. They are followed by South and Central America with 20.4%. Europe and Africa accounted for 15.9% and 4.5%, respectively (Agriculture and Agri-Food Canada, 2006).

Asian countries have potentials to increase chicken production and consumption, due to the rapid development and industrialization of these countries, and to demographic and lifestyle changes (Scanes, 2007). It is likely that an improvement in living condition increases demand for a better source of protein, and chicken meat provides good source of protein with reasonable price. The Organization for Economic Co-operation and Development (2006) expected that Latin America would maintain the highest export growth, an estimated 5.25% increase between 2006 and 2015. Overall, poultry meat has great potential to increase its market share due to high quality broiler products, economical prices and short harvesting time (Goodwin, 2005).

1.2.2 A brief overview of the importance of broiler production in Canada

The Canadian poultry industry operates under a supply management system, by which the production is tightly controlled through a quota system based on the balance of supply and demand (Agriculture and Agri-Food Canada, 2006). Broiler production was estimated to increase by 1.63% (17,000 metric tonnes) between 2009 and 2011 (Statistics Canada, 2012a). According to the Global Agriculture Information Network (2011), Canada would expect to import 76,950 metric tonnes of chicken meat in 2012, increased by 246 metric tonnes from 2010. Income generated by sales of chicken meat was $2.25 billion in 2011, increased by 14.1% from 2010 (Statistics Canada, 2012a). In comparison with other animal protein sources, broiler meat has highest consumption per capita, approximately 31.22 kg in 2011, which was unchanged since 2004 (Statistics Canada, 2012b). The ethnic diversification of the Canadian population is one of the key factors for the increased demand for broiler meat because this type of meat is likely not restricted to most ethnic groups and religions (Research and Markets, 2011).

1.2.3 Prophylactic antibiotic use and its benefits to poultry industry

Antibiotics were approved as animal feed additives without prescription by the United States Food and Drug Administration in 1951 (Torok et al., 2011). A significant growth-promoting effect was observed in broilers fed with zinc bacitracin and salinomycin, compared to broilers fed no antibiotic diet (Engberg et al., 2000). The author explained that the supplementation of antibiotics not only decreased lactobacilli and coliform bacteria in the ileum of broilers, but also

increased the activities of amylase and lipase in pancreas homogenates (Engberg et al., 2000). Guban et al. (2006) reported that prophylactic antibiotic reduced *Lactobacillus salivarius* populations, a main causative agent of increasing deconjugated bile salts in the proximal ileum of broilers, subsequently reduced fat absorption. Antibiotics inhibit the RNA transcription of microbes, or disrupt the cell wall of invading bacteria, impairing bacterial proliferation (Torok et al., 2011). Miles et al. (2006) reported that prophylactic antibiotics not only suppressed the proliferation of bacteria, but also saved energy from tissue maintenance and increased nutrient absorption, achieved by reducing intestinal cell proliferation, mucosae, and thinner lamina propia. Prophylactic antibiotics had positive effects on environmental impact due to increased nutrient absorption in the intestinal tract by reducing the nutrient competition between microflora and the host (Russell, 2003). Furuse and Yokota (1985) reported that prophylactic antibiotics reduced the level of nitrogen, phosphorus, and residual nutrients that are excreted in the environment.

Despite the positive effect of prophylactic antibiotics on nutrient retention, their use has been contended. According to Roe and Pillai (2003), antibiotic-resistant microorganisms from animals could be transferred to humans through several pathways such as: natural water, irrigation water, drinking water, and foods. Because of those potential risks, regulation 1831/2003 of the European Parliament determined that only coccidiostats and histomonostats could be used as feed additives after December 31, 2005 (European Food Safety Authority, 2003). From then onward, antibiotics administered to animals required a

prescription from a veterinarian, and they could be only used therapeutically. Prophylactic use of ionophores, a popular and effective anticoccidial substance, would be disallowed after December 31, 2012 (European Food Safety Authority, 2003). However, the ban has not been put in place by July 31, 2013.

Bacitracin methylene disalicylate (BMD) is among several prophylactic antibiotics that have been commonly supplemented in poultry feed to enhance growth performance and control enteric diseases (Chapman and Johnson, 2002). BMD targets both gram positive and negative bacteria (Presscott et al., 2000). The mode of action of BMD is to interfere with the formation of peptidoglycan strands and inhibit protein synthesis of bacteria (Torok et al., 2011). BMD weakens the cell wall of bacteria by interfering with its synthesis (Torok et al., 2011). Therefore, BMD can suppress the growth of bacteria in the gastrointestinal tract, producing a reduction in the thickness of the mucosa (Stutz et al., 1982). Engberg et al. (2000) reported fewer coliform bacteria in the ileum of broilers fed BMD (20 ppm) compared to the control broilers. Roura et al. (1992) reported that prophylactic antibiotics promoted growth of broilers by preventing innate immunologic stress, subsequently conserving energy for broilers to partition toward growth.

1.2.4 Chicken innate immunity

1.2.4.1 Overview of chicken innate immunity

Similar to mammals, chickens have developed an immune system which can protect the chickens from invading pathogens (Bar-Shira et al., 2006). An

efficient immune response would help the birds to clear the pathogens at the least energetic cost (Swaggerty et al., 2005). The immune system of chickens is composed of innate immunity and humoral immunity. Heterophils, monocyte-macrophages and natural killer cells are among the main leukocytes. These cells are the primary response which protects the body from invading pathogens (Kogut, 2009). Heterophils, monocytes and macrophages secrete cytokines (hormone-like substances) to communicate between cells (Swaggerty et al., 2005). These phagocytic cells involves in killing bacteria by a process of adhesion, chemotaxis, and phagocytosis (Kogut et al., 1998). The role of phagocytic cells is crucial during an infection since they can limit the proliferation of invading bacteria to a level that can be eliminated by the antibodies of the humoral system (Kogut et al., 1998). The humoral immunity of chickens consists of B and T cells, which are derived from the bursa of Fabricius and lymphatic tissues, respectively (Bar-Shira et al., 2006). Humoral immunity plays an important role in immune homeostasis of the host. For example, Bar-Shira et al. (2006) reported that T helper cells, and cytotoxic T-cells, two sub-populations of T cells could initiate and suppress specific immune response, respectively. The humoral response is initiated when stimulant agents are primed, and presenting the internalized bacteria on the surface of the antigen presenting cells.

1.2.4.2 Heterophils

Heterophils in the chicken are equivalent to neutrophils in mammals (Kogut, 2009). Although they have several functions in common with neutrophils,

heterophils have some characteristics. For example, heterophils do not produce an effective oxidative burst during bacterial killing process (Penniall and Spitznagel, 1975). Heterophils are important in the defensive system of chicks at post-hatch, when acquired immunity is still under-developed (Kogut et al, 1994). The function and efficiency of heterophils can be inheritable, but there are variations in heterophil effciency among chicken lines (Swaggerty et al., 2003). Heterophil mRNA gene expression can be used as a biomarker to indicate the pro-inflammatory response of chickens. For example, Swaggerty et al. (2006) reported that heterophils collected from chicken lines that were resistant to *Salmonella* had greater pro-inflammatory gene expression than a susceptible line.

Heterophils are able to engulf and kill invading bacteria via a sequence of mechanisms known as phagocytosis, the final step of which is destruction of the bacterium via degranulation, a primary bacterial killing mechanism in chickens (Swaggerty et al., 2003). Heterophils can kill bacteria via oxygen-independent mechanisms, involving cationic antimicrobial peptides. This non-oxidative antimicrobial activity was first reported by Brune and Spitznagel (1973). When heterophils undergo degranulation as a response to inflammatory stimulation, they release enzymes and antimicrobial peptides. Three isolated cationic antimicrobial peptides, called gallinacins (Gal-1 α, Gal-1, Gal-2), from chicken heterophils were found to be homologs to the human β-defensins (Harwig et al., 1994). Two other cationic antimicrobial peptides (chicken heterophil peptides 1 and 2) were also extracted from chicken heterophils (Evans et al., 1994). Mageed

et al. (2008) reported that mammal cationic antimicrobial peptides have a wide spectrum of bacterial killing ability, and even are able to target cancer cells. The interactions between the positive charge of antimicrobial peptides and negative charge of bacteria cause damage to the bacterial membrane (Scott et al., 2000; Gudmundsson and Agerberth, 1999). Some cationic antimicrobial peptides are capable of neutralizing endotoxin lipopolysaccharides (LPS), a component of the outer membrane of Gram-negative bacteria (Scott et al., 1999). Cationic antimicrobial peptides reduce the production of *IL-1β, IL-6* and *TNF-α* cytokines that are secreted by a macrophage when it is stimulated by LPS, and subsequently reduce the inflammatory response (Mageed et al., 2008).

Pattern recognition receptors (PRR) are present on the surface of heterophils, and recognize pathogen-associated molecular patterns (PAMP) on bacteria (Farnell et al., 2003a). Toll like receptors (TLR), scavenging receptors, complement receptors, C-type receptors and integrins are all types of PRR (Yilma et al., 2005). Each TLR is responsible for distinct types of bacteria. For example, TLR4 can recognize the PAMP on gram negative bacteria (Farnell et al, 2003b), whereas TLR5 can target PAMPs on flagellin (Iqbal et al., 2005).

Heterophils are activated by chemotactic mediators such as cytokines and chemokines (Kogut et al., 1995). Cytokines are mediator proteins, secreted by immune cells to inform and orchestrate the immune response (Kaiser et al., 2000). Chemokines are also mediator proteins but they have a limited scope of action, targeting only the surrounding cells (Hamal et al., 2010). Interleukin 1 beta (*IL-1β*), interleukin 6 (*IL-6*) and tumor necrosis-like factor alpha (*TNF-α*)

are the three major chemotactic mediators of the pro-inflammatory response, and are activated following the stimulation of lipopolysaccharides (Rautenschlein et al., 1999; Zhang et al., 2012). Rautenschlein et al. (1999) reported that chicken *TNF-α* has a different structure from *TNF-α* in mammals, but they share similar functions such as inducing macrophage nitric oxide (NO) production and causing maturity in a transformed chicken macrophage cell line (MQ-NCSU). The up-regulation of those cytokines results in recruiting more lymphocytic cells to the infected site (Kaiser et al., 2000). Monocyte and macrophage populations invaded the infected area approximately 3 hours after the cells were challenged with *Salmonella enteritidis* (Van Immerseel et al., 2002).

1.2.4.3 Macrophages

Macrophages are derived from bone marrow stem cells through three steps. Initially, bone marrow differentiates into promonocytes, which become monocytes under catalysis from colony stimulating factors (Qureshi et al., 1990; Heggen-Peay et al., 2000). Monocytes then enter the blood stream where they become a major phagocytic cellular component of the innate immune system. It takes approximately 6 days for the promonocytes to mature and established in various tissues since they are derived from bone marrow tissue (Stabler et al., 1994). Macrophages can be found in the blood stream and in various body tissues (Qureshi, 1990). Macrophages acquire chemotactic, adherence, phagocytic capacities, as well as the ability to produce cytokines (Heggen-Peay et al., 2000). These functions can be modulated by nutrition (Kogut 2009),

genetics (Qureshi, 1990), and a variety of environmental factors (Qureshi et al., 1993).

The primary characteristic of macrophages is their capacity for phagocytosis. The pattern recognition molecules on the surface of the macrophage can bind to bacteria via mannose or fructose present on the surface of bacteria (Qureshi, 1990). Scavenger receptors and Fc receptors on the macrophages are also involved in cleaning up apoptotic cells or internalizing pathogens (Kogut, 2001). Bacteria can be internalized, and then carried into the cytoplasm in the form of a phagosome, where it is fused with lysosome enzyme to become a phagolysosome (Rieger et al., 2010). This process leads to the degradation of internalized bacteria, and the presentation of the processed bacterial peptides to humoral cells such as B or T lymphocytes. This is the route by which the macrophages become the bridge between innate immunity and humoral immunity.

Macrophages are able to produce nitric oxide, a toxic chemical that is detrimental to bacteria (Qureshi, 1990). MQ-NCSU chicken macrophages are able to produce NO when stimulated with LPS (Rautenschlein et al., 1999). Dil and Qureshi (2002) reported that the NO production increased almost 3 times when macrophages were exposed to LPS. NO is released via the process of converting L-arginine to L-citruline (Qureshi, 1990), and NO production could be enhanced by increasing the amount of L-arginine in the diet (Leshchinsky and Klasing, 2001). Macrophage phagocytic functions could be also modulated by nutritional strategies (Kogut, 2009). For example, Aslam et al. (1998) reported

that phagocytic capacity of macrophages was reduced in broiler chicks fed deficient in vitamin D_3. Konjufca et al. (2004) reported that broilers fed 110 or 220 mg/kg of vitamin E had greater phagocytic activities compared to broilers fed 16 mg/kg of vitamin E at 3 weeks of age.

1.2.4.4 Reactive nitrogen intermediates

Reactive nitrogen intermediates (RNI) are one of four important metabolite categories that determine the progress of immune response (Qureshi et a., 1993). The others are: the reactive oxygen species (Golemboski et al., 1990a), the ecosanoids (Golemboski et al., 1990b) and the xenobiotics (Lorr et al., 1992). RNIs are capable of killing bacteria, causing significant local or systemic biological effects (Li et al., 2009). RNIs consist of NO, NO_2 and NO_3 and are the major category of macrophage metabolites (Marletta *et al.,* 1988). Qureshi (1990) reported that RNIs are produced during the process of converting L-arginine to L-citruline by the nitric oxide synthase enzyme (*iNOS*). The production of NO depends on the amount of arginine intake from the diet (Sung and Dietert, 1994). After being activated by immunogens, macrophages can release the pro-inflammatory cytokines *IL-1β, IL-6*, and innate antimicrobial *iNOS* cytokines (Kaiser et al., 2012). Chang et al. (1996) indicated that LPS is able to stimulate a significant NO concentration, but repeated exposure of chicken cells to LPS significantly reduced NO concentration.

In chickens, the *iNOS* gene has successfully been cloned and sequenced, and there was approximately 70.4% similarity to the mammalian *iNOS* gene (Lin et al., 1996). NO is produced after reactive oxygen species (ROS) are triggered,

and it requires the activation of the *iNOS* (Qureshi et al., 1993). NO is a neurotransmitter (Knowles et al., 1998), a vasoactive compound which is able to bring more blood to the infected site (Moncada et al., 1991; Bermudez, 1993), an anti-parasitic (Liew et al., 1990) and a destructive agent to tumor cells (Keller et al., 1990). Other studies also indicated that NO is involved in apoptosis processes (Albina et al., 1993) and immunoregulation (Mills, 1991; Denham and Roland, 1992).

1.2.5 Nutritional factors that potentially affect chicken immunity

1.2.5.1 Cholecalciferol

Cholecalciferol (Vitamin D_3) is one of two popular forms of vitamin D, a fat soluble vitamin that most animals are able to produce by absorbing ultraviolet rays from sunlight. Vitamin D_3 is the main source of vitamin D required by chickens because vitamin D_2 can only perform approximately 3% of the biological activity of vitamin D_3 in chickens (Sunde et al., 1978). Lund and DeLuca (1966) reported that vitamin D_3 needs to be converted to its hormone-like chemical to be functional. The transformation of vitamin D_3 to 25(OH)-D_3 in the liver is catalyzed by cytochrome P450 (Soares et al., 1995). Then this metabolite migrates to the kidney, where it can be converted to the steroid hormone 1, 25$(OH)_2D_3$ by 25-hydroxy-D_3-1α-hydroxylase (Norman , 1990).

Vitamin D_3 is fat soluble, and absorbed with the aid of conjugated bile salts; however, the absorption capacity may be limited in young chickens due to the presence of *Lactobacillus salivarius* populations (Guban et al., 2006). The proximal jejunum has been observed as the main absorption area for vitamin D_3

and 25-OH-D_3 in chickens (Bar et al., 1980). Wasserman et al. (1968) reported that calcium binding protein receptors (CaBP), one of proteins in the intestinal wall rely on vitamin D for the optimal intestinal absorption of calcium. CaBP can be found in a variety of chicken cells, including shell gland, kidney, brain, blood, pancreas, and bone. By binding to vitamin D binding protein, vitamin D_3 is able to circulate in the blood (Norman, 1990).

Vitamin D_3 can also be attached by cells and tissue via vitamin D receptors (VDR), which are present on the surface of many cell types of chickens (Norman, 1990). McCarthy et al. (1983) reported that 1,25-dihydroxy vitamin D_3, the active form of vitamin D increased cell proliferation and differentiation in human. VDR are found on antigen presenting cells, B cells and T cells (Norman, 1990). These immune cells are capable of secreting 1-α-hydroxylase to convert 25-OH-D_3 to 1, 25$(OH)_2D_3$, the most active form of vitamin D (Blunt et al., 1968). 1, 25$(OH)_2D_3$ has been shown to promote the differentiation of monocyte precursors towards monocytes, macrophages, and aid antigen presentation function of monocytes (Manolagas et al., 1985; Prowedini et al., 1986). The presence of VDRs on a variety of cells suggests a high demand of vitamin D during the host development (Bhalla, 1983; Praslickova et al., 2008). Supplementation of 25-OH-D_3 in the diet increased the number of innate immune cells, enhancing the maturity of phagocytic cells; however, it simultaneously suppressed the proliferation of phagocytic cells (Chou et al., 2009). Chou et al. (2009) postulated that reduced of cell-mediated response could indicate a premature shift from innate immunity (Th1 cells) to humoral

immunity (Th2 cells). Shanmugasundaram and Selvaraj (2012) reported that vitamin D_3 can modulate both innate immunity and adaptive immunity.

1.2.5.2 Early nutrition

Early high nutrient density diet could increase body weight and feed efficiency of broilers, and have residual effect on growth performance at the finisher phase as well. Quentin et al. (2005) suggested that high nutrient density in the starter diet had carry effects until d 40 of age, and supported the birds to express their growth potential. For example, Quentin et al. (2005) reported that breast development was positively correlated with high nutrient density in the starter diet (3,121 kcal/kg ME and 22.9% CP vs. 2,902 kcal/kg ME and 20.9% CP). Providing broiler chicks a good start at the first 14 d of life could increase the viability and performance of broilers at later stages (Nitsan et al., 1991a). The immaturity of the digestive tract and particularly the insufficient production of digestive enzymes could be a factor impairing digestion of broiler chicks (Nitsan et al., 1991b). Guban et al. (2006) reported that broiler chicks could not digest and absorb dietary lipid efficiently due to the limitation of the conjugated bile salt, caused by the bile salt hydrolase activity from *Lactobacillus salivarius* populations.

1.2.5.3 Protein for broiler chicks during the starting phase

Because body proteins are constantly being synthesized and degraded (Zhang et al., 2002), it is postulated that fast growing of broilers would require higher dietary amino acids than slow growing strain. Dozier et al. (2009) reported that high-yielding broilers are more responsive to a greater level of sulfur amino

acids. High dietary amino acids are correlated with high level of muscle satellite cell expressions during the first week post-hatch (Halevy et al., 2003; Velleman et al., 2010). Powell et al. (2013) reported that *pectoralis major* satellite cell mitotic activity was significantly increased when the *pectoralis major* satellite cells were cultured with 30/96 mg/L of Met/Cys, compared to cells that were cultured with 7.5/24, 3/9.6 or 1/3.2 mg/L of Met/Cys. Skeletal muscle mRNA expression of chicks was seen to increase when broiler chicks were fed high crude protein diet associated with high dietary amino acids (Collin et al., 2003). Birds fed low protein density required higher feed intake to meet protein requirements (Dairo et al., 2010). Growth, feed conversion, and carcass yield of broilers can be affected by dietary nutrient density (Quentin, 2005). Suitable nutrition levels are required for both proliferation and differentiation of breast muscle satellite cells, and affect subsequent breast muscle building (Powell et al., 2013).

1.2.5.4 Energy for broiler chicks at starting phase

Broilers fed low dietary energy had a higher feed conversion ratio, and lower body weight compared to broiler received higher dietary energy levels (Leeson et al., 1996; Plumstead et al., 2007). However, if a diet has high energy in relationship to its protein content, abdominal fat pad weight increases (Summers et al., 1992). Thus, dietary energy should be formulated in balance with protein level to optimize growth rate and meat yield. The dietary energy provided to broilers comes from carbohydrate, fat and protein. Leeson et al. (1996) reported that chickens are able to meet energy requirement by adjusting feed intake.

However, Plumstead et al. (2007) reported that broiler chickens at d 21 were inefficient in adjusting feed intake when broilers were fed dietary levels ranging from 3,000 to 3,200 kcal/kg ME. Hosseini-Vashan et al. (2010) reported that a dietary energy of 3,200 kcal/kg increased nutrient intake, carcass characteristics, and broiler performance, compared with broilers fed a dietary energy of 2,800 kcal/kg in the starter phase. Although the primary sources of dietary energy do not provide the building blocks for protein synthesis, they do contribute the energy required to make proteins (Plumstead et al., 2007). Birds fed diets high in energy relative to protein, have increased fat pad weight, while meat accretion is proportional to the density of protein relative to energy level (Summers et al., 1992).

1.2.6 Impact of nutritional supplementations on gastrointestinal ecology

Broiler chicks at hatch have a sterile gastrointestinal tract, and microbial colonization of the chicken gut starts immediately after hatch (Geyra et al., 2001). Apajalahti et al. (2001) reported that ileum and cecum of broiler chicks could contain approximately 10^8 and 10^9 of bacterial cells per gram of digesta, respectively. Within 3 days, the bacterial density could increase by 10 times, compared to the first day posthatch, and remain relatively stable during the following 30 days. Gut microflora affects the nutrient absorption capacity in broiler chicks by increasing the intestinal cell turn over, resulting from the colonization of the microflora. The increased cell turnover could increase thickness of mucosa and the rate of digesta passage, increasing nutrient requirements. Engberg et al. (2000) reported that intestinal microbes increased

nutritional requirements and reduced nutrient absorption efficiency in fast growing broilers, compared to germ-free broilers. Defining what is an ideal microflora for the intestinal tract of broilers still remains a puzzle (Leusink et al., 2010). The natural microflora once established tends to be very stable; however, this establishment can be affected by diet, diseases and environmental factors (Apajalahti et al., 2001).

Nutrition influences the diversity of the gut microflora (Bjrrum et al., 2005). Several products, including live bacteria, prebiotics, organic acids, trace minerals, enzymes, herbs and spices, as well as antibiotics have been considered as methods to manipulate the gut microflora (Hofacre et al., 2003). Geyra et al. (2002) reported that nutrition can change the development of the crypt, the cavity between two adjacent villi, where enterocytes migrate up to the existing villus structure. Prophylactic BMD and yeast product (comprising dehydrated distiller grains, torula yeast, fermented *Aspergillus oryzae*, and *Bacillus subtilis*) increased ileal maturation, measured by the level of intestinal alkaline phosphatase activity (Fasina and Thanissery, 2011). Chou et al. (2009) reported that broilers fed with 69 µg (2,760 IU) of 25-OH-D_3 increased the ratio between villus height and crypt depth, which resulted in a higher absorption area in the proximal ileum of broiler chicks, compared with chicks fed 3,000 IU of vitamin D_3 in the starter diet.

1.3 Research application and research objectives

Necrotic enteritis caused by *Clostridium perfringens* remains a main threat to the poultry industry. As prophylactic antibiotic use has already been banned in

several countries, it is necessary to find alternatives to help broiler chickens defend against intestinal infectious diseases, particularly necrotic enteritis.

The overall goal of the current research was to investigate the effects of early nutrition density, 25-OH-D_3, and the possible interaction among them with BMD on the performance of broilers, gut morphology, *ex vivo* innate immune response and pro-inflammatory gene expression when chicks were undergoing an inflammatory response when challenged with *Salmonella typhimurium* lippopolysacharide. These goals were archived in this thesis by testing the following hypotheses:

1) It was hypothesized that supplemental early nutrition density, 25-OH-D_3 and BMD would influence performance and meat yield of broilers.

This hypothesis is addressed in Chapter 2, by investigating the performance of broilers, including body weight, carcass yield and feed conversion ratio.

2) It was hypothesized that supplemental early nutrition, 25-OH-D_3 and BMD would change the intestinal morphology of broilers.

This hypothesis is addressed in Chapter 3, by evaluating ileal morphology (villus height, villus width, crypt depth, ratio villus height:crypt depth) of broilers at d 7, d 21 and d 35 of age.

3) It was hypothesized that supplemental early nutrition density diets, 25-OH-D_3 and BMD would influence the innate immune response of broiler chicks when they were undergoing an inflammatory response.

This hypothesis is addressed in Chapter 4, by measuring several aspects of innate immunity, including *ex vivo* bacterial killing capacity, phagocytosis, and the concentration of NO when chicks were challenged with *Salmonella typhimurium* lippopolysaccharide.

4) It was hypothesized that supplemental early nutrition diets, 25-OH-D_3 and BMD would alter the expression of pro-inflammatory genes that were extracted from spleen tissues when chicks were undergoing an inflammatory response.

This hypothesis is addressed in Chapter 5, by investigating the expression of pro-inflammatory cytokine mRNA including interleukin-1 beta (*IL-1β*), LPS-induced tumor necrosis factor alpha (*LITAF*), and innate immune related *iNOS* when broiler chicks were injected *Salmonella typhimurium* lippopolysacharide.

1.4 Approach

A broiler experiment was performed using Ross 708 (3,200 chicks) at the Poultry Research Centre. Each pen contained 100 mixed-sex chicks at a stocking density of 10 birds/m^2. A 20L:4D photoperiod was used, and all temperature and ventilation management followed commercial primary breeder recommendations. A 2 x 2 x 2 factorial arrangement was used, with two feeding strategies included 2 nutrient density starter diets (HIGH: 3,250 kcal/kg and 22% CP; LOW: 2,858 kcal/kg and 21% CP), and 25-OH-D_3 supplementation (0 or 69 µg/kg of feed), and bacitracin methylene disalicylate (BMD® 110: 0 or 0.5 g/kg of feed). From d 14 onward, the two levels of 25-OH-D_3 and BMD were continued, while all groups received the same basal diet for grower (3,150

kcal/kg, 21.71% CP) and finisher phases (3,200 kcal/kg, 20.03% CP). Individual body weights of all birds were recorded at 0, 14, 28, 35, and 40 d. At d 35, 8 birds per pen were randomly selected, wing banded, and processed to determine yield. Prior to processing, feed and water were withheld from the birds to prevent fecal contamination of carcasses. Feed intake was measured at these points in time, and feed conversion ratios were calculated (CHAPTER 2). At 7, 21, and 35 d of age, an additional 4 birds per treatment were humanely euthanized for histological examination of ileal tissues to quantify villus characteristics (CHAPTER 3).

To determine the innate immune response of broiler chicks, 20 birds per treatment (n=160) were selected at d 13 of age. Half were injected intra-abdominally with 3 mL of 100 µg/mL lipopolysaccharide (LPS) to produce an immune response; the other half served as controls. Three hours after injection with LPS, 2 mL blood samples were collected for innate immune function assay including *ex vivo* bacterial killing assay, phagocytosis and nitric oxide assay (CHAPTER 4). After blood collection, the injected and control birds were humanely euthanized to collect spleen tissue samples to evaluate treatment effects on expression of mRNA pro-inflammatory cytokines including *IL-1β*, *LITAF*, and innate immune related *iNOS* (CHAPTER 5).

1.5 REFERENCES

Agriculture and Agri-Food Canada, 2006. Profile of the Canadian poultry industry. http://www.agr.gc.ca/poultry/prindc2006_e.pdf. Accessed July 2013.

Albina, J. E., S. Cui, R. B. Mateo, and J. S. Reichner. 1993. Nitric oxide-mediated apoptosis in murine peritoneal macrophages. J. Immunol. 150: 5080-5085.

Apajalahti, J.H., A. Kettunen, M. R. Bedford, and W. E. Holben. 2001. Percent G+C profiling accurately reveals diet-related differences in the gastrointestinal microbial community of broiler chickens. Appl. Environ. Microbiol. 67: 5656-5667.

Aslam, S. M., J. D. Garlich, and M. A. Qureshi. 1998. Vitamin D deficiency alters the immune responses of broiler chicks. Poult. Sci. 77:842-849.

Bar, A., M. Sharvit, D. Noff, S. Edelstein, and S. Hurwitz. 1980. Absorption and excretion of cholecalciferol and of 25-hydroxycholecalciferol and metabolites in birds. J. Nutr. 110:1930-1934.

Bar-Shira, E., and A. Friedman. 2006. Development and adaptations of innate immunity in the gastrointestinal tract of the newly hatched chick. Dev. Comp. Immunol. 30:930-41.

Bermudez, L. E. 1993. Differential mechnism of intracellular killing of *Mycobacterium avium* and *Listeria monocytogenes* by activated human and murine macrophages: the role of nitric oxide. Clin. Exp. Immunol. 91:277-281.

Bhalla, A. K., E. P. Amento, T. L. Clemens, M. F. Holick, and S.M. Krane. 1983. Specific high-affinity receptors for 1, 25-dihydroxyvitamin D_3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. J. Clin. Endocrinol. Metab. 57:1308–1310.

Bjerrum, L., A. B. Pedersen, and R. M. Engberg. 2005. The influence of whole wheat feeding on *Salmonella* infection and gut flora composition in broilers. Avian Dis. 49:9–15.

Blunt, J. W., H. F. DeLuca, H. K. Schnoes. 1968. 25-hydroxycholecalciferol. A biologically active metabolite of vitamin D_3. Biochemistry. 7:3317-3322.

Brune, K., and J. K. Spitznagel. 1973. Peroxidaseless chicken leukocytes: isolation and characterization of antibacterial granules. J. Infect. Dis. 127: 84-94.

Cantorna, M. T., Y. Zhu, M. Froicu, and A. Wittke. 2004. Vitamin D status, 1,25-dihydroxyvitamin D_3 and the immune system. Am. J. Clin. Nutr. 80:1717S-1720S.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52:159-161.

Chang, C. C., C. C. McCormick, A. W. Lin, R. R. Dietert, and Y. J. Sung. 1996. The inhibition of nitric oxide synthase gene expression *in vivo* and in vitro by repeated doses of endotoxin. Am. J. Physiol. 271:539-548.

Chapman, H., and Z. Johnson. 2002. Use of antibiotics and roxarsone in broiler chickens in the USA: analysis for the years 1995 to 2000. Poult. Sci. 81:356-364.

Chou, S. H., T. K. Chung, and B. Yu. 2009. Effects of supplemental 25-hydroxycholecalciferol on growth performance, small intestinal morphology, and immune response of broiler chickens. Poult. Sci. 88: 2333-2341.

Collin A, V. M. B. Moraes, P. Van As, V. M. Darras, M. Taouis, E. Decuypere, and J. Buyse. 2003. Effects of dietary macronutrient content on energy metabolism and uncoupling protein mRNA expression in broiler chickens. Br. J. Nutr. 90:261-269.

Dairo, F. A. S, Adesehinwa, A. O. K., Oluwasola and J. A. Oluyemi. 2010. High and low dietary energy and protein levels for broiler chickens. Afr. J. Agric. Res. 5:2030-2038.

Denham, S., and I. J. Rowland. 1992. Inhibition of the reactive proliferation of lymphocytes by activated macrophages: the role of nitric oxide. Clin. Exp. Immunol. 87:157-162.

Dil, N., and M. A. Qureshi. 2002. Differential expression of inducible nitric oxide synthase is associated with differential Toll-like receptor-4 expression in chicken macrophages from different genetic backgrounds. Vet. Immunol. Immunopathol. 84: 191-207.

Dozier, W. A. III, A. Corzo, M. T. Kidd, P. B. Tillman, and S. L. Branton. 2009. Digestible lysine requirements of male and female broilers from fourteen to twenty-eight days of age. Poult. Sci. 88:1676-1682.

Engberg, R. M., M. S. Hedemann, T. D. Leser, and B. B Jensen. 2000. Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. Poult. Sci. 79:1311-1319.

European Food Safety Authority. 2003. European legislation on animal nutrition. ec.europa.eu/food/food/animalnutrition/feedadditives/legisl_en.htm. Accessed Apr. 2013.

Evans, E. W., G. G. Beach, J. Wunderlich, and B. G. Harmon. 1994. Isolation of antimicrobial peptides from avian heterophils. J. Leukocyte Biol. 56:661-665.

Farnell, M. B., T. L. Crippen, H. He, C. L. Swaggerty, and M. H. Kogut. 2003a. Oxidative burst mediated by toll like receptors (TLR) and CD14 on avian heterophils stimulated with bacterial toll agonists. Dev. Comp. Immunol. 27:423-429.

Farnell, M. B., H. He, and M. H. Kogut. 2003b. Differential activation of signal transduction pathways mediating oxidative burst by chicken heterophils in response to stimulation with lipopolysaccharide and lipoteichoic acid. Inflammation. 27:225-231.

Fasina, Y. O., and R. R. Thanissery. 2011. Comparative efficacy of a yeast product and bacitracin methylene disalicylate in enhancing early growth and intestinal maturation in broiler chicks from breeder hens of different ages. Poult. Sci. 90: 1067-1073.

Furuse, M., and H. Yokota. 1985. Effect of the gut microflora on chick growth and utilization of protein and energy at different concentrations of dietary protein. Br. Poult. Sci. 26:97-104.

Geyra, A., Z. Uni, and D. Sklan. 2001. Enterocyte dynamics and mucosal development in the posthatch chick. Poult. Sci. 80:776-782.

Geyra, A., Z. Uni, O. Gal-Garber, D. Guy, and D. Sklan. 2002. Starving affects CDX expression during small intestinal development in the chick. J. Nutr. 132:911-917.

Global Agriculture Information Network. 2011. Canada poultry annual. http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Poultry%20and%20Products%20Annual_Ottawa_Canada_09-17-2010.pdf. Accessed May 2013.

Golemboski, K. A., S. E. Bloom, R. R. Dietert. 1990a. Dynamics of avian inflammatory response to cross-linked dextran. Changes in avian blood leukocyte populations. Inflammation. 14:31-40.

Golemboski, K., J. Whelan, S. Shaw, J. Kinsella, and R. Dietert, 1990b. Avian inflammatory macrophage function: shifts in arachidonic acid metabolism respiratory burst and cell surface phenotype during the response to Sephadex. J. Leuk. Biol. 48:495–501.

Goodwin, H. L. 2005. Location of production and consolidation in the processing industry: the case of poultry. J. Agric. Appl. Econ. 32:339-346.

Guban, J., D. R. Korver, G. E. Allison, and G. W. Tannock. 2006. Relationship of dietary antimicrobial drug administration with broiler performance, decreased population levels of *Lactobacillus salivarius*, and reduced bile salt deconjugation in the ileum of broiler chickens. Poult. Sci. 85: 2186-2194.

Gudmundsson, G. H., and B. Agerberth. 1999. Neutrophil antibacterial peptides, multifunctional effector molecules in the mamalian immune system. J. Immunol. Methods. 232:45-54.

Harwig, S. L., K. M. Swiderek, J. N. Kokryakov, L. Tan, T. D. Lee, E. A. Panyutich, G. M. Aleshina, O. V. Shamova, and R. I. Lehrer. 1994. Gallinacins: cysteine-rich antimicrobial peptides of chicken leukocytes. FEBS Lett. 342:281-285.

Heggen-Peay, C. L., M. A. Qureshi, F. W. Edens, and H. J. Barnes. 2000. Alterations in macrophage-produced cytokine and nitrite associated with poult enteritis and mortality syndrome. Avian Dis. 44:69-65.

Hofacre, C. L., T. Beacorn, S. Collet, and G. Mathis. 2003. Using competitive exclusion, mannan-oligosaccharide and other intestinal products to control necrotic enteritis. J. Appl. Poult. Res. 12:60-64.

Holick, M. F., L. A. Baxter, P. K. Schraufrogel, T. E. Tavela, and H. F. DeLuca. 1976. Metabolism and biological activity of 24,25-dihydroxyvitamin D_3 in the chick. J. Biol. Chem. 251:397-402.

Hosseini-Vashan, S.J., A. Golian, Gh. Motaghinia, M. Namvari and M. Hamedi. 2010. Comparison of growth performance and carcass characteristics of broiler chickens fed diets with various energy and constant energy to protein ratio. J. Anim. Vet. Adv. 9:2565-2570.

Iqbal, M., V. J. Philbin, G. S. K. Withanage, P. Wigley, R. K. Beal, M. J. Goodchild, P. Barrow, I. McConnell, D. J. Maskell, J. Young, N. Bumstead, Y. Boyd, and A. L. Smith. 2005. Identification and functional characterization of chicken toll-like receptor 5 reveals a fundamental role in the biology of infection with *Salmonella enterica serovar typhimurium*. Infect. Immun. 73:2344-2350

Kaiser, P., L. Rothwell, E. E. Galyov. 2000. Differential cytokine expression in avian cells reponse to invasion by *Salmonella typhymurium, Salmonella enteritidis*, and *Salmonella gallinarium*. Microbiology. 146:3217-3226.

Kaiser, M. G., S. S. Block, C. Ciraci, W. Fang, M. Sifri, and S. J. Lamont. 2012. Effects of dietary vitamin E type and level on lipopolysaccharide-induced cytokine mRNA expression in broiler chicks. Poult. Sci. 91: 1893-1898.

Keller, R., M. Geiges, and R. Keist, 1990. L-arginine dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. Cancer Res. 50: 1421-1425.

Klasing, K. C., D. E. Laurin, R. K. Peng, and D. M. Fry. 1987. Immunologically mediated growth depression in chicks: influence of feed intake, corticosterone and interleukin-1. J. Nutr. 117:1629-1637.

Knowles, R. G., M. Salter, S. L. Brools, and S. Moncada. 1990. Anti-inflammatoty glucucorticoids inhibit the induction by endotoxin of nitric oxde synthase in the lung, liver and aorta of the rat. Bichem. Biophys. Res. Commun. 172:1042-1048.

Kogut M. H., K. J. Genovese, D. J. Nisbet. 2001. Signal transduction pathways activated by engaging immunoglobulin Fc receptors on chicken heterophils. Dev. Comp. Immunol. 25:639-46.

Kogut, M. H., E. D. McGruder, B. M. Hargis, D. E. Corrier, and J. R. DeLoach. 1994. Dynacmics of avian inflammatory response to *Salmonella*-immune lymphokines. Changes in avian blood leukocyte populations. Inflammation. 18:373-388.

Kogut, M. H., E. D. McGruder, B. M. Hargis, D. E. Corrier, and J. R. DeLoach. 1995. Characterization of the pattern of inflammatory cell influx in chicks following the intraperitoneal administration of live Salmonella enteritidis and Salmonella enteritidis-immune lymphokines. Poult. Sci. 74:8-17.

Kogut, M. H., E. D. McGruder, B. M. Hargis, D. E. Corrier, and J. R. DeLoach. 1995. Characterization of the pattern of inflammatory cell influx in chicks following the intraperitoneal administration of live Salmonella enteritidis and Salmonella enteritidis-immune lymphokines. Poult. Sci. 74:8-17.

Kogut, M. H., V. Lowry, R. Moyes, L. Bowden, R. Bowden, K. Genovese, and Deloach. 1998. Lymphokine-augmented activation of avian heterophils. Poult. Sci. 77:964-971.

Kogut, M. H. 2009. Impact of nutrition on the innate immune response to infection in poultry. J. Appl. Poult. Res. 18:111-124.

Konjufca, V. K., W. G. Bottje, T. K. Bersi, and G. F. Erf. 2004. Influence of dietary vitamin E on phagocytic functions of macrophages in broilers. Poult. Sci. 83:1530-1534.

Korver, D. R., and K. C. Klasing. 1997. Dietary fish oil alters specific and inflammatory immune response in chicks. J. Nutri. 127:2039-2046.

Korver, D. R, E. Roura, and K. C. Klasing. 1998. Effect of dietary energy level an oil source on broiler performance and response to an inflammatory challenge. Poult. Sci. 77:1217:1227.

Leeson, S., L. Caston, and J. D. Summers. 1996. Broiler response to diet energy. Poult. Sci. 75:529-535.

Leshchinsky, T. V., and K. C. Klasing. 2001. Divergence of the inflammatory response in two types of chickens. Dev. Com. Immunol. 25:629-638.

Leusink, G. H. Rempel, B. Skura, M. Berkyto, W. Whyte, Y. Yang, J. Y. Rhee, S. Y. Xuan, S. Chiu, F. Silversides, D. Fitzpatrick, and M. S. Diarra. 2010. Growth performance, meat quality, and gut microflora of broiler chickens fed with cranberry extract. Poult. Sci. 89:1514-1523.

Li, H., Y. Zhang, S. F. Zuo, Z. X. Lian, and N. Li. 2009. Effects of methyltestosterone on immunity against *Salmonella pullorum* in dwarf chicks. Poult. Sci. 88:2359-2548.

Liew, F. Y., S. Millott, C. Parkison, R. M. J. Palmer, and S. Moncada. 1990. Macrophage killing of *Leishmania* parasite *in vivo* mediated by nitric oxide from L-arginine. J. Immunol. 144:4794-7.

Lin, A., C. Chang, and C. C. McCormicl. 1996. Molecular cloning and expression of an avian inducible nitric oxide synthase cDNA and the analysis the genomic 5' flanking region. J. Biol. Chem. 271:11911-11919.

Lorr, N. A., K. A. Golemboski, R. A. Hemendinger, R. R. Dietert and S. E. Bloom, 1992. Distribution and inducibility of a P450I activity in cellular components of the avian immune system. Arch. Toxicol. 60:560–566.

Lund, J., and H. F. DeLuca, 1966. Biologically active metabolite of vitamin D_3 from bone, liver and blood serum. J. Lipid Res. 7:739-744.

Mageed, A. M., N. Isobe, Y. Yoshimura. 2008. Expression of avian beta-defensins in the oviduct and effects of lipopolysaccharide on their expression in the vagina of hens. Poult. Sci. 87:979-984.

Hamal, K. R., R. F. Wideman, N. B. Anthony, and G. F. Erf. 2010. Differential gene expression of proinflammatory chemokines and cytokines in lungs of ascites-resistant and susceptible broiler chickens following intravenous cellulose microparticle injection. Vet. Immunol. Immunopathol. 133:250-255.

Manolagas, S. C., D. M. Prowdini, and C. D. Tsoukas. 1985. Interactions of 1,25-dihydroxyvitamin D_3 and the immune system. Mol. Cell Endocrinol. 43:113-122.

Marletta, M. A., P. S. Yoon, Iyengar, C. D. Leaf, and J. S. Wishnok. 1988. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry. 27:8706-8711.

McCarthy, D. M., J. F. San Miguel, and H. C. Freake, 1983. 1,25-dihydroxyvitamin D_3 inhibits proliferation of human promyelocytic leukemia (HL60) cells and induces monocyte-macrophage differentiation in HL60 and normal human bone marrow cells. Leukemia Res. 7:51–55.

Miles, R., G. Butcher, P. Henry, and R. Littell. 2006. Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. Poult. Sci. 85:476-485.

Mills, C. D., 1991. Molecular basis suppressor macrophages arginine metabolism via nitric oxide synthase pathway. J. Immunol. 146:2719-2723.

Moncada, S., D. D. Rees, R. Schulz, and R. M. J. Palmer. 1991. Development and mechanism of a specific supersensitivity to nitrovasodialators after inhibition of vascular nitric oxide syntheis *in vivo*. Proc. Natl. Acad. Sci. USA. 88:2166-2170.

Nitsan, Z., E. A. Dunnington, and P. B. Siegel. 1991a. Organ growth and digestive enzyme levels to fifteen days of age in lines of chickens differing in body weight. Poult. Sci. 70:2040–2048.

Nitsan, Z., G. Ben-Avraham, Z. Zoref, and I. Nir. 1991b. Growth and development of the digestive organs and some enzymes in broilers chicks after hatching. Br. Poult. Sci. 32:515-523.

Norman, A. W. 1990. The avian as an animal model for the study of vitamin D endocrine system. J. Exp. Zool. Suppl. 4:37-45.

Organization for Economic Co-operation and Development. 2006. OECD-FAO Agriculture outlook 2006-2015. ftp://ftp.fao.org/docrep/fao/009/a0621e/a0621e00.pdf. Accessed Apr. 2013.

Penniall, R., and J. K Spitzanagel. 1975. Chicken neutrophils: oxiadtive metabolism in phagocytic cells devoid of myeloperoxidase. Proc. Natl. Acad. Sci. USA. 72:5012-5015.

Plumstead, P. W., H. Romero-Sanchez, B. D. Paon, J. W. Spears, and J. Brake. 2007. Effects of dietary metabolizable energy and protein on early growth responses of broilers to dietary lysine. Poult. Sci. 86:2639-2648.

Powell, D. J., D. C. McFarland, A. J. Cowieson, W. I. Muir, and S. G. Velleman. 2013. The effect of nutritional status on myogenic satellite cell proliferation and differentiation. Poult. Sci. 92: 2163-2173.

Praslickova, D., S. Sharif, A. Sarson, M. F. Abdul-Careem, D. Zadworny, A. Kulenkamp, G. Ansah, and U. Kuhnlein. 2008. Association of a marker in the vitamin D receptor gene with Marek's disease resistance in poultry. Poult. Sci. 87:1112-1119.

Presscott, M. G., A. C. Vreugdenhil, W. A. Buurman, R. E. Hancock, and M. R. Gold. 2000. Cutting edge: cationic antimicrobial peptides block the binding of lipopolysaccharide (LPS) to LPS binding protein. J. Immunol. 164:549-553.

Prowedini, D. M., L. J. Deftos, and S. C. Manolagas. 1986. 1, 25-dihydroxyvitamin D_3 promotes in vitro morphologic and enzymatic changes in normal human monocytes consistent with their differentiation into macrophages. Bone. 7:23-28.

Quentin, M., Bovarel I., and M. Picard. 2005. Effects of the starter diet, light intensity, and essential amino acids level on growth and carcass composition of broilers. J. Appl. Poult. Res. 14:69-76.

Qureshi, M. A., L. Miller, H. S. Lillehoj, and M. D. Ficken. 1990. Establishment and characterization of a chicken mononuclear cell line. Vet. Immunol Imunnopathol. 26:237-250.

Qureshi, M. A., J. N. Petitte, S. M. Laster, and R. R. Dietert. 1993. Avian macrophages: contribution to cellular microenvironment and changes in effector functions following activation. Poult. Sci. 72:1280–1284.

Rautenschlein, S., A. Subramanian, and J. M. Sharma. 1999. Bioactivities of a tumour necrosis-like factor released by chicken macrophages. Dev. Comp. Immunol. 23:629-640.

Research and Markets. 2011. Canada Agribusiness report Q1. http://www.researchandmarkets.com/reports/1533606/canada_agribusiness_report_q1_2011. Accessed Apr 2013.

Rieger, A. M., B. E. Hall, and D. R. Barreda. 2010. Macrophage activation differently modulates particle binding, phagocytosis and downstream antimicrobial mechanism. Dev. Comp. Immunol. 34:1144-1159.

Roe, M., and S. Pillai. 2003. Monitoring and identifying antibiotic resistance mechanisms in bacteria. Poult. Sci. 82:622-626.

Roura, E., J. Homedes, and K. C. Klasing. 1992. Prevention of immunologic stress contributes to the growth-permitting ability of dietary antibiotics in chicks. J. Nutri. 122:2382-2390.

Russell, S. M. 2003. The effect of airsacculitis on bird weights, uniformity, fecal contamination, processing errors, and populations of *Campylobacter spp*. and *Escherichia coli*. Poult. Sci. 82:1326–1331.

Scanes, C. G. 2007. The global importance of poultry. Poult. Sci. 86:1057-1058.

Scott, M. G., A. C. Vreugdenhil, W. A. Buurman, R. E. Hancock, M. R. Gold. 2000. Cutting edge: cationic antimicrobial peptides block the binding of lipopolysaccharide (LPS) ro LPS binding protein. J. Immunol. 164:549-553

Shanmugasundaram, R., and R. K. Selvaraj. 2012. Vitamin D-1α-hydroxylase and vitamin D-24hydroxylase mRNA studies in chickens. Poult. Sci. 91:1819-1824.

Skinner, J. T., S. Bauer, V. Young, G. Pauling, and J. Wilson. 2010. An economic analysis of the impact subclinical (mild) necrotic enteritis in broiler chickens. Avian Dis. 54:1237:1240.

Soares, J. H., J. M. Kerr, and R. W. Gray. 1995. 25-hydroxycholecalciferol in poultry nutrition. Poult. Sci. 74:1919-1934.

Stabler, J. G., T. W. McCormick, K. C. Powell, and M. H. Kogut. 1994. Avian heterophils and monocytes: phagocytic and bactericidal activities against *Salmonella enteritidis*. Vet. Microbiol. 38:293-305.

Statistics Canada. 2012a. Poultry and egg statistics – April to June 2012. Statistic Canada, Ottawa, Ontario, Canada. http://www.statcan.gc.ca/pub/23-015-x/2012002/t002-eng.pdf. Accessed Mar. 2013.

Statistics Canada. 2012b. Per capita poultry consumption comparison with other animal protein sources. Table 002-0010, 002-0019, 051-0001. Statistic Canada, Ottawa, Ontario, Canada. http://www.agr.gc.ca/poultry/consm_eng.htm. Accessed Mar. 2013.

Stutz, M. W., S. L. Johnson, and F. R. Judith. 1983. Effects of diet, bacitracin, and body weight restrictions on the intestine of broiler chicks. Poult. Sci. 62: 1626-1632.

Stutz, M. W., and G. C. Lawton. 1984. Effects of diet and antimicrobials on growth, feed efficiency, intestinal *Clostridium perfringens*, and ileal weight of broiler chicks. Poult. Sci. 63: 2036-2042.

Summers, J. D., D. Spratt, and J. L. Atkinson. 1992. Broiler weight gain and carcass composition when fed diets varying in amino acid balance, dietary energy, and protein level. Poult. Sci. 71:263-273.

Sunde, M. L. C. M. Turk, and H. F. DeLuca. 1978. The essentiality of vitamin D metabolites for embryonic chick development. Science. 200:1067-1069.

Sung, Y. J., and R. R. Dietert. 1994. Nitric oxide (NO)-induced mitochondrial injury among chicken NO-generating and target leukocytes. J. Leukocyte Biol. 56:52–58.

Swaggerty, C. L., I. Y. Pevzner, V. K. Lowry, M. B. Farnell, and M. H. Kogut. 2003. Functional comparison of heterophils isolated from commercial broiler chickens. Avian. Pathol. 32:95-102.

Swaggerty, C. L., P. J. Ferro, I. Y. Pevzner, M. H. Kogut. 2005. Heterophils are associated with resistance to systemic *Salmonella enteritidis* infection in genetically distinct lines of chickens. FEMS Immunol. Med. Microbiol. 43:149-154.

Swaggerty, C. L., P. Kaiser, L. Rothwell, I. Y. Pevzner, and M. H. Kogut. 2006. Heterophil cytokine mRNA profiles from genetically distinct lines of chickens with differential heterophil-mediated innate immune responses. Avian. Pathol. 35: 102-108.

Torok, V. A., G. E. Allison, N. G. Percy, K. Ophel-Keller, and R. J. Hughes. 2011. Influence of antimicrobial feed additives on broiler commensal posthatch gut microbiota development and performance. Appl. Environ. Microbiol. 77:3380-3390.

Van Immerseel, F., J. De Buck, I. De Smet, J. Mast, F. Haesebrouck, and R. Ducatelle. 2002. Dynamics of immune cell infiltration in the caecal lamia propria of chickens after neonatal infection with a *Salmonella enteritidis* strain. Dev. Comp. Immunol. 26:355-364.

Wasserman, R. H., R. A. Corradino, and A. N. Taylor. Vitamin D-dependent calcium-binding protein: purification and some properties. 1968. J. Biol. Chem. 243:3978-3986.

World Health Organization. 2002. The medical impact of the use of antimicrobials in food animals: report of a WHO meeting, Berlin, Germany.http://whqlibdoc.who.int/hq/1997/WHO_EMC_ZOO_97.4.pdf. Accessed August 2013.

Yilma, A., S. Shen, D. L. Adeson, S. Xavier, and J. J. Zhu. 2005. Identification and sequence analysis of chicken toll-like receptors. Immunogenetics. 56:743-753.

Zhang, M., T. Nii, N. Isobe, and Y. Yoshimura. 2012. Expression of Toll-like receptors and effects of lipopolysaccharide on the expression of proinflammatory cytokines and chemokine in the testis and epididymis of roosters. Poult. Sci. 91:1997-2003.

Zhang, X., D. L. Chinkes, and R. R. Wolfe. 2002. Measurement of muscle protein fractional synthesis and breakdown rates from a pulse tracer injection. Am. J. Physiol. Endocrinol. Metab. 283:E753–E764.

CHAPTER 2: Effect of early nutrition, 25-hydroxy vitamin D_3 and bacitracin methylene disalicylate on broiler performance

2.1 INTRODUCTION

Antibiotics have been added to animal feed at low dosages for prophylaxis and growth promotion since the 1940s (Torok et al., 2011). Bacitracin methylene disalicylate (BMD) is among several types of antibiotics that have been commonly supplemented in feeds of broilers (Chapman and Johnson, 2002). Prophylactic addition of 0.5 g/kg BMD increased lipid absorption in the proximal ileum of broiler chicks by reducing *Lactobacillus salivarius* populations (Guban et al., 2006). A reduction in gut microflora could mitigate the nutrient competition between the host and intestinal microflora, subsequently more nutrients are partitioned toward growth (Miles et al., 2006). However, prophylactic antibiotic use has been banned by several European countries (Casewell et al., 2003). The publics concerned that prophylactic antibiotic use in the long run could increase bacteria with resistant genes to the therapeutic antibiotics, and could pose a potential threat to human if there was a bacteria cross-contamination between human and livestock (World Health Organization, 2002). Currently, it is unlikely that prophylactic antibiotics can be replaced by single alternative supplementation in controlling enteropathogens (Hofacre et al., 2003).

Nutritionists have been searching for alternative substances that could mitigate the absence of prophylactic antibiotics in broiler industry. One method that has the potential to alleviate the reliance on prophylactic antibiotic is to increase

intrinsic health of broilers by nutritional regimens (Kogut, 2009). Cheema et al. (2003) observed an increase in T cell proliferation when broilers were provided 21.9% CP compared with those fed 20.1% CP in isoenergetic starter diets, although the high protein diet had inconsistent effects on monocyte-macrophage functions. Nutrient densities not only affect broiler metabolism (Scott, 2002), but also impact growth performance and carcass quality (Jones and Wiseman, 1985, Kidd et al., 2005). For example, broilers fed 3,059 kcal/kg and 23% CP had greater body weight, feed efficiency and breast yield than those fed 2,527 kcal/kg and 19% CP in the starter diet (Zhao et al., 2009).

Supplementation of vitamin D is necessary for broiler production since natural synthesis of vitamin D is UV-dependent and broilers are often raised in a barn environment, with little or no direct sunlight (Norman, 1990). The most biologically active form of vitamin D for birds is 1, α-25 $(OH)_2D_3$ (Norman, 1990). This metabolite is synthesized in the kidney from 25-OH-D_3 which is hydroxylated in the liver after cholecalciferol (vitamin D_3) is absorbed from the gut lumen (Soares et al., 1995). Natural hepatic 25-OH-D_3 concentration of broilers may decline due to either heat stress condition (Shim et al., 2006), or diseases (Whitehead et al., 2004). The hydroxylated vitamin D_3 metabolite is approximately 4 times more potent than conventional cholecalciferol (Haussler and Rasmussen, 1972; Soares et al., 1978). The aim of the current study was to investigate the effects and interactions among nutrient density at the starter phase, 25-OH-D_3, and BMD on the performance, and meat yield of broiler

chickens raised to 40 d of age. We hypothesized that reduced nutrient density would decrease broiler performance and meat yield.

2.2 MATERIALS AND METHODS

The experimental protocol was approved by the Animal Care and Use Committee of the University of Alberta and was performed in accordance with the guidelines of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

2.2.2 Experimental design.

The current experiment was conducted as a 2 x 2 x 2 factorial arrangement of treatments. Two levels of nutrient density were provided in the starter diets from 0 to 14 d of age, containing high protein and energy (HIGH: 24% CP and 3,025 kcal/kg) or low protein and energy (LOW: 22% CP and 2,858 kcal/kg). The ratios of tryptophan, threonine, histidine, methionine, arginine and total sulfur amino acids to lysine were the same in the HIGH and LOW diets. Grower and finisher diets were subsequently provided from 15 to 28 d, and 29 to 40 d, respectively. During all three dietary phases, two levels of 25-OH-D_3 (HyD®, 0 or 69 µg/kg, equivalent to 2,760 IU/kg) and two levels of antibiotic (BMD® 110, 0 or 0.5 g/kg) were applied, according to the manufacturer's recommendations for preventing necrotic enteritis disease and improving broilers' performance. The HIGH diet was based on nutritional recommendations of the primary breeder (Aviagen, 2009). The wheat-based diets were formulated to meet or

exceed nutrient requirements specified by the National Research Council (1994) as shown in Table 2.1.

2.2.3 Stocks and management.

A total of 3,200 Ross 708 mixed-sex broiler chicks were used in this study. At day of hatch, 100 chicks were placed randomly into each of 32 floor pens at a stocking density of 10 birds/m^2. A lighting program of 20L:4D was used for the entire 40 d growing period. Temperature was managed according to commercial primary breeder recommendations (Aviagen, 2009). Chicks had ad libitum access to feed and water. Mortality increased by 2.2% in the broilers which were fed non-antibiotic diets at d 28. Postmortem examination revealed that the higher mortality was attributable to a necrotic enteritis outbreak in the broilers that were not supplemented with BMD. All pens were identically treated with penicillin (Pot-Pen 500,000,000 I.U., Vétoquinol Canada Inc.) via the drinking water for 2 consecutive days until the mortality rate was reduced.

2.2.4 Data collection.

Group BW were measured for all pens at weekly intervals. BW gain and FCR were calculated weekly. Mortality was recorded with post-mortem determination of cause of death. Feed conversion ratios (feed:gain) were corrected for mortality and culled birds.

On d 40, 256 broilers were processed (32 per treatment) after a 12 h feed withdrawal period. The birds were electrically stunned, and then bled for 2 min. After scalding (63°C) for 45 s, carcasses were mechanically defeathered and

manually eviscerated before being cut up. Carcass, breast (*Pectoralis major and Pectoralis minor*), wing and leg weights were recorded. Breast yield and carcass yield were determined by the ratio of carcass and breast to live BW before feed withdrawal.

2.2.5 Economic analysis

A brief economic analysis was done based on cost per kg of live body weight. In the current study, the cost for early HIGH nutrient density diet was $309.74/tonne, and the cost for the LOW nutrient density diet was $275.13/tonne. Costs for supplementation of BMD and 25-OH-D_3 were $3.5/tonne and $1.5/tonne, respectively. Feed costs were calculated by multiplying the total feed intake by the total cost of feed price, antibiotic and 25-OH-D_3. Cost per kg of meat produced was calculated by dividing the feed cost to live body weight per chicken.

2.2.6 Statistical analysis.

Broiler performance, meat yield, and cost per kg of meat produced were analyzed as a 3-way ANOVA with dietary nutrient density from d 0 to d 14 of age, 25-OH-D_3, and BMD treatments as main effects by using the MIXED procedure of SAS (SAS Institute, 2002). The pen was the experimental unit for broiler performance and individual broilers were the experimental unit for meat yield data. Sex was determined at processing and was included in the model as a random term to account for the variations between male and female broilers. Differences between main effects, means, and their interactions were classified

by pairwise comparisons, and unless otherwise noted, differences were considered significant at $P< 0.05$.

2.3 RESULTS AND DISCUSSION

2.3.1 Broiler Performance.

There were no significant interactions between early nutrition, 25-OH-D_3 and bacitracin methylene disalicylate (BMD) on broiler performance and meat yield. Therefore, only the means of the main effects are presented.

2.3.1.1 Body weight. Birds fed BMD had lower BW than the control birds at d 7 and d 22 of age (Table 2.2). Several reports on antibiotic use showed greater final body weight when prophylactic antibiotics were supplemented in the diets for broiler chickens (Henry et al., 1987; Miles et al., 2006). However, Baurhoo et al. (2007) reported that prophylactic virginamycin fed birds exhibited decreased commensal bacteria as well as harmful bacteria, reducing digestion ability of broilers, and leading to reduced BW gain. This could be an explanation for the reduced BW in birds receiving BMD at d 7, although the mode of action is different for BMD and virginamycin (Miles et al., 2006). How prophylactic antibiotic BMD influences the intestinal microflora populations would require further study. The reason for the unexpected result at d 22 is not clear in this trial. In the current study, a necrotic enteritis (NE) outbreak occurred at d 28. Because the immunity of broilers was engaged by the *Clostridium perfringens*, normal broiler growth could have been compromised since the third week, particularly during the feed change period on d 28. In the subclinical form, *Clostridium perfringens* can damage the intestinal mucosa, causing reduced

weight gain and increased FCR (Hofacre et al., 2003). Roura et al. (1992) reported that BW and FCR of broilers could be compromised by immune challenge, and the inflammatory stress could be reduced with the supplementation of prophylactic antibiotic. However, there was no significant difference in growth rate between broilers fed antibiotic diet and non-antibiotic diet. The mechanism by which this occurred is unclear in the current study.

Broiler chickens fed the HIGH diet from d 0 to d 14 had greater BW than those fed the LOW diet on days 7, 14, 21, 28 and 40 of age (Table 2.2). The results from the current study were in line with Golian et al. (2010), who reported that broiler chicks fed a high energy and protein diet (3,200 kcal/kg and 23% CP) had greater BW and lower FCR than broilers fed a low nutrient diet (2,900 kcal/kg and 20% CP). The authors also reported that the high nutrient density diet is important for early growth and feed efficiency (Golian et al., 2010). Jahanian (2009) reported that 22.35% CP in the diet increased BW gain of broilers compared with broilers fed 19% CP when 3,100 kcal/kg of ME was fed during a 21-day feed trial. Young broiler chickens are less adaptable than older broilers in adjusting feed intake due to the physical limitation of the intestinal tract (Jones and Wiseman, 1985; Brickett et al., 2007). Therefore, the HIGH diet from d 0 to d 14 of age could have provided greater amount of nutrients with a lower feed intake for the young birds. In the current study, broilers fed the LOW diet consumed an extra 97 g of feed (data not shown) compared with broilers fed the HIGH diet by d 22 of age. Although broilers were only fed the HIGH diet from d 0 to d 14, the greater BW persisted at d 40 compared with broilers fed the LOW

diet. Quentin et al. (2005) reported that a high nutrient density diet in the starter phase could result in a long term enhancement of growth performance of broilers. That could be due to an increase of muscle satellite cell proliferation at postnatal period, which subsequently increased the muscle growth at later stages (Halevy et al., 2000). Increased amino acid levels and nutrient intake in the HIGH relative to the LOW nutrient density may have increased protein synthesis and protein deposition (Tesseraud et al., 1996).

2.3.1.2 Feed conversion ratio. Prophylactic BMD increased FCR compared with birds fed the non-antibiotic diet at d 7 (Table 2.3). It is not clear why this occurred. Croswell et al. (2009) reported that Bacitracin disrupted normal microbiota in the mouse colon, increased the colonization of *Salmonella typhimurium*, and subsequently reducing feed absorption efficiency. In the current study, broilers fed BMD had lower BW than broilers fed the non-antibiotic diet, causing the increased FCR in broilers fed BMD at d 7. The effect was small and transient because BMD did not affect FCR at later phases in the study.

Broilers fed the HIGH diet had a lower FCR than those fed the LOW diet during all experimental periods, except for d 29 to d 35 (Table 2.3). In the current experiment, the greatest reduction in FCR due to the HIGH diet was 12.5% and 12.8% at d 14 and d 22, respectively. This result was consistent with Holsheimer and Ruesink (1993), who reported that feed conversion ratio was 5.3% lower in birds fed 3,250 kcal/kg ME and 22% CP compared with those fed 3,000 kcal/kg and 20.3% CP from d 0 to d 14 of age. In the current study, broilers fed the

HIGH diet had lower feed intake compared with broilers fed the LOW nutrient density (725.8 vs. 822.6 g of feed, P< 0.0001) at d 22, contributing to decreased FCR. There was no significant effect of early HIGH nutrient diet on FCR of broilers at d 35. The growth rate of broilers could have been interfered by the NE outbreak at d 28, because the intestinal mucosa might be damaged by the colonization of *Clostridium perfringens* during the time of sub clinical infection (Hofacre et al., 2003). The result reflected that *Clostridium perfringens* affected all experimental broilers, disregard broilers in the HIGH or LOW diet. Broilers were treated with penicillin antibiotic for two days, and the treatment could stop the development of necrotic lesions, helping broilers to recover their normal feed intake (Lanckriet et al., 2010).

2.3.1.3 Mortality. The effect of BMD on cumulative mortality was seen clearly at d 35 and d 40, when broiler chickens fed BMD had lower mortality than broilers fed antibiotic-free diets (Table 2.4). Riddle and Kong (1992) reported that wheat-based diets caused an increased susceptibility to necrotic enteritis, compared to broilers fed corn based diets. In the current experiment, wheat was the primary ingredient (60-70%), and may be a predisposed factor the broilers to the proliferation of *Clostridium perfringens* (Dänicke et al., 2007). The inclusion of xylanase (Avizyme, 50 g/kg of feed) could aid digestion of broilers (Engberg et al., 2004), but could not prevent broilers from the colonization of *Clostridium perfringens.* In the current study, broilers fed diets without BMD supplementation had higher mortality compared with broilers fed prophylactic BMD. It is important to note that therapeutic penicillin (Pot-Pen

500,000,000 I.U., Vétoquinol Canada Inc.) was administered to all birds in the experiment to control a necrotic enteritis outbreak on d 28 and d 29. Although the intestinal counts of *Clostridium perfringens* were not assessed in the current study, the inclusion of therapeutic penicillin reduced the mortality, which was observed to be lower in broilers fed prophylactic BMD (Table 2.4). In the current study, feeding BMD increased the profit of poultry meat produced by $0.10/kg. A reduction in mortality would increase profits for broiler producers by decreasing the costs per kg of poultry meat produced.

In the current experiment, dietary 25-OH-D_3 had no effect on any parameters of broiler performance, including BW, FCR, and mortality. The result was consistent with previous studies (Ledwaba and Roberson, 2003; Applegate et al., 2003). Ledwaba and Roberson (2003) reported that 25-OH-D_3 had no effect on BW and FCR when the birds were provided 55 µg/kg (2,200 IU/kg) of cholecalciferol in the basal diet. Yarger et al. (1995) reported that supplementation of 25-OH-D_3 in the range between 50 to 70 µg/kg of feed (2,000 to 2,800 IU/kg of feed) without supplemental cholecalciferol increased BW, feed efficiency, and meat yield of broiler chickens. In the current study, dietary 25-OH-D_3 was fortified to 4,000 IU of cholecaliferol in the basal diet in the groups fed 25-OH-D_3. It is likely that the supplementation of 25-OH-D_3 (69 µg/kg) did not provide further increasing broiler performance in the current experiment.

2.3.2 Meat yield.

BMD did not have any effect on broiler meat yield at d 40 (Table 2.5). The HIGH diet increased breast yield compared with the broilers fed the LOW diet. Although the HIGH diet was only provided to birds from d 0 to d 14, it increased breast yield by 2.2%, compared with those fed the LOW diet at d 40 (Table 2.5). Sklan and Noy (2003) reported that a high nutrient density diet at the first week post-hatch increased the number of satellite cells in the breast muscle, which subsequently increased breast muscle development. This observation was consistent with Kidd et al. (2005), although it was not clear whether the increased in breast growth was due to hypertrophy or hyperplasia. In this current experiment, birds fed 25-OH-D_3 had lighter pectoralis major and minor, resulting in lower breast muscle yield compared to broilers fed without 25-OH-D_3 (Table 2.5). There were varying reports about the effect of 25-OH-D_3 on breast yield. Most studies which reported a superior effect of 25-OH-D_3 over cholecalciferol were conducted when 25-OH-D_3 and cholecalciferol were not combined or 25-OH-D_3 was replaced partially for cholecalciferol (Yarger et al, 1995, Mitchell et al., 1997; Fritts et al., 2003). In the current study, 4,000 IU of cholecalciferol was provided in the basal diet following the recommendation of the primary breeder, and 69 µg of 25-OH-D_3 (equivalent to 2, 760 IU of cholecalciferol) were supplemented to the basal diet. Therefore, birds on all treatments should have received adequate vitamin D_3. It is not known at this time why 25-OH-D_3 fortified to vitamin D_3 had a negative effect on BW and breast muscle. Results from the current study do not support the hypothesis that 25-OH-D_3 would

increase broiler performance. Michalczuk et al. (2010) reported that replacement of 1,500 IU out of 4,000 IU of total vitamin D activity with 25-OH-D_3 numerically increased BW at d 42 compared to those fed the basal diet, whereas replacement of 2,760 IU out of 4,000 IU of total vitamin D activity with 25-OH-D_3 numerically decreased BW compared to the broilers received 1,500 IU of 25-OH-D_3 supplementation. 25-OH-D_3 supplementation was also expected to increase the innate immune response of broiler chicks due to the increase in biological activities of 25-OH-D_3 with vitamin D receptors on the phagocytic cells, compared to that of cholecalciferol (Norman, 1990). However, it is important to note that an NE outbreak at d 28 might have caused the unexpected effect of 25-OH-D_3 on breast yield. The trigger of an innate immune response is a costly energy expense, and it may take away the energy, which could be shifted toward muscle development (Roura et al., 1992).

The current study demonstrated that prophylactic BMD decreased mortality but did not increase body weight gain and FCR of broilers. The HIGH nutrient diet increased broiler performance with no interactions between the factors. Prophylactic BMD did not increase final BW of broilers at d 40, and had no effect on FCR from d 14 to d 40. Feeding BMD did not increase yield, but decreased FCR and reduced bird mortality, with a resultant increase in profit of $0.10/kg of poultry meat produced. Reducing nutrient density to reduce feed cost is not recommended for Ross 708 broilers to obtain the desired level of productivity and yield.

Table 2.1 Composition and analysis of experimental diets. Control diets contained similar amount of nutrients, but without supplementation of antibiotic and HyD premix at starter phase

	Starter		Grower	Finisher
	(0 to 14 d)		(15 to 28 d)	(29 to 40 d)
Ingredient (%)	HIGH	LOW		
Wheat	61.16	70.11	66.17	70.66
Soybean meal	22.89	17.49	17.37	12.98
Canola meal	4.5	4.5	4.5	4.5
Canola oil	3.48	0.05	4.62	4.82
Fish meal- menhaden	3.00	3.00	3.00	3.00
Calcium carbonate	1.14	1.16	0.89	0.86
Dicalcium phosphate	1.43	1.43	1.19	1.05
Salt	0.22	0.22	0.22	0.22
L - Lysine	0.32	0.28	0.26	0.22
DL - Methionine	0.30	0.22	0.23	0.17
L-Threonine	0.11	0.09	0.09	0.07
Amprolium	0.05	0.05	0.05	0.05
Vitamin & mineral premix[1]	0.50	0.50	0.50	0.50
Choline chloride premix[2]	0.50	0.50	0.50	0.50
Vitamin E premix[3]	0.30	0.30	0.30	0.30
Avizyme 1302[4]	0.05	0.05	0.05	0.05
HyD premix[5]	0 or 0.05	0 or 0.05	0 or 0.05	0 or 0.05
Antibiotic growth promoter[6]	0 or 0.05	0 or 0.05	0 or 0.05	0 or 0.05
Calculated nutrients				
ME (kcal/kg)	3,025	2,858	3,150	3,200
CP (%)	23.90	22.30	21.71	20.03
Calcium (%)	1.05	1.05	0.90	0.85
Available phosphorus (%)	0.50	0.50	0.45	0.42
Total methionine + cysteine (%)	1.07	0.96	0.95	0.86
Total methionine (%)	0.68	0.59	0.59	0.52
Total lysine (%)	1.43	1.27	1.24	1.09

[1]Provided following per kg of diet: vitamin A (retinyl acetate), 10,000 IU; cholecalciferol, 4,000 IU; vitamin E, 35 IU; vitamin K, 4.0 mg; pantothenic acid, 15 mg; riboflavin, 10 mg; folic acid, 0.2 mg; niacin, 65 mg; thiamine, 4.0 mg; pyridoxine, 5.0 mg; vitamin B_{12}, 0.02 mg; biotin, 0.2 mg; iodine, 1.65 mg; Mn, 120 mg; Cu, 20 mg; Zn, 100 mg, Se, 0.3 mg; Fe, 80 mg and choline, 2.63 mg.

[2] Provided 100 mg choline per kg of diet.

[3] Provided 15 IU vitamin E per kg of diet.

[4] Xylanase enzyme; Danisco Animal Nutrition, Marlborough, Wiltshire, UK.

[5]Added to feed as Rovimix HyD; DSM Nutritional Products; 0 or 69 µg/kg of 25-OH-D_3 (equivalent to 2,760 IU of vitamin D_3/kg) were added in the control or experimental diets respectively.

[6]Bacitracin Methylene Disalicylate (BMD® 110): 0 or 0.5 g/kg, containing 55 mg of BMD per kg.

Table 2.2 Effect of starter nutrient density, 25-OH-D_3 and antibiotic on body weight of mix-sexed broiler chickens from 0 to 40 d

	Age (days)						
Effect	0	7	14	22	28	35	40
		g					
A[1]							
0	37.2	135.0[a]	351.5	834.1[a]	1,370	2,029	2,524
0.5g/kg	38.7	129.9[b]	347.3	805.8[b]	1,387	2,044	2,557
N[2]							
HIGH	37.4	138.1[a]	368.4[a]	836.7[a]	1,404[a]	2,035	2,567[a]
LOW	38.6	126.7[b]	330.3[b]	803.2[b]	1,353[b]	2,010	2,514[b]
D[3]							
0	38.6	133.9	348.5	832.9	1,385	2,027	2,551
69µg/kg	37.3	130.9	350.3	817.2	1,359	2,037	2,530
Pooled SEM	0.8	1.7	2.5	8.4	8	17	18
Source of variation	P-values						
A	0.175	0.041	0.244	0.024	0.142	0.248	0.198
N	0.293	<0.001	<0.001	0.011	<0.001	0.501	0.045
D	0.230	0.219	0.623	0.330	0.278	0.603	0.408
A x N	0.401	0.829	0.339	0.340	0.742	0.160	0.889
A x D	0.178	0.977	0.086	0.553	0.472	0.373	0.874
N x D	0.563	0.284	0.339	0.340	0.742	0.160	0.889
A x N x D	0.471	0.503	0.782	0.142	0.491	0.914	0.293

[a,b] Treatment means within the same column within effect with no common superscripts are significantly different ($P < 0.05$).

[1]Bacitracin Methylene Disalicylate (BMD® 110): 0 or 0.5 g/kg, containing 55 mg of BMD per kg.

[2]Starter nutrient density: HIGH (3,025 kcal/kg; 23.9% CP), or LOW (2,858 kcal/kg; 22.3% CP) fed from 0 to 14 d.

[3]Broilers fed a diet supplemented with 0 or 69 µg of 25-OH-D_3 (all basal diets contained an additional 4,000 IU/kg of vitamin D_3).

Table 2.3 Effect of starter nutrient density, 25-OH-D_3 and antibiotic on feed conversion ratio (FCR)

	Age (days)					
Effect	0-7	0-14	0-22	0-28	0-35	0-40
	kg/kg					
A[1]						
0	1.24[b]	1.43	1.53	1.56	1.67	1.73
0.5g/kg	1.37[a]	1.42	1.52	1.53	1.65	1.72
N[2]						
HIGH	1.24[b]	1.33[b]	1.43[b]	1.49[b]	1.62	1.69[b]
LOW	1.37[a]	1.52[a]	1.64[a]	1.61[a]	1.68	1.76[a]
D[3]						
0	1.29	1.44	1.54	1.56	1.66	1.73
69µg/kg	1.32	1.41	1.53	1.54	1.65	1.72
Pooled SEM	0.04	0.01	0.05	0.01	0.03	0.01
Source of variation	P-values					
A	0.032	0.677	0.363	0.107	0.476	0.557
N	0.029	<0.001	<0.001	<0.001	0.095	0.001
D	0.501	0.076	0.622	0.254	0.896	0.893
A x N	0.297	0.458	0.951	0.509	0.827	0.201
A x D	0.524	0.260	0.429	0.435	0.383	0.499
N x D	0.297	0.458	0.951	0.509	0.827	0.201
A x N x D	0.635	0.138	0.086	0.759	0.514	0.329

[a,b]Treatment means within the same column within effect with no common superscripts are significantly different ($P < 0.05$).

[1]Bacitracin Methylene Disalicylate (BMD® 110): 0 or 0.5 g/kg, containing 55 mg of BMD per kg.

[2]Starter nutrient density: HIGH (3,025 kcal/kg; 23.9% CP), or LOW (2,858 kcal/kg; 22.3% CP) fed from 0 to 14 d.

[3]Broilers fed a diet supplemented with 0 or 69 µg of 25-OH-D_3 (all basal diets contained an additional 4,000 IU/kg of vitamin D_3).

Table 2.4 Effect of starter nutrient density, 25-OH-D_3 and antibiotic on cumulative mortality

	Age (days)					
Effect	7	14	22	28	35	40
	%					
A[1]						
0	2.5	3.7	4.8	6.6	10.3[a]	12.6[a]
0.5g/kg	2.2	2.8	3.6	4.0	4.6[b]	5.5[b]
N[2]						
HIGH	2.3	3.3	4.5	6.2	8.9	10.1
LOW	2.4	3.3	3.9	4.5	6.0	7.9
D[3]						
0	2.3	3.3	4.7	5.9	7.0	8.3
69µg/kg	2.4	3.3	3.8	4.8	7.8	9.7
Pooled SEM	0.29	0.4	0.63	1.01	1.6	1.76
Source of variation	P-values					
A	0.508	0.164	0.179	0.082	0.018	0.009
N	0.847	0.976	0.457	0.254	0.179	0.392
D	0.709	0.923	0.277	0.437	0.714	0.574
A x N	0.460	0.287	0.546	0.422	0.364	0.480
A x D	0.897	0.301	0.711	0.813	0.244	0.158
N x D	0.306	0.690	0.611	0.562	0.827	0.593
A x N x D	0.182	0.286	0.845	0.768	0.639	0.906

[a,b]Treatment means within the same column within effect with no common superscripts are significantly different ($P < 0.05$).

[1]Bacitracin Methylene Disalicylate (BMD® 110): 0 or 0.5 g/kg, containing 55 mg of BMD per kg.

[2]Starter nutrient density: HIGH (3,025 kcal/kg; 23.9% CP), or LOW (2,858 kcal/kg; 22.3% CP) fed from 0 to 14 d.

[3]Broilers fed a diet supplemented with 0 or 69 µg of 25-OH-D_3 (all basal diets contained an additional 4,000 IU/kg of vitamin D_3).

Table 2.5 Effect of starter nutrient density, 25-OH-D_3 and antibiotic treatments on broiler carcass yield at d 40

Effect	Carcass	Leg	*Pectoralis*		Wing	Breast	Carcass	Breast
			Major	*Minor*				
	g						% BW	% BW
A[1]								
0	1,624	519.9	384.0	86.8	195.1	472.3	63.8	18.4
0.5g/kg	1,626	520.9	381.5	86.4	194.0	466.3	63.9	18.4
N[2]								
HIGH	1,643	524.6	392.5[a]	87.2	196.2	479.9[a]	64.1	18.6[a]
LOW	1,611	516.1	373.0[b]	86.0	192.9	458.7[b]	63.4	18.2[b]
D[3]								
0	1,653	527.8	391.2[a]	89.1[a]	193.7	482.5[a]	64.0	18.6[a]
69µg/kg	1,602	513.0	374.3[b]	84.5[b]	192.9	456.1[b]	63.6	18.3[b]
Pooled SEM	18.6	6.5	5.4	1.1	2.2	8.7	0.4	0.2
	P-values							
A	0.747	0.914	0.748	0.653	0.732	0.954	0.386	0.702
N	0.244	0.367	0.019	0.630	0.298	0.023	0.054	0.005
D	0.063	0.123	0.039	0.008	0.593	0.014	0.169	0.033
A x N	0.784	0.301	0.965	0.823	0.917	0.607	0.435	0.157
A x D	0.139	0.164	0.085	0.104	0.771	0.057	0.308	0.203
N x D	0.148	0.212	0.228	0.117	0.385	0.209	0.364	0.302
A x N x D	0.607	0.964	0.395	0.233	0.957	0.814	0.883	0.329

[a,b] Treatment means within the same column within effect with no common superscripts are significantly different ($P < 0.05$).

[1]Bacitracin Methylene Disalicylate (BMD® 110): 0 or 0.5 g/kg, containing 55 mg of BMD per kg.

[2]Starter nutrient density: HIGH (3,025 kcal/kg; 23.9% CP), or LOW (2,858 kcal/kg; 22.3% CP) fed from 0 to 14 d.

[3]Broilers fed a diet supplemented with 0 or 69 µg of 25-OH-D_3 (all basal diets contained an additional 4,000 IU/kg of vitamin D_3).

2.4 REFERENCES

Applegate, T., R. Angel, and H. Classen. 2003. Effect of dietary calcium, 25-hydroxycholecalciferol, or bird strain on small intestinal phytase activity in broiler chickens. Poult. Sci. 82:1140-1148.

Aviagen. 2009. Ross broiler management manual. http://www.poultryhub.org/wp-content/uploads/2012/06/Ross_US_broiler_Manual_09.pdf. Accessed January 2013.

Baurhoo, B., L. Phillip, and C. A. Ruiz-Feria. 2007. Effects of purified lignin and mannan oligosaccharides on intestinal integrity and microbial populations in the ceca and litter of broiler chickens. Poult. Sci. 86:1070-1078.

Brickett, K. E., J. P. Dahiya, H. L. Classen, and S. Gomis. 2007. Influence of dietary nutrient density, feed form, and lighting on growth and meat yield of broiler chickens. Poult. Sci. 86:2172-2181.

Canadian Council on Animal Care. 1993. A guide to the care and the use of experimental animals. 2nd ed. CCAC, Ottawa, ON, Canada.

Chapman, H., and Z. Johnson. 2002. Use of antibiotics and roxarsone in broiler chickens in the USA: analysis for the years 1995 to 2000. Poult. Sci. 81:356-364.

Cheema, M. A., M. A. Qureshi, and G. B. Havenstein. 2003. A comparison of the immune profile of commercial broiler strains when raised on marginal and high protein diets. Int. J. Poult. Sci. 2:300-312.

Croswell, A., E. Amir, P. Teggatz, M. Barman, and N. H. Salzman. 2009. Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric salmonella infection. Infect. Immun. 77:2741-2753.

Dänicke, S., H. Valenta, and S. Matthes. 2007. On the interactions between Fusarium toxin-contaminated wheat and nonstarch polysaccharide hydrolyzing enzymes in diets of broilers on performance, intestinal viscosity, and carryover of deoxynivalenol. Poult. Sci. 86:291-298.

Engberg, R. M., M. S. Hedemann, S. Steenfeldt, and B. B. Jensen. 2004. Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poult. Sci. 84:925-938.

Fritts, C. A., and P. W. Waldroup. 2003. Effect of source and level of vitamin D on live performance and bone development in growing broilers. J. Appl. Poult. Res. 12:45-52.

Golian, A., M. A. Azghadi, and M. Pilevar. 2010. Influence of various levels of energy and protein on performance and humoral immune responses in broiler chicks. Global Veterinaria. 4:434-440.

Guban, J., D. R. Korver, G. E. Allison, and G. W. Tannock. 2006. Relationship of dietary antimicrobial drug administration with broiler performance, decreased population levels of *Lactobacillus salivarius*, and reduced bile salt deconjugation in the ileum of broiler chickens. Poult. Sci. 85:2186-2194.

Halevy, O., A. Geyra, M. Barak, Z. Uni, and D. Sklan. 2000. Early posthatch starvation decreases satellite cell proliferation and skeletal muscle growth in chicks. J Nutr. 130:858-64.

Haussler, M. R., and H. Rasmussen. 1972. The metabolism of vitamin D_3 in the chick. J. Biochem. 247:2328-2335.

Henry, P. R., C. B. Ammerman, D. R. Campbell, and R. D. Miles. 1987. Effect of antibiotics on tissue trace mineral concentration and intestinal tract weight of broiler chicks. Poult. Sci. 66:1014-1018.

Hofacre, C. L., T. Beacorn, S. Collett, and G. Mathis. 2003. Using competitive exclusion, mannan-oligosaccharide and other intestinal products to control necrotic enteritis. J. App. Poult. Res. 12:60-64.

Holsheimer, J. P., and E. W. Ruesink. 1993. Effect on performance, carcass composition, yield, and financial return of dietary energy and lysine levels in starter and finisher diets fed to broilers. Poult. Sci. 72:806-815.

Jahanian, R. 2009. Immunological responses as affected by dietary protein and arginine concentrations in starting broiler chicks. Poult. Sci. 88:1818-1824.

Jones, R. L., and J. Wiseman. 1985. Effect of nutrition on broiler carcase composition: Influence of dietary energy content in the starter and finisher phases. Br. Poult. Sci. 26:381-388.

Kidd, M. T., C. D. McDaniel, S. L. Branton, E. R. Miller, B. B. Boren, and B. I. Fancher. 2004. Increasing amino acid density improves live performance and carcass yields of commercial broilers. J. Appl. Poult. Res. 13:593-604.

Kogut, M. H. 2009. Impact of nutrition on the innate immune response to infection in poultry. J. Appl. Poult. Res. 18:111-124.

Lanckriet, A., L. Timbermont, M. De Gussem, M. Marien, D. Vancraeynest, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2010. The effect of commonly used anticoccidials and antibiotics in a subclinical necrotic enteritis model. Avian Pathol. 39:63-68.

Ledwaba, M., and K. Roberson. 2003. Effectiveness of twenty-five-hydroxycholecalciferol in the prevention of tibial dyschondroplasia in Ross cockerels depends on dietary calcium level. Poult. Sci. 82:1769-1777.

Michalczuk, M., J. Niemiec, D. Pietrzak, and J. Mroczek. 2010. Effectiveness of vitamin D_3 and calcidiol (25-OH-D_3) application in feeding broiler chickens - Production performance and meat quality. Pol. J. Food Nutr. Sci. 60:121-126.

Miles, R., G. Butcher, P. Henry, and R. Littell. 2006. Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. Poult. Sci. 85:476-485.

Mitchell, R., H. Edwards, G. McDaniel, and G. Rowland. 1997. Dietary 1,25-dihydroxycholecalciferol has variable effects on the incidences of leg abnormalities, plasma vitamin D metabolites, and vitamin D receptors in chickens divergently selected for tibial dyschondroplasia. Poult. Sci. 76:338-345.

National Research Council. 1994. Nutrient requirements of poultry. 9th rev. ed. National academy press, Washington, DC, USA.

Norman, A. W. 1990. The avian as an animal model for the study of vitamin D endocrine system. J. Edp. Zool. Suppl. 4:37-45.

Quentin, M., I. Bouvarel, and M. Picard. 2005. Effects of the starter diet, light intensity, and essential amino acids level on growth and carcass composition of broilers. J. Appl. Poult. Res. 14:69-76.

Riddle, C., and X. Kong, 1992. The influence of diet on necrotic enteritis in broiler chicks. Avian Dis. 36:499–503.

Roura, E., J. Homedes, and K. C. Klasing. 1992. Prevention of immunologic stress contributes to the growth-permitting ability of dietary antibiotics in chicks. J. Nutri. 122:2382-2390.

SAS Institute. 2002. SAS/STAT User's Guide Release 9.02. SAS Inst. Inc, Cary, NC.

Scott, T. A. 2002. Evaluation of lighting programs, diet density, and short-term use of mash as compared to crumbled starter to reduce incidence of

sudden death syndrome in broiler chicks to 35 days of age. Can. J. Anim. Sci. 82:375-383.

Shim, K. S., K. T. Hwang, M. W. Son, and G. H. Park. 2006. Lipid metabolism and peroxidation in broiler chicks under chronic heat stress. Asian-Aust. J. Anim. Sci. 19:1206-1211.

Sklan, D., and Y. Noy. 2003. Crude protein and essential amino acid requirements in chicks during the first week posthatch. Br. Poult. Sci. 44:266-274.

Soares, J. H., Jr., J. M. Kerr, and R. W. Gray. 1995. 25-hydroxycholecalciferol in poultry nutrition. Poult. Sci. 74:1919-1934.

Soares, J. H., M. R. Swerdel, and E. H. Bossard. 1978. Phosphorus availability. 1. The Effect of chick age and vitamin D metabolites on the availability of phosphorus in defluorinated phosphate. Poult. Sci. 57:1305-1312.

Tesseraud, S., N. Maaa, R. Peresson, and A. M. Chagneau. 1996. Relative responses of protein turnover in three different skeletal muscle to dietary lysine. Poult. Sci. 75:641–650.

Torok, V. A., G. E. Allison, N. G. Percy, K. Ophel-Keller, and R. J. Hughes. 2011. Influence of antimicrobial feed additives on broiler commensal posthatch gut microbiota development and performance. Appl. Environ. Microbiol. 77:3380-3390.

Whitehead, C. C., H. A. McCormack, L. McTeir, and R. H. Fleming. 2004. High vitamin D_3 requirements in broilers for bone quality and prevention of tibial dyschondroplasia and interactions with dietary calcium, available phosphorus and vitamin A. Br. Poult. Sci. 45:425-436.

World Health Organization. 2002. The medical impact of the use of antimicrobials in food animals: report of a WHO meeting, Berlin, Germany.http://whqlibdoc.who.int/hq/1997/WHO_EMC_ZOO_97.4.pdf. Accessed August 2013.

Yarger, J. G., C. A. Saunders, J. L. Mcnaughton, C. L. Quarles, B. W. Hollis, and R. W. Gray. 1995. Comparison of dietary 25-hydroxycholecalciferol and cholecalciferol in broiler chickens. Poult. Sci. 74:1159-1167.

Zhao, J. P., J. L. Chen, G. P. Zhao, M. Q. Zheng, R. R. Jiang, and J. Wen. 2009. Live performance, carcass composition, and blood metabolite responses to dietary nutrient density in two distinct broiler breeds of male chickens. Poult. Sci. 88:2575-2584.

CHAPTER 3: Effect of early nutrition, 25-hydroxy vitamin D_3 and bacitracin methylene disalicylate on ileal morphology of broilers.

3.1 INTRODUCTION

Gastrointestinal health plays a key role in the overall health and performance of broiler chickens. The small intestine performs the majority of nutrient absorption in the gastrointestinal tract in chickens (Speier et al., 2012). Antibiotic growth promoters (AGP) have been administered in poultry feed to increase broiler performance because AGP reduce nutrient competition between the host and intestinal microflora, increase feed efficiency, and reduce mortality rate in broilers (Torok et al., 2011). These effects are attributable to a reduction in bacteria populations within the small intestine (Engberg et al., 2000). However, prophylactic antibiotic use has been scrutinized and banned in several countries (Casewell et al., 2003). Currently, supplementation of non-antibiotic feed additives is a new trend in poultry production which is intended to reduce the negative impact on broiler performance caused by the absence of prophylactic antibiotics (Mountzouris et al., 2007).

Other types of nutritional supplementation can also alter the intestinal microflora population and subsequently affect intestinal morphology (Van Leeuwen, 2004). For example, probiotics reduced villus height and thickness of muscularis in the duodenum and jejunum of broiler chickens (Awad et al., 2006; Tsirtsikos et al., 2011). Laudadio et al. (2012) reported that broilers fed 20.5% protein from d 14 to d 49 had a greater ratio of villus height to crypt depth than broilers fed 18.5% protein. However, little research has been conducted to study the effect of early

nutrient densities on the morphometric indices of the ileum, including villus length, villus width, and crypt depth.

Recently, 25-OH-D_3 has been shown to reduce the weight of the small intestine in broilers while increasing the ratio of villus height to crypt depth, potentially reducing the energy required for intestinal tract maintenance (Chou et al., 2009). Investigating the effects of dietary supplementations on ileal morphology would contribute to the understandings the mechanism of improved broiler performance and meat yield in the CHAPTER 1. We hypothesized that reduced early nutrient diets would increase crypt depth, but supplementation of BMD and 25-OH-D_3 would increase villus height of broilers in the current experiment.

3.2 MATERIALS AND METHODS

The experimental protocol was approved by the Animal Care and Use Committee of the University of Alberta and was performed in accordance with the guidelines of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

3.2.1 Experimental design.

A subset of broilers (n=96) were randomly selected for morphologic assays. These broilers received the same dietary nutrients and supplementations of BMD and 25-OH-D_3 as broilers in the experiment 1 (refer to CHAPTER 2, page 36).

3.2.2 Stocks and management.

A total of 3,200 Ross 708 mixed-sex broiler chicks were used in the current study. At day of hatch, 100 chicks were placed randomly into each of 32 floor pens. A lighting program of 20L:4D was used for the entire 40 d growing period.

Temperature was managed according to the primary breeder recommendations (Aviagen, 2009). Chicks had ad libitum access to feed and water. At d 28, mortality rate increased, and postmortem examination revealed that the increased mortality was attributable to a necrotic enteritis outbreak in the broilers that were not supplemented with BMD. The infection subsequently spread through other experimental pens. All pens were identically treated with penicillin (Pot-Pen 500,000,000 I.U., Vétoquinol Canada Inc.) via the drinking water for 2 consecutive days.

3.2.3 Tissue sampling and morphological examination.

Thirty-two birds (4 birds/treatment) were euthanized for gut morphology assessment at each of d 7, d 21 and d 35 of age. A segment (approximately 5 cm) was collected from the distal jejunum and proximal ileum, using Meckel's diverticulum as the segment's midpoint. These segments were placed immediately in 10% buffered neutral formalin for fixation, and subsequently embedded in paraffin. Embedded tissue samples were sliced into cross sections (approximately 5 μm thick) with a microtome, placed onto glass slides and stained with hematoxylin and eosin for examination (Sakamoto et al., 2000). The slides were coded and all slices examined and measurements conducted with a Zeiss Axiovert 200M inverted microscope using a light microscope at 10X magnification (Carl Zeiss MicroImaging Inc., Thornwood, NY). Only intact and well-oriented villus-crypt units were selected for measurements. The variables measured were villus height, villus width and crypt depth, ascertained by using the image analysis software MetaMorph (version 6.2, Universal Imaging, Downington, PA). Five

replicate measurements for each variable studied were taken from each chicken. These 5 measurements were then averaged to generate a mean value for each variable for each individual chicken (Nain et al., 2012). Crypt depth was determined from the base upward to the region of transition between the crypt and villus (Aptekmann et al., 2001). Villus height was determined from the tip of the villus to the villus-crypt junction. The average villus width was determined from two width measurements, one taken approximately 100 μm from the villus tip and the other at the villus-crypt junction (Geyra et al., 2001). The ratio between villus height and crypt depth was calculated by dividing villus height by crypt depth (Kettunen et al., 2001).

3.2.4 Statistical analysis.

Villus height, villus width, crypt depth and ratio of villus height to crypt depth were analyzed as a 3-way ANOVA, with dietary nutrient density from d 0 to d 14 of age, 25-OH-D_3, and BMD as main effects, using the MIXED procedure of SAS (SAS Institute, 2002). The individual broiler was the experimental unit in the model. Pairwise differences between means were determined with the PDIFF option of the LSMEANS statement. Differences between means were considered significant at $P< 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Villus height.

At d 7, broiler chicks fed the HIGH nutrient density diet without 25-OH-D_3 had longer villi than broilers fed either the HIGH nutrient density diet with 25-OH-D_3 or the LOW nutrient density diet without 25-OH-D_3 (Table 3.1). Chou et al.

(2009) reported that villus height was reduced in the ileum at d 14, d 21 and d 35 in broilers fed 25-OH-D_3, compared to birds fed cholecalciferol (the conventional means of vitamin D_3 supplementation). The authors postulated that 25-OH-D_3 supplementation could allow broilers to conserve energy normally required for the proliferation of epithelial cells (Chou et al., 2009). This could explain the reduction in villus height observed in correlation with 25-OH-D_3 in the current study. However, whether the 25-OH-D_3 supplementation would result in an overall increase or decrease in production efficiency was not mentioned by Chou et al. (2009). The effect of 25-OH-D_3 was investigated in the current study (reported in CHAPTER 2), and was found to have no effect on any parameter of broiler performance. This result suggested that the reduction of villus height observed in broilers fed the HIGH nutrient diet with 25-OH-D_3 neither impaired nor promoted the growth of the broilers. Vaezi et al. (2011) reported that broiler chicks fed 1.4% of lysine had greater epithelial cell protrusion on the tip of individual villi, compared to broilers fed diet containing 1.2% of lysine.

There was a nutrient density by 25-OH-D_3 by BMD interaction at d 21, showing a decreased villus height in broilers fed the LOW nutrient diet with BMD, compared to broilers fed the LOW diet without supplementation of either BMD or 25-OH-D_3. However, a greater villus height was recorded for broilers fed the HIGH nutrient diet with either BMD or 25-OH-D_3, compared to broiler fed the HIGH diet and received both BMD and 25-OH-D_3 (Table 3.1). It is unclear why the combination of the HIGH diet, BMD and 25-OH-D_3 reduced villus height in the current experiment. BMD was expected to decrease villus height. Previous

studies have shown that prophylactic antibiotics reduced the nutrient competition between microflora and the host, so that intestinal villi do not need to proliferate to catch nutrients in the gut lumen (Baurhoo et al., 2009, Viveros et al., 2011). Krinke and Jamroz (1996) reported that broilers fed prophylactic avorpacin had lower cell proliferation in the duodenum compared to birds fed a control diet. The reduced cell renewal could be attributed to increased efficiency of nutrient absorption by villi, because the major effect of AGP is to suppress enteric bacteria, resulting in a reduced bacterial colonization of the surface of the villi (Geyra et al., 2001). In the current experiment, the HIGH nutrient diet had greater impact on the villus height than supplementation of either BMD or 25-OH-D_3.

3.3.2 Villus width.

Although diets with different nutrient densities were only utilized until d 14, broilers fed the HIGH nutrient density diet had greater villus width than broilers fed the LOW density diet at d 35 (Table 3.1). The greater villus width could have been due to a higher lysine level (1.43%) in the HIGH nutrient density diet, compared to the LOW nutrient density diet lysine level (1.27%). Vaezi et al. (2011) reported that broilers fed 1.4% lysine had greater villus width in the ileum at d 14 than broilers fed 1.2% lysine. The NE outbreak at d 28 could have damaged the broiler villi in the current experiment. Golder et al. (2011) reported that NE reduced villus length, and increased crypt depth and villus width at d 18 in broilers which had been challenged with *Eimeria* and *Clostridium perfringens* at d 9 and d 15 of age. Imondi and Bird (1966) reported that the intestinal epithelial cells are replaced and extruded into the intestinal lumen within 48 to 96

h, suggesting that enterocyte renewal could have occurred by d 35 when the ileal tissues were collected in the current experiment. Iji et al. (2001) reported that increased villus width provided an increased absorptive area in broiler chicks, resulting in more nutrients being absorbed from the small intestine. In the current study, the HIGH nutrient diet increased BW at d 40, consistent with Iji et al. (2001).

3.3.3 Crypt depth.

Intestinal epithelial cells originate in the crypt (Cheng et al., 1974; Wilson et al., 1998). The dividing cells migrate upwards from the base to the luminal surface of the crypt, eventually forming the wall of the villus (Imondi and Bird, 1966). Crypt depth has been used as an indication of mucosal cell turnover (Xu et al., 2003). Nain et al. (2012) postulated that greater villus height resulted in greater nutrient absorption from the gut lumen, while smaller crypt depth required fewer nutrients for cell turnover. At d 7 in the current study, there was no difference in crypt depth for broilers fed the HIGH nutrient density diet, whether or not they were supplemented with 25-OH-D_3. However, crypt depth was significantly reduced by supplementation of 25-OH-D_3 in broilers fed the LOW nutrient density diet (Table 3.1 and Figure 3.1). Chou et al. (2009) found that dietary 25-OH-D_3 reduced crypt depth in the duodenum at d 14 and in the jejunum at d 21 and d 28, suggesting a lower rate of epithelial cell migration from the crypt to the villus. It should be noted that villus height was reduced at a lower rate than crypt depth. For example, at d 14 villus height was reduced by 8.6% and crypt depth by 34.3% in birds fed 25-OH-D_3, compared with birds fed conventional vitamin D_3 (Chou et

al., 2009). Reduced cell production conserves energy, allowing it to be directed toward alternate biological purposes such as growth and immunity (Klasing et al., 1987).

3.3.4 Villus height to crypt depth ratio.

In broilers fed the LOW nutrient density diet, the ratio of villus height to crypt depth was significantly increased at d 7 with 25-OH-D_3 supplementation (Figure 3.2). Chou et al. (2009) also reported that the ratio of villus height to crypt depth was increased by supplementation of 25-OH-D_3, finding that the metabolite increased the ratio by decreasing the crypt depth in the ileum. 25-OH-D_3 may have also influenced cellular differentiation and migration along the cell wall, in conjunction with its effect on the crypt because chicken enterocytes differentiate in both the crypt and along the length of the villus (Uni et al., 1998). At d 21, a greater ratio was observed for broilers fed the HIGH nutrient diet with BMD compared to broilers fed the HIGH nutrient diet with 25-OH-D_3 and BMD or the LOW nutrient diet with BMD only (Figure 3.3). The HIGH nutrient diet could have increased proliferation of the enterocytes along the villi, resulting in longer villi (Uni et al., 1998). The mechanism by which the combination of 25-OH-D_3 and BMD decreased the ratio of villus height to crypt depth in broilers fed the HIGH nutrient diet was not determined in the current experiment.

Effects of dietary supplementations on ileal morphology were transient, only being observed at d 7 and d 21 for villus length, crypt depth and at d 35 for villus width. The HIGH nutrient diet provided readily digestible nutrients for broilers, resulting in shorter crypt depth and villi that were both wider and longer. 25-OH-

D_3 and BMD had varying effects on villus height, depending upon nutrient levels, and whether the two treatments were combined. The decrease in villus height caused by 25-OH-D_3 did not negatively affect BW of broilers, suggesting that the supplementation of 25-OH-D_3 or BMD in broilers fed the HIGH nutrient diet may help broilers to conserve energy from generating new enterocytes.

Table 3.1 Effect of starter nutrient density, 25-OH-D_3 and BMD on the ileal morphology of broiler chickens at d 7, d 21, d 35

Effect	N	D	A	Villus height (µm)			Villus width (µm)			Crypt depth (µm)		
Day				7	21	35	7	21	35	7	21	35
N[1]	HIGH			488	752	977	111	101	120[a]	124	134	153
	LOW			461	711	853	90	106	78[b]	129	148	146
D[2]		0		488	741	904	109	104	103	135	144	156
		69		461	722	926	94	103	94	118	139	143
A[3]			0	447	763	912	101	114	95	126	137	149
			0.5	501	700	918	101	94	102	127	146	150
N x D	HIGH	0		534[a]	787	995	123	106	124	119[b]	129	153
		69		441[b]	718	958	100	97	115	129[ab]	139	153
	LOW	0		442[b]	695	813	94	102	82	150[a]	158	158
		69		480[ab]	727	894	86	110	73	107[b]	138	134
N x A	HIGH		0	453	772	944	121	108	117	121	139	140
			0.5	522	732	1009	102	95	122	128	129	166
	LOW		0	442	754	880	81	120	72	131	134	159
			0.5	480	668	826	99	94	83	126	163	133
D x A		0	0	434	761	897	106	114	93	133	134	154
			0.5	542	722	910	111	95	113	136	153	158
		69	0	461	766	927	96	114	96	118	139	145
			0.5	461	679	925	90	94	92	117	138	142
N x D x A	HIGH	0	0	448	673[abc]	892	132	116	110	121	133	129
			0.5	620	901[a]	1098	114	96	138	118	125	178
		69	0	458	872 [a]	996	109	101	125	120	146	151
			0.5	424	564[bc]	920	90	93	106	138	132	154
	LOW	0	0	421	848[ab]	903	80	112	77	146	135	179
			0.5	463	543[c]	723	108	93	87	155	181	138
		69	0	463	660[abc]	858	83	128	68	117	132	139
			0.5	497	794[abc]	930	90	94	78	97	145	129
SEM				25.8	67.9	83.3	10.1	10.8	10.7	9.6	9.4	13.2
Source of variation				---------- Prob > F ----------								
N				0.348	0.576	0.180	0.066	0.668	0.001	0.681	0.171	0.614
D				0.331	0.790	0.803	0.167	0.967	0.449	0.115	0.627	0.383
A				0.063	0.395	0.951	0.968	0.098	0.514	0.909	0.370	0.988
N x D				0.025	0.497	0.516	0.475	0.454	0.965	0.016	0.158	0.411
N x A				0.584	0.754	0.518	0.104	0.580	0.824	0.554	0.061	0.083
D x A				0.067	0.743	0.932	0.624	0.951	0.320	0.859	0.339	0.812
N x D x A				0.081	0.007	0.148	0.658	0.559	0.322	0.236	0.514	0.192

[a, b, c] Means within the same column within effect with no common superscripts are significantly different ($P < 0.05$)

[1]Starter nutrient density: HIGH (3,025 kcal/kg; 23.9% CP), or LOW (2,858 kcal/kg; 22.3% CP); fed from 0 to 14 d.

[2]Broilers fed a diet containing 4,000 IU/kg of vitamin D_3 or supplemented with 69 µg of 25-OH-D_3 in addition to 4,000 IU/kg of vitamin D_3.

[3]Bacitracin Methylene Disalicylate (BMD® 110): 0 or 0.5 g/kg premix, containing 55 mg of BMD per kg.

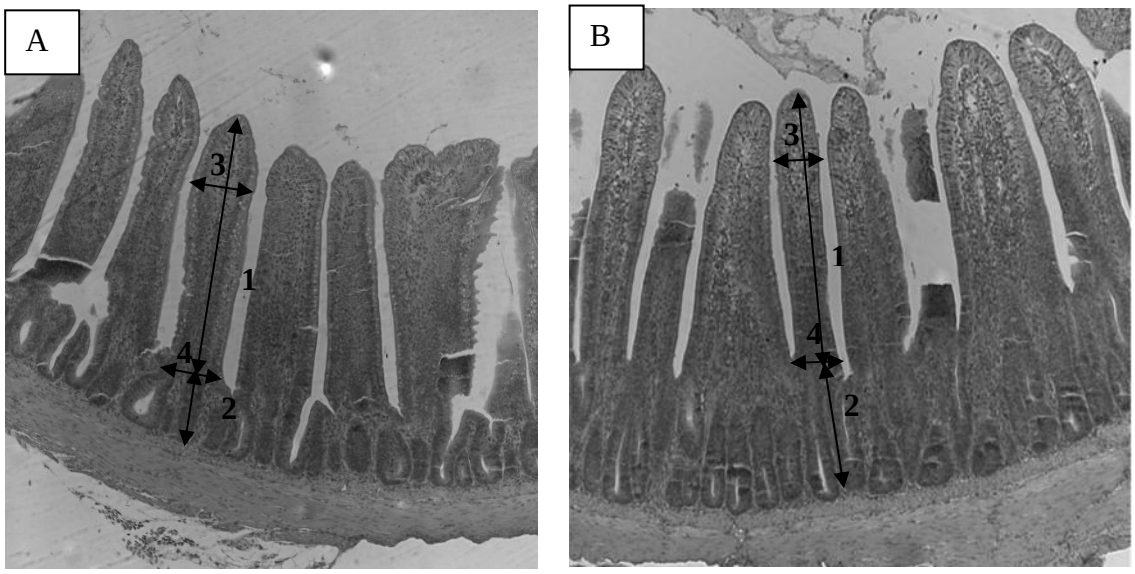

Figure 3.1 Villus height and crypt depth in the ileum of broiler chicks at d 7 of age, fed Low nutrient (22% CP and 2,858 kcal/kg) with 25-OH-D_3 (A), or Low nutrient without 25-OH-D_3 (B). (1) villus height, (2) crypt depth, (3) apical width, (4) basal width, magnification: X 10.

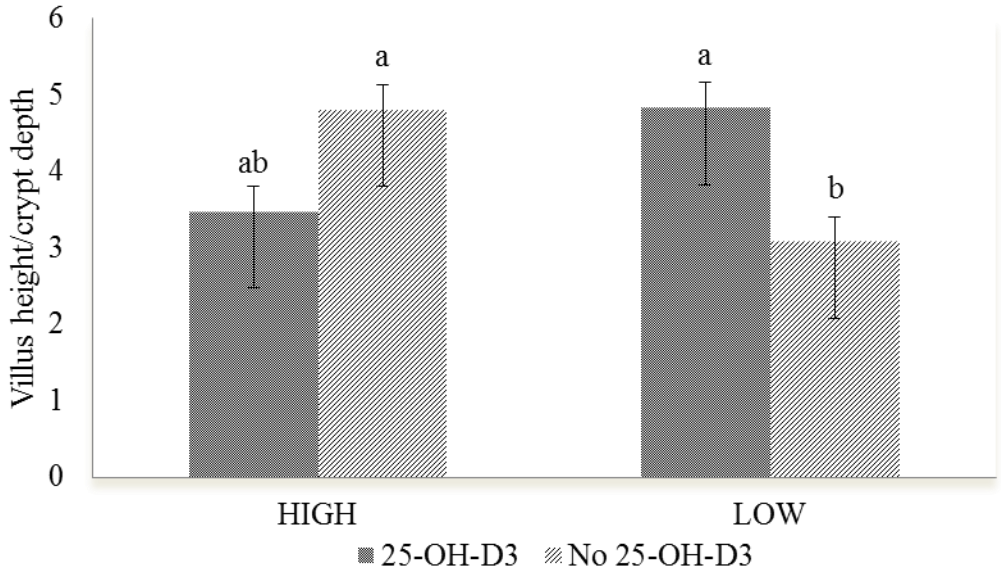

Figure 3.2 Dietary nutrient density x 25-OH-D_3 2-way interaction for ratio of villus height to crypt depth at d 7 ($P < 0.05$). Broiler chicks were fed either a HIGH (3,025 kcal/kg; 23.9% CP), or LOW (2,858 kcal/kg; 22.3% CP) nutrient density diet from 0 to 14 d, with 0 or 69 µg/kg of 25-OH-D_3.

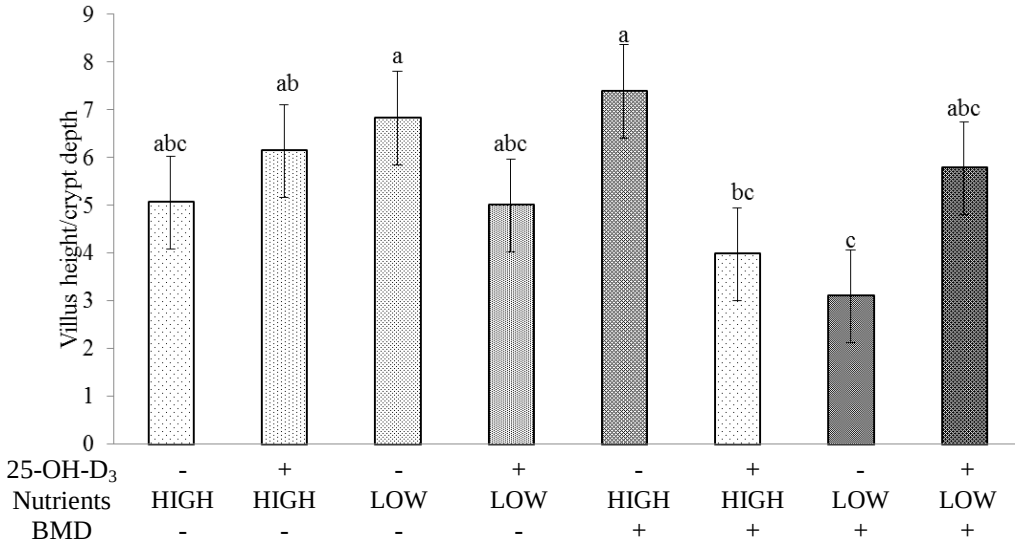

Figure 3.3 Dietary nutrient density x 25-OH-D_3 x BMD 3-way interactions observed for ratio of villus height to crypt depth at d 21 ($p < 0.05$). From 0 to 14 d of age, broiler chicks were fed either a HIGH (24% CP and 3,025 kcal/kg) or LOW (22% CP and 2,858 kcal/kg) nutrient density diet, with 0 or 69 µg/kg 25-OH-D_3, and 0 or 0.5g/kg bacitracin methylene disalicylate (BMD® 110).

3.4 REFERENCES

Aptekmann, K. P., S. M. B. Artoni, M. A. Stefanini, and M. A. Orsi. 2001. Morphometric analysis of the intestine of domestic quails (*Coturnix coturnix japonica*) treated with different levels of dietary calcium. Anat. Histol. Embryol. 30:277-280.

Aviagen. 2009. Ross broiler management manual. http://www.poultry hub.org/wp-content/uploads/ 2012/06/Ross US broiler manual 09. pdf. Accessed January 2013.

Awad, W. A, J. Bohm, E. Razzazi-Fazeli, K. Ghareeb, and J. Zentek. 2006. Effect of addition of a probiotic microorganism to broiler diets contaminated with deoxynivalenol on performance and histological alterations of intestinal villi of broiler chickens. Poult. Sci. 85:974-979.

Baurhoo, B., P. R. Ferket, and X. Zhao. 2009. Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. Poult. Sci. 88:2262-2272.

Canadian Council on Animal Care. 1993. A guide to the care and the use of experimental animals. 2nd ed. CCAC, Ottawa, ON, Canada.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52:159-161.

Cheng, H., and C. P. Leblond. 1974. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial cell types. Am. J. Anat. 141:537-561.

Chou, S. H., T. K. Chung, and B. Yu. 2009. Effects of supplemental 25-hydroxycholecalciferol on growth performance, small intestinal morphology, and immune response of broiler chickens. Poult. Sci. 88:2333-2341.

Engberg, R. M., M. S. Hedemann, T. D. Leser, and B. B. Jensen. 2000. Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. Poult. Sci. 79:1311-1319.

Geyra, A., Z. Uni, and D. Sklan. 2001. Enterocyte dynamics and mucosal development in the posthatch chick. Poult. Sci. 80:776-782.

Golder, H. M., M. S. Geier, R. E. Forder, P. I. Hynd, R. J. Hughes. 2011. Effects of necrotic enteritis challenge on intestinal micro-architecture and mucin profile. Br. Poult. Sci. 52:500-506.

Iji, P. A., A. Saki, and D. R. Tivey. 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. Br. J. Nutr. 42:505-513.

Imondi, A. R., and F. H. Bird. 1966. The turnover of intestinal epithelium in the chick. Poult. Sci. 45:142-147.

Kettunen, H., K. Tiihonen, S. Peuranen, M. T. Saarinen, and J. C. Remus. 2001. Dietary betaine accumulates in the liver and intestinal tissue and stabilizes the intestinal epithelial structure in healthy and coccidia-infected broiler chicks. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 130:759-769.

Klasing, K. C., D. E. Laurin, R. K. Peng, and D. M. Fry. 1987. Immunologically mediated growth depression in chicks: influence of feed intake, corticosterone and interleukin-1. J. Nutr. 117:1629-1637.

Krinke, A. L., and D. Jamroz. 1996. Effects of feed antibiotic avoparcine on organ morphology in broiler chickens. Poult. Sci. 75:705–710.

Laudadio, V., L. Passantino, A. Perillo , G. Lopresti, A. Passantino, R. U. Khan, and V. Tufarelli. 2012. Productive performance and histological features of intestinal mucosa of broiler chickens fed different dietary protein levels. Poult. Sci. 91:265-270.

Mountzouris, K. C., P. Tsistsikos, E. Kalamara, S. Nitsh, G. Schatzmayr, and K. Fegeros. 2007. Evaluation of the efficacy of a probiotic containing *Lactobacillus, Bifidobacterium, Enterococcus,* and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poult. Sci. 86:309-317.

Nain, S., R. A. Renema, M. J. Zuidhof, and D. R. Korver. 2012. Effect of metabolic efficiency and intestinal morphology on variability in n-3 polyunsaturated fatty acid enrichment of eggs. Poult. Sci. 91:888-898.

National Research Council. 1994. Nutrient requirements of poultry. 9th rev. ed. National Academy Press, Washington, DC, USA.

Sakamoto, K., H. Hirose, A. Onizuka, M. Hayashi, N. Futamura, Y. Kawamura, and T. Ezaki. 2000. Quantitative study of changes in intestinal morphology and mucus gel on total parenteral nutrition in rats. J. Surg. Res. 94:99-106.

SAS Institute. 2002. SAS/STAT User's Guide Release 9.02. SAS Inst. Inc, Cary, NC.

Speier, J. S., L. Yadgary, Z. Uni, and E. A. Wong. 2012. Gene expression of nutrient transporters and digestive enzymes in the yolk sac membrane and small intestine of the developing embryonic chick. Poult. Sci. 91:1941–1949.

Torok, V. A., G. E. Allison, N. G. Percy, K. Ophel-Keller, and R. J. Hughes. 2011. Influence of antimicrobial feed additives on broiler commensal posthatch gut microbiota development and performance. Appl. Environ. Microbiol. 77:3380-3390.

Tsirtsikos, P., K. Fegeros, C. Balaskas, A. Kominakis, and K. C. Mountzouris. 2011. Dietary probiotic inclusion level modulates intestinal mucin composition and mucosal morphology in broilers. Poult. Sci. 91:1860-1868.

Uni, Z., R. Platin, and D. Sklan. 1998. Cell proliferation in chicken intestinal epithelium occurs both in the crypt and along the villus. J. Comp. Physiol. B. 168: 241-247.

Vaezi, G., M. Teshfam, S. Bahadoran, H. Farazyan, and S. Hosseini. 2011. Effects of different levels of lysine on small intestinal villous morhology in starter diet of broiler chickens. Global Veterinaria. 6:523-526.

Van Leeuwen P., J. M. Mouwen, J. D. Van Der Klis, M.W. Verstegen. 2004. Morphology of the small intestinal mucosal surface of broilers in relation to age, diet formulation small intestinal microflora and performance. Br. Poult. Sci. 45:41-8.

Viveros, A., S. Chamorro, M. Pizarro, I. Arija, C. Centeno, and A. Brenes. 2011. Effects of dietary polyphenol-rich grape products on intestinal microflora and gut morphology in broiler chicks. Poult. Sci. 90:566–578.

Wilson, J. W., D. M. Pritchard, J. A. Hickman, and C. S. Potten. 1998. Radiation-induced p53 and p21WAF-1/CIP1 expression in the murine intestinal epithelium: apoptosis and cell cycle arrest. Am. J. Pathol. 153:899-909.

Xu, Z., C. Hu, M. Xia, X. Zhan, and M. Wang. 2003. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poult. Sci. 82:1030-1036.

CHAPTER 4: Effect of early nutrition, 25-hydroxy vitamin D_3 and bacitracin methylene disalicylate on broiler innate immunity

4.1 INTRODUCTION

Necrotic enteritis (NE), a disease caused by *Clostridium perfringens,* remains a threat to the poultry industry (Watkins et al., 1997). NE usually occurs in broiler chickens at about 4 weeks after hatching and may cause substantial damage in the intestinal tract of broilers, causing mortality up to 50% in the infected flocks (Palliyeguru et al., 2010). Skinner et al. (2010) reported that NE caused approximately a $2 billion annual loss to the poultry industry internationally, mainly attributable to costs of medication and impaired growth. Prophylactic antibiotics are among the most common methods of controlling NE, however, prophylactic antibiotic use has been banned in several countries (Casewell et al., 2003). The elimination of prophylactic antibiotics resulted in a two-fold increase in therapeutic antibiotic use in response to NE outbreaks in European countries (Emborg et al., 2001). Providing nutrients that can optimize immune functions could be an alternative strategy to increase resistance of broiler chicks to infectious diseases (Kogut, 2009).

Innate immunity is the first defensive line against invading pathogens. Similar to mammals, broiler chickens have developed both innate and humoral immunity against invading pathogens (Swaggerty et al., 2009). Heterophils, monocytes and macrophages are among the primary types of leukocytes (He et al., 2003). These mediating cells are able to engulf antigens, and secrete antimicrobial peptides and signal chemicals, such as acute phase proteins and pro-inflammatory

cytokines. The activation of these phagocytic cells results in a pro-inflammatory response, causing high body temperature and increased metabolic rate (Klasing and Korver, 1997). These events do not favor growth, feed intake or feed efficiency in the growing broilers (Klasing and Korver, 1997). However, an effective innate immune response to immunogens enables broilers to mount a humoral response, resulting in down regulation of the inflammatory response (Grindstaff, 2008). For example, chickens that have stronger innate immune response could limit the infection, caused by parasites, and had greater body weight gain than the susceptible line (Swaggerty et al., 2011).

High dietary nutrient density has been shown to increase the innate immune response to antigens by supporting the maturation of phagocytic cells (Kidd et al., 2005). This could enhance the biological functions of innate immune cells including adherence, chemotaxis, bacterial killing and phagocytosis (Kogut et al., 1995; Moyes et al., 1998). Aslam et al. (1998) reported that vitamin D is an immunomodulating substance, which can regulate cellular immune response. 25-OH-D_3 is a hydroxylated product of cholecalciferol known to be as much as 4 times more potent than cholecalciferol (Soares et al., 1978). However, natural serum 25-OH-D_3 may decline in broilers due to either stress conditions (Shim et al., 2006) or diseases (Whitehead et al., 2004).

Bacitracin methylene disalicylate (BMD) is among several prophylactic antibiotics that have been used as supplements in poultry diets in order to control disease and improve growth (Chapman and Johnson, 2002). BMD has been shown to increase average daily feed intake, average daily weight gain, feed

efficiency, and growth, while decreasing the mortality associated with necrotic enteritis (NE) in broiler chickens, compared with birds fed the same diet without BMD (Brennan et al., 2003). Miles et al. (2006) reported that BMD could limit the proliferation of intestinal microflora, subsequently reduce the nutrient competition between the host and gut microflora. A reduction in microflora population could reduce the nutrient competition between the host and microflora populations and decrease the immunologic stress as well.

The objective of the current experiment was to investigate the effects of nutrient density in early nutrition, 25-OH-D_3 and BMD on several indicators of innate immunity of broiler chicks, including *ex vivo* bacterial killing capacity, phagocytosis and nitric oxide concentration. We hypothesized that feeding the LOW diet could decrease the innate immune response of broilers.

4.2 MATERIALS AND METHODS

The experimental protocol was approved by the Animal Care and Use Committee of the University of Alberta and was performed in accordance with the guidelines of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

4.2.1 Experimental design.

A subset of broilers were randomly selected for innate immune assays (n= 72), and nitric oxide assays (n= 128). These broilers were selected from the initial trial population, received the same dietary nutrient densities and supplementation of 25-OH-D_3 and BMD as broilers in the CHAPTER 2 (page 36).

4.2.2 Stocks and management.

A total of 3,200 Ross 708 mixed-sex broiler chicks were used in this study. At day of hatch, 100 chicks were placed randomly into each of 32 floor pens. A lighting program of 20L:4D was used for the entire 40 d growing period. Temperature was managed according to the primary breeder recommendations (Aviagen, 2009). Chicks had ad libitum access to feed and water.

4.2.3 Blood sampling.

On d 13, 9 birds per treatment were randomly selected for the investigation of bactericidal capacity and phagocytosis of phagocytic cells present in chicken blood. Whole blood samples were collected from individual chicks by brachial venipuncture. The blood was collected in a tube with EDTA to prevent coagulation. For NO assay, on d 14, 4 broilers per pen were randomly selected and half (2 replicates/pen) were injected intra-abdominally with 3 mL of a 100 µg/mL lipopolysaccharide (LPS) solution to induce an inflammatory response and assess the effect of the treatments on the innate immune response. The other half served as non-injected control following methodology of Korver et al. (1998). After LPS injection, chicks were monitored every hour for 3 h before 2 mL blood samples were collected to measure NO concentration in the plasma. Chick plasma was separated from the whole blood by centrifugation at 2,000 rpm for 15 min and frozen at -20°C for subsequent analysis of total NO concentration using the Griess reagent (Cayman Chemical Co., Ann Arbor, MI, US) according to methodology of Bowen et al. (2007).

4.2.4 Bacterial killing capacity.

Ex vivo bacterial killing capacity was assessed by comparing the growth of *Escherichia coli*, mixed with whole blood on a medium, relative to bacterial growth on a medium that did not contain whole blood (Millet et al., 2007). One *E. coli* pellet (MicroBiologics Inc., Saint Cloud, MN, USA) was diluted in 40 ml of sterile phosphate saline buffer (pH 7.4) to serve as a stock solution. This *E. coli* stock solution was again diluted with 8 ml of PBS to obtain the working solution. The amount of bacteria used to challenge the white blood cells of broiler chicks was determined from a duplicate of fresh working solution. Forty µl of heparinized blood were diluted with Gibco CO_2 independent media supplemented with 4 mM of L-Glutamine at the ratio of 1:10. Each sample of diluted blood was mixed with the working *E. coli* solution. The mixture solution contained approximately 250 *E. coli* particles per 50 µl of diluted blood. Fifty µl of each sample were incubated for 90 min at 41°C and then plated on Petri dishes with tryptic soy agar. Plates were incubated overnight at 37°C and the percentage of bacteria killed was determined as follows: [(total *E. coli* particles added to the working solution - surviving *E. coli* colonies counted)/ total *E. coli* particles added to the working solution] x 100.

4.2.5 Phagocytosis.

Phagocytosis activity was measured using flow cytometry and fluorescent bacteria in accordance to Millet et al. (2007) with some modifications. Briefly, whole blood was diluted with CO_2 - independent media (Gibco, Invitrogen Corportion, Burlington, ON, CA) at the ratio of 1:20, and supplemented with

100 µg/ml of penicilin, 100 µg/ml of streptomycin and 4 mM L-Glutamine. *E. coli* particles (K-12 strain; Molecular Probes (E-2864), Invitrogen Incorporation, Burlington, ON, CA) were mixed with diluted blood at a ratio of 1:100. The mixture was incubated at 41°C for 15 min. The phagocytic reaction was inactivated by placing the mixture on ice for 5 min. Non-phagocytized bacteria were removed from the mixed solution by washing it twice with 300 µl of CO_2 - independent media supplemented with penicillin (100 µg/ml), 100 µg/ml of streptomycin (100 µg/ml) and L-Glutamine (4 mM). Red blood cells were removed by a lysing reaction using the solution dd H_2O + 0.83% w/v NH_4Cl + 0.17% w/v $NaHCO_3$ + 0.2% EDTA. A clear wash buffer (0.5g bovine serum albumin, BSA), 1 ml EDTA and 500 ml Hanks balanced salt solution, HBSS) was added to each sample, then transferred to a sterile tube and stored on ice for subsequent fluorescence measurement by flow cytometry (Becton-Dickinson FacScan Flowcytometry, Sunnyvale, CA). Percentage of cells that engulfed *E. coli*, and the average number of *E. coli* per cell (phagocytic capacity) were assessed.

4.2.6 Plasma nitric oxide assay.

In vitro nitric oxide production was assessed. Total nitrate and nitrite were measured in the plasma using the Cayman Chemical Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). Chick plasma was analyzed following the kit instructions. Briefly, the plasma was thawed at room temperature and centrifuged at 10,000 g for 10 min through Microcon YM 30 Centrifugal Filters (Millipore Corporation, Bedford, MA). The filtered plasma

was then diluted (1:2) with deionized distilled water. Eighty µl of diluted plasma was added per well in a 96-well plate, followed by 10 µl of the Enzyme Cofactor mixture, and subsequently 10 µl of the Nitrate Reductase mixture. A nitrite standard curve was also generated in each plate to determine total nitrite concentration (µM) by using the mixture of 0.9 ml of Assay Buffer and 0.1 ml of reconstituted nitrate standard. The plate was covered and incubated at room temperature for 1 h before adding 50 µl of Griess Reagent R1 and Griess Reagent R2, respectively, to individual wells (standards and unknowns). The final assay volume after addition of Griess Reagent was 200 µl. The plate was placed at room temperature for 10 min to allow the colour to develop. The absorbance was read at 540 nm (A_{540}) using a plate reader (SpectraMax M2 Fluorescence Plate Reader, Sunnyvale, CA), and the total concentration of Nitrate and Nitrite was determined as follows: [Nitrate + Nitrite] (µM)= [(A_{540} - $y_{intercept}$)/slope] x [(200/ volume of sample used)] x dilution.

4.2.7 Statistical analysis.

Bacterial killing and phagocytosis were analyzed as a 3-way ANOVA with dietary nutrient density, 25-OH-D_3, and BMD as main effects by using the MIXED procedure of SAS. Nitric oxide concentration was analyzed as a 4-way ANOVA, with LPS treatment, dietary nutrient density, 25-OH-D_3, and BMD as main effects (SAS Institute, 2002). Pen was considered as a random effect in the model. Differences between main effects, means, and their interactions were classified by pairwise comparisons, and unless otherwise noted, differences were considered significant at $P< 0.05$.

4.3 RESULTS AND DISCUSSION

4.3.1 Bacterial killing capacity and phagocytosis.

Broiler chicks fed the HIGH nutrient density diet showed a nearly significant increase (10%) in *ex vivo E. coli* killing capacity in the whole blood as compared to blood from chicks that were fed the LOW nutrient density (P= 0.058). However, there was no difference in the percentage of phagocytosis, and the number of *E. coli* engulfed per cell (Table 4.1). The increased bacterial killing capacity of the whole blood could be due to increased phagocytic acitivities, although the cell population was not classified in the current experiment. Cheema et al. (2003) reported that broiler chicks fed 21.9% CP had greater phagocytic response to phytohaemagglutinin-P than those fed 20.1% CP at 4 weeks of age.

There was no difference in the percentage of phagocytosis, and the number of *E. coli* engulfed per cell between broilers fed BMD and the non-antibiotic group (Table 3.1). Miles et al. (2006) reported that the main beneficial effect of BMD is to suppress gastrointestinal bacteria population. A reduction in bacteria population alleviates the competition for nutrients between enteric microflora and the host (Engberg et al., 2000). The *ex vivo* bactericidal result from the current study suggested that prophylactic antibiotic BMD did not affect the phagocytic activities of the innate immune cells. The increased in feed efficiency or reduced mortality could have been due to the effect of BMD on the microflora populations in the intestinal tract of broilers.

In the current study, we did not detect an effect of either 25-OH-D_3 or BMD on bacterial killing capacity (Table 3.1). 25-OH-D_3 has been shown to modulate the immune response (Chou et al., 2009). Golemboski et al. (1990) reported that chicken phagocytic cells are capable of converting 25-OH-D_3 into 1α, 25-$(OH)_2D_3$, a hormone that supports immune cell response to pathogens. Bar et al. (1980) reported that 1α, 25-$(OH)_2D_3$ increased leukemic hematopoietic cells to differentiate into monocytes-macrophages. Thus, an increased in bacterial killing capacity was expected when chicks were supplemented with 25-OH-D_3. However, the inclusion rate could influence the conversion of 25-OH-D_3 metabolite because of a very tight regulation of the 1α, 25-$(OH)_2D_3$. For example, Mitchell et al. (1997) reported that increased level of 1α, 25-$(OH)_2D_3$ could decrease plasma 25-OH-D_3, suggesting that there could be a conversion limit between 25-OH-D_3 and 1α, 25-$(OH)_2D_3$. Chou et al. (2009) reported that supplemental 25-OH-D_3 had no effect on the innate immunity, but increased T-cell proliferation in broiler chickens. For example, supplemental 25-OH-D_3 reduced total serum IgA and IgG in uninfected birds at d 14, but increased total serum IgG in infected birds on d 21, suggesting that 25-OH-D_3 had varied effects, depending upon the health status of broilers (Chou et al., 2009). Thus, the exact regulatory role of 25-OH-D_3 remains nebulous, and the non-effect observed in the current experiment might be attributable to the types of assays that we targeted in the innate immunity. Andreasen et al. (1993) reported that heterophils isolated from 6 to 8 week old broiler chicks performed greater chemotaxis to a pathogenic strain of *Staphylococcus aureus* compared with a

non-pathogenic strain of *Staphylococcus aureus*. In the current experiment, a non-pathogenic strain of *E. coli* was used for both the bacterial killing capacity assay, and the phagocytosis assay. Chick heterophils may be able to react differently to different bacteria (Andreasen et al., 1993). Therefore, it would be of interest to conduct bactericidal assays with different strains of bacteria in future experiments.

4.3.2 Nitric oxide concentration.

In broiler chicks which were challenged with LPS, NO level was three times greater than in non-injected control chicks (63.34 vs. 22.36 mM, Table 4.2). This result was in agreement with the findings of Bowen et al. (2007), who reported that LPS can elicit an inflammatory response from monocytes and macrophages. There was no effect of dietary treatments on nitric oxide concentration (Table 3.2). Mature monocytes or macrophages are capable of releasing nitric oxide when challenged with *Salmonella typhimurium* LPS (Dil and Qureshi, 2002a; Dil and Qureshi, 2002b). In the current experiment, we did not find the effect of 25-OH-D_3 on the nitric oxide level, and this result was in agreement with Fritts et al. (2004), who reported that neither dietary source nor level of vitamin D_3 metabolites affected innate immunity of broiler chicks. However, 25-OH-D_3 was seen to increase the proliferation of lymphocytes including T and B cells rather than leukocyte compartments (heterophils, monocytes, macrophages) when 69 µg of 25-OH-D_3 (2,760 IU/kg of feed) was fed to broiler chicks from 0 to 21 d (Chou et al., 2009).

In conclusion, BMD did not affect any indices of innate immune function. The HIGH nutrient density diet nearly increased the *ex vivo E. coli* killing capacity relative to the LOW nutrient density diet (P= 0.058). If the *ex vivo* result holds true *in vivo,* this finding could benefit the broiler industry as the chicks might be more resistant to infectious diseases when fed a HIGH nutrient density diet. Supplementation of 25-OH-D_3 (69 µg/kg, equivalent to 2,760 IU/kg) in addition to cholecalciferol (4000 IU/kg) had no effect on the innate immunity of broilers within the parameters measured. It is possible that an effect of 25-OH-D_3 would have been observed if this metabolite was partially replaced for total amount of cholecalciferol in broiler diets.

Table 4.1 Effect of early nutrient density, 25-OH-D_3 and antibiotic on bacterial killing capacity and phagocytosis

Effect	% *E. coli* killed[4]	% of cells phagocytising[5]	Phagocytic capacity[6]
N[1]			
HIGH	76.56	40.85	256
LOW	65.39	34.98	267
D[2]			
0	74.10	39.17	263
69 µg/kg	68.20	36.70	261
BMD[3]			
0	71.25	35.92	269
0.5 g/kg	71.14	39.94	255
Pooled SEM	7.99	4.42	11.99
Source of variation	P-values		
N	0.058	0.311	0.516
D	0.328	0.670	0.880
BMD	0.985	0.490	0.407
N x D	0.541	0.961	0.769
N x BMD	0.666	0.780	0.663
D x BMD	0.175	0.520	0.221
N x D x BMD	0.214	0.654	0.630

[1] Starter nutrient density: HIGH (3,025 kcal/kg; 23.9% CP), or LOW (2,858 kcal/kg; 22.3% CP) fed from 0 to 14 d.

[2] Broilers fed a diet supplemented with 0 or 69 µg of 25-OH-D_3 (all basal diets contained an additional 4,000 IU/kg of vitamin D_3).

[3]Bacitracin Methylene Disalicylate (BMD® 110): 0 or 0.5 g/kg, containing 55 mg of BMD per kg.

[4] Percentage of killing bacteria of broiler when the whole blood was plate cultured and incubated with *E. coli* bacteria.

[5] The number of cells which contain at least one *E. coli* bioparticle.

[6] Average number of *E. coli* per cell.

Table 4.2 Effect of early nutrition, 25-OH-D_3 and antibiotic on nitric oxide concentration in plasma of chicks

Treatments	NO level (µM)	SEM	Probability
N[1]			
HIGH	34.95	1.09	0.2509
LOW	40.52	1.09	
D[2]			
0	37.44	1.09	0.9338
69µg/kg	37.84	1.09	
BMD[3]			
0	35.49	1.09	0.3622
0.5g/kg	39.89	1.09	
LPS[4]			
Non-injected	22.36[b]	1.08	<0.0001
Injected	63.34[a]	1.08	

[a, b] Mean within the same column within effect with no common superscripts are significantly different ($P < 0.05$).

[1] Broiler chicks fed nutrient density diet containing 2,858Kcal/kg and 22% CP or 3,025 Kcal/kg and 24% CP from d 0 to d 14.

[2] Broilers fed a diet supplemented with 0 or 69 µg of 25-OH-D_3 (all basal diets contained an additional 4,000 IU/kg of vitamin D_3).

[3]Bacitracin Methylene Disalicylate (BMD® 110): 0 or 0.5g/kg, containing 55 mg of BMD per kg.

[4]Lipopolysaccharide (LPS) individually injected to half of the birds (3 ml of a 100 µg/ml) *Salmonella typhimurium* LPS solution, the other half were non-injected (control).

4.4 REFERENCES

Andreasen, J. R., Jr., C. B. Andreasen, M. Anwer, and A. E. Sonn. 1993. Heterophil chemotaxis in chickens with natural staphylococcal infections. Avian Dis. 37:284-290.

Aslam, S., J. Garlich, and M. A. Qureshi. 1998. Vitamin D deficiency alters the immune responses of broiler chicks. Poult. Sci. 77:842-849.

Aviagen. 2009. Ross broiler management manual. http://www.poultryhub.org/wpcontent/uploads/2012/06/Ross_US_broiler_Manual_09.pdf. Accessed January 2013.

Bar, A., M. Sharvit, D. Noff, S. Edelstein, and S. Hurwitz. 1980. Absorption and excretion of cholecalciferol and of 25-hydroxycholecalciferol and metabolites in birds. J. Nutr. 110:1930-1934.

Bowen, O. T., G. F. Erf, M. E. Chapman, and R. F. Wideman. 2007. Plasma nitric oxide concentrations in broilers after intravenous injections of lipopolysaccharide or microparticles. Poult. Sci. 86:2550–2554.

Brennan, J., J. Skinner, D. Barnum, and J. Wilson. 2003. The efficacy of bacitracin methylene disalicylate when fed in combination with narasin in the management of necrotic enteritis in broiler chickens. Poult. Sci. 82:360-363.

Canadian Council on Animal Care. 1993. A guide to the care and the use of experimental animals. 2nd ed. CCAC, Ottawa, ON, Canada.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52:159-161.

Chapman, H., and Z. Johnson. 2002. Use of antibiotics and roxarsone in broiler chickens in the USA: analysis for the years 1995 to 2000. Poult. Sci. 81:356-364.

Cheema, M. A., M. A. Qureshi, and G. B. Havenstein. 2003. A comparison of the immune profile of commercial broiler strains when raised on marginal and high protein diets. Int. J. Poult. Sci. 2:300-312.

Chou, S. H., T. K. Chung, and B. Yu. 2009. Effects of supplemental 25-hydroxycholecalciferol on growth performance, small intestinal morphology, and immune response of broiler chickens. Poult. Sci. 88:2333-2341.

Dil, N., and M. A. Qureshi. 2002a. Differential expression of inducible nitric oxide synthase is associated with differential Toll-like receptor-4 expression in chicken macrophages from different genetic backgrounds. Vet. Immunol. Immunopathol. 84:191-207.

Dil, N., and M. A. Qureshi. 2002b. Involvement of lipopolysaccharide related receptors and nuclear factor κB in differential expression of inducible nitric oxide synthase in chicken macrophages from different genetic backgrounds. Vet. Immunol. Immunopathol. 88:149-161.

Emborg, H., A. Ersboll, O. Heuer, and H. C. Wegener. 2001. The effect of discontinuing the use of antimicrobial growth promotors on the productivity in the Danish broiler production. Prev. Vet. Med. 50:53-70.

Engberg, R., M. Hedemann, T. Leser, and B. Jensen. 2000. Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. Poult. Sci. 79:1311-1319.

Fritts, C. A., G. F. Erf, T. K. Bersi, and P. W. Waldroup. 2004. Effect of source and level of vitamin D on immune function in growing broilers. J. Appl. Poult. Res. 13:263-273.

Golemboski, K. A., S. E. Bloom, R. R. Dietert. 1990. Dynamics of avian inflammatory response to cross-linked dextran. Changes in avian blood leukocyte populations. Inflammation. 14:31-40.

Grindstaff, J. L. 2008. Maternal antibodies reduce costs of an immune response during development. J. Exp. Biol. 211:654-660.

He, H., M. B. Farnell, and M. H. Kogut. 2003. Inflammatory agonist stimulation and signal pathway of oxidative burst in neonatal chicken heterophils. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 135:177-184.

Kidd, M., A. Corzo, D. Hoehler, E. Miller, and W. Dozier. 2005. Broiler responsiveness (Ross x 708) to diets varying in amino acid density. Poult. Sci. 84:1389-1396.

Klasing, K., and D. R. Korver. 1997. Leukocytic cytokines regulate growth rate and composition activation of the immune system. J. Anim. Sci. 1997. 75(Suppl. 2):58-67.

Kogut, M. H., E. D. McGruder, B. M. Hargis, D. E. Corrier, and J. R. DeLoach. 1995. *In vivo* activation of heterophil function in chickens following injection with *Salmonella enteritidis*-immune lymphokines. J. Leukoc. Biol. Suppl. 57:56-62.

Kogut, M. H. 2009. Impact of nutrition on the innate immune response to infection in poultry. J. Appl. Poult. Res. 18:111-124.

Korver, D., E. Roura, and K. Klasing. 1998. Effect of dietary energy level and oil source on broiler performance and response to an inflammatory challenge. Poult. Sci. 77:1217-1227.

Long, J. R, ad R. B. Truscott. 1976. Necrotic enteritis in broiler chickens. III. Reproduction of the disease. Can. J. Comp. Med. 37:302-308.

Miles, R., G. Butcher, P. Henry, and R. Littell. 2006. Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. Poult. Sci. 85:476-485.

Millet, S., J. Bennett, K. A. Lee, M. Hau, and K. C. Klasing. 2007. Quantifying and comparing constitutive immunity across avian species. Dev. Comp. Immunol. 31:188-201.

Mitchell, R., H. Edwards, G. McDaniel, and G. Rowland. 1997. Dietary 1,25-dihydroxycholecalciferol has variable effects on the incidences of leg abnormalities, plasma vitamin D metabolites, and vitamin D receptors in chickens divergently selected for tibial dyschondroplasia. Poult. Sci. 76:338-345.

Moyes, R. B., M. H. Kogut, R. E. Droleskey, and J. R. DeLoach. 1998. Differential expression of adhesion molecules by chicken heterophils activated *in vivo* with *Salmonella enteritidis*-immune lymphokines. Vet. Immunol. Immunopathol. 62:83-95.

National Research Council. 1994. Nutrient requirements of poultry. 9th rev. ed. National Academy Press, Washington, DC, USA.

Palliyeguru, M., Rose S. P., and Mackenzie A. M. 2010. Effect of dietary protein concentrates on the incidence of subclinical necrotic enteritis and growth performance of broiler chickens. Poult. Sci. 89:34-43.

SAS Institute. 2002. SAS/STAT User's Guide Release 9.02. SAS Institute Inc., Cary, NC.

Shim, K. S., K. T. Hwang, M. W. Son, and G. H. Park. 2006. Lipid metabolism and peroxidation in broiler chicks under chronic heat stress. Asian-Aust. J. Anim. Sci. 19:1206-1211.

Skinner, J. T., S. Bauer, V. Young, G. Pauling and J. Wilson. 2010. An economic analysis of the impact subclinical (mild) necrotic enteritis in broiler chickens. Avian Dis. 54:1237:1240.

Soares, J. H. Jr., M. R. Swerdel, and E. H. Bossard. 1978. Phosphorus availability. 1. The effect of chick age and vitamin D metabolites on the availability of phosphorus in defluorinated phosphate. Poult. Sci. 57:1305-1312.

Swaggerty, C. L., I. Y. Pevzner, H. He, K. J. Genovese, D. J. Nisbet, P. Kaiser, and M. H. Kogut. 2009. Selection of broilers with improved innate immune responsiveness to reduce on-farm infection by foodborne pathogens. Foodborne Pathog. Dis. 6:777-783.

Swaggerty, C. L., K. J. Genovese, H. He, S. E. Duke, I. Y. Pevzner, and M. H. Kogut. 2011. Broiler breeders with an efficient innate immune response are more resistant to *Eimeria tenella*. Poult. Sci. 90 :1014–1019.

Watkins, K. L., T. R. Shryock, R. N. Dearth, and Y. M. Saif. 1997. In-vitro antimicrobial susceptibility of *Clostridium perfringens* from commercial turkey and broiler chicken origin. Vet. Microbiol. 54:195-200.

Whitehead, C. C., H. A. McCormack, L. McTeir, and R. H. Fleming. 2004. High vitamin D_3 requirements in broilers for bone quality and prevention of tibial dyschondroplasia and interactions with dietary calcium, available phosphorus and vitamin A. Br. Poult. Sci. 45:425-436.

CHAPTER 5: Effect of early nutrition, 25-hydroxy vitamin D3, and bacitracin methylene disalicylate on the expression of pro-inflammatory cytokines and inducible nitric oxide synthase mRNA

5.1 INTRODUCTION

Prophylactic antibiotics have been administered to broilers to control infectious diseases. It is estimated that prophylactic antibiotics are present in two thirds of the poultry diets in the United States because of their growth promoting effect (Chapman and Johnson, 2002). Antibiotic growth promoters could increase growth of broiler from 3% to 5% (Thomke and Elwinger, 1998). However, prophylactic antibiotic use has been banned by the European Union, and the pressure from activists and consumers will likely lead to a decrease in the non-therapeutic use of the drugs in other countries (Casewell et al., 2003). According to the World Health Organization (2002), long term inclusion of prophylactic antibiotics has the potential to increase the incidence of bacteria with gene characteristics, of which are resistant therapeutic antibiotics. This may impose a potential threat to human health if there was bacterial cross-contamination to humans through the ingestion of livestock meat. However, without the use of prophylactic antibiotics, Skinner et al. (2010) estimated that a necrotic enteritis outbreak could cause approximately $2 billion in annual losses to the poultry industry internationally. Currently, evidence suggests that prophylactic antibiotics cannot be replaced by any single alternative supplementation in controlling enteropathogens and growth promotion (Hofacre et al., 2003). Alternatives to antibiotics would be in urgent demand for chicken producers to

mitigate the substantial loss of broiler performance due to the ban of prophylactic antibiotics in poultry diets (Mountzouris et al., 2007).

Nutrient density impacts growth performance as well as immunological characteristics in broilers (Kogut, 2009). Cholecalciferol (Vitamin D_3) is well known for its support of proper skeletal development and growth in poultry (Edwards, 1993). Vitamin D_3 is hydroxylated in the liver to become 25-OH-D_3, a precursor of 1α, 25-dihydroxycholecalcipherol, the active form of vitamin D_3 (Soares et al., 1995). Recent research has addressed the positive impact of 25-OH-D_3 supplementation on several parameters of broiler performance, in which chicks fed 25-OH-D_3 had higher body weight and lower feed conversion ratio than those fed vitamin D_3 (Chou et al., 2009; Gómez-Verduzco et al., 2013). Bar et al. (1980) reported that 25-OH-D_3 has a higher metabolite conversion to 1α, $25(OH)_2D_3$ than vitamin D_3. According to Manolagas et al. (1985), antigen-presenting cells have vitamin D_3 receptors on their surface and 1α, $25(OH)_2D_3$ promotes the maturation of monocytes into macrophages and enhances the cells' ability to present antigens. Thus, the supplementation of 25-OH-D_3 could increase the inflammatory response of broilers to invading pathogens.

Lipoplysaccharide (LPS) is able to trigger a systemic inflammatory response in chickens (Korver and Klasing, 1997; Korver et al., 1998; Kogut et al., 2005). Kumar et al. (2011) reported that the imflammatory process is mediated by a cascade of pro-inflammatory cytokines including interleukin-1 beta (*IL-1β*), interleukin 6 (*IL-6*), tumor necrosis factor alpha (*TNF-α*), and innate immune related inducible nitric oxide synthase (*iNOS*). Chicken *IL-1β* belongs to the *IL-1*

superfamily of cytokines and is able to initiate a crosstalk among immune cells, causing an inflammatory response and increased antibody production (Sterneck et al., 1992). Nitric oxide (NO) is produced through the process of converting L-arginine to L-citruline using *iNOS* as a catalyst (Qureshi, 1990), and is extremely toxic to microbes (Blanchette et al., 2003). *TNF-α* plays a key role in the regulation of immune cells, where it induces fever and inflammation (Hong et al., 2006). Chicken *TNF-α* has not been successfully sequenced. However, LPS-induced tumor necrosis factor *α* (*LITAF*) has been sequenced in chickens, and it has similar functions to *TNF-α* (Hong et al., 2006). The up-regulation of those cytokines increased the inflammatory response, which is necessary to fight against invading pathogens, but excessive immune response decreased feed intake and body weight of broilers (Klasing, 1987).

We hypothesized that the LOW nutrient diet could decrease the expression of pro-inflammatory cytokine genes (*IL1-β* and *LITAF*) and the *iNOS* gene. A better understanding of nutrient density, 25-OH-D_3 and BMD supplementation at the level of gene expression may provide more insight for nutritionists and poultry producers to formulate diets that can enhance the immunity of broilers, while providing sufficient nutrients for optimized growth.

5.2 MATERIALS AND METHODS

The experimental protocol was approved by the Animal Care and Use Committee of the University of Alberta and was performed in accordance with the guidelines of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

5.2.1 Experimental birds, diets and tissue sampling.

For the gene expression experiment, 128 broiler chicks (n= 4 birds/pen) were randomly selected for tissue collection on d 14 post hatch. Half of the selected chicks (n= 64, 2 birds/pen) were injected with 3 mL of 100 µg (300 µg total) of *Salmonella typhimurium* lipopolysaccharide (LPS) on d 14 of age, while the other half were not injected following methodology of Korver et al. (1998). There was no LPS injection in the uninjected LPS group because previous studies demonstrated a significant change in inflammtory response between the LPS injected and the uninjected chicks (Koutsos et al., 2006). Broiler chicks were euthanized at 3 hours after the LPS injection by cervical dislocation, and whole spleens were removed from 4 chicks within each nutrient density level x 25-OH-D_3 x BMD treatment (2 LPS-injected and 2 control). Collected tissues were immediately stored in RNAlater buffer (Ambion, Austin, TX, USA). Subsequently, all samples were stored at -20°C until RNA was isolated.

5.2.2 RNA extraction.

Total cellular RNA was isolated from approximately 20 mg of spleen tissue of individual broiler chicks using the TRI reagent (Ambion, Austin, TX, USA), modified to optimize RNA integrity. Briefly, each spleen tissue sample was quickly cut and weighed (20 mg), then transferred to a 2-mL homogenization tube containing metal beads (CK 14 Precellys® lysing) on dry ice. One mL of TRI Reagent Solution was added to each homogenization tube, and then the tube was placed immediately into the Precellys® 24 homogenizer (Bertin Technologies, Montigny, France) for homogenization. The homogenization

program consisted of two 30 s shaking cycles of at 5,500 rpm, with 10 s rest between each cycle. After incubating the homogenized solution was kept at room temperature for 5 min. The separation phase was begun by adding 0.2 ml chloroform and shaking vigorously for 15 s. The solution was incubated for 15 min at room temperature, followed by centrifugation at 12,000 x g for 10 min at 4°C, and then the aqueous phase was transferred to a fresh tube. RNA precipitation was performed by adding 0.5 ml of 100% isopropanol to the aqueous phase, vortexing for 5 s, and incubation at room temperature for 5 min. Precipitated RNA formed a gel-like or white pellet after a centrifugation of the mixture solution at 12,000 x g for 10 min at 4°C, after which the supernatant was removed. The precipitated RNA was washed by adding 1 ml of 75% ethanol with a quick vortex and 5 min of centrifugation at 7,500 x g at 4°C, subsequently the excess ethanol was pipetted away before the RNA pellets were air-dried for approximately 2 min and re-suspended to 30 µL of RNase-DNase free water (Ambion, Austin, TX, USA).

The amount and quality of total RNA were measured based on absorbance at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies, NC, USA). RNA samples were accepted if the ratio of the UV absorbance between 260 and 280 nm was between 1.8 and 2.0, indicating a higher percentage of RNA than protein in the dissolved RNA solution. Isolated RNA was diluted until it reached a range of 200 to 250 ng/µl with RNase-DNase free H_2O. RNA samples were placed on dry ice, and subsequently stored at -80°C.

5.2.3 RNA integrity.

To confirm the quality of isolated RNA, each individual sample was electrophoresed on a RNA-denaturing gel. Four µl of each RNA sample was added to 24 µl of RNA-denaturing sample buffer (60 µl of 10X MOPS (3[N-morpholino] propanesulfonic acid), 120 µl of formaldehyde (37% w/v), supplied by Fisher Scientific (NH, US) and 300 µl of deionized formamide (Ambion, Austin, TX, USA). The mixture of RNA and buffer was denatured in a thermocycler (Bio-Rad, Hercules, CA, USA) at 65°C for 15 min. Denatured samples were placed on regular ice before loading into the wells on the RNA-denaturing gel. The gel was created by adding 21.6 ml of formaldehyde (37% w/v; Fisher Scientific) into a 1.2% agarose gel solution (120 ml of mili Q water and 1.44 g of agarose). Before casting the gel, 10 µl of SyBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) was added. The denatured samples were individually loaded into each well on the firmed gel, which was then covered with 1X MOPS buffer (pH 7.0), and electrophoresed at 70 V for 45 minutes. After the SyBR® Safe stained denatured RNA had migrated approximately two thirds the distance down the gel, the RNA smear was visualized using a Typhoon TRIO^{+} Variable Mode Imager (Amersham Biosciences, San Jose, CA, USA). An emission filter of 520 BP 40, and pixel size of 250 microns were used to yield clear gel images. The quality of total RNA was accepted if the 28S ribosomal band of the RNA was 1 to 2 times brighter than the 18S. Samples that did not meet this criterion were re-extracted until the sufficient quality RNA was obtained.

5.2.4 DNase treatment and cDNA transcription.

Total RNA samples were treated with DNase to ensure that only mRNA would be reverse transcribed in the following cDNA reaction. One micro-liter of both RQ1 RNase-free DNase 10X Reaction buffer and RQ1 RNase-free DNase (Promega, Madison, WI, USA) were added to 8 µl of RNA (250 ng/µl), mixed, and then incubated at 37°C for 30 min. One micro-liter of RQ1 stop solution (Promega, Madison, WI, USA) was added to the incubated solution to terminate the reaction, followed by a final incubation at 65°C. Following the DNase reaction, a total of 10 µl of each RNA (now 200 ng/µl) was then reverse transcribed into cDNA by an addition of 10 µl of reverse-transcription reagent form the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). The reverse transcription stage was performed in accordance to the manufacturer's guide, and consisted of 25°C for 15 s, 37°C for 120 min, and 85°C for 5 min. The reaction was kept at 4°C before further analysis. Subsequently, 5 µl of cDNA (100 ng/µl) from each sample was diluted to 1 ng/µl, and used as a template in real-time PCR reactions. Similar procedures from DNase treatment to the end of the cDNA reaction were applied to a pooled RNA sample, which was created from combining 2 µl of individual RNA. The pooled sample cDNA served as the standard curve template for the real-time PCR reaction, and a 10X serial dilution of standard curve was made by diluting the pooled cDNA sample (100 ng/µl) into a serial dilution of 10, 1, 0.1, 0.01 ng/µl for the *LITAF* gene. Due to low expression of *IL-1β* and *iNOS* genes, the

standard curves for those two genes consisted of a serial dilution of 25, 2.5, 0.25, and 0.025 ng/µl.

5.2.5 Primer design.

The mRNA sequence of 6 house-keeping genes, beta-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ubiquitin B (*UBB*), hexose-6-phosphate dehydrogenase (*H6PD*), ribosomal protein L19 (*RPL19*), and eukaryotic translation elongation factor 1 alpha 2 (*EEF1A2*), and the 3 genes of interest (*IL-1β, LITAF* and *iNOS*) were obtained from NCBI (http://www.ncbi.nlm.nih.gov/gene/) and were submitted to the UCSC genome browser database (Kent et al., 2002) to determine the position of introns and exons within each gene. The exon-intron annotated gene sequence of each gene was then submitted to Primer 3 software. Each set of primers was designed to amplify a qRT-PCR product size (amplicon length) of between 100-200 bp with 18-23 nucleotides/primer on the exon-exon junction as only exon sections are expressed. Once a primer pair was designed, OligoAnalyzer 3.1 (Owczarzy et al., 2008) was used to check for primer-dimers and other possible self-affinities. The nucleotide sequence of selected primers were then blasted against the chicken genome to test the compatibility between the designed primers and the desired gene sequence (Altschul et al., 1990), and to make sure the primers did not amplify other parts of the chicken genome. Primer sequences were sent to Integrated DNA Technologies Incorporated (IA, US) for synthesis. Primers that were used for real-time PCR in this experiment are listed in Table 5.1.

5.2.6 Selection of house-keeping genes.

Expression levels of house-keeping genes were used to normalize gene-of-interest expression for technical variation due to RNA isolation and cDNA synthesis. There were six candidate house-keeping genes tested for suitability as normalization factors: *ACTB, GAPDH, H6PD, RPL19*, and *EEF1A2*. Initially, the primer amplification of these genes was tested on the standard curve of cDNA. House-keeping genes were selected based on stable and efficient amplification, plus the identification of a specific amplified product as determined by the presence of a single peak on dissociation curves generated during the melt portion of the real-time PCR run. The efficiency of selected primers ranged between 83.9% and 100.1% (Table 5.2). The cycle threshold (Ct) value is defined as the cycle number in which there is a stable exponential increase in the amount of amplified PCR product. The best primer sets were selected based on the R^2 of a line drawn through the plot of Ct against sample concentration of two replicate amplifications over the entire range of the standard curve. Three genes (*ACTB, GAPDH* and *UBB*), out of 6 house-keeping candidates qualified in all of the above requirements, and were selected to serve as house-keeping genes in this experiment.

5.2.7 Identification of the best combination of house-keeping genes.

The average of the duplicate quantity values from each sample per housekeeping gene were submitted to geNorm and Normfinder (Andersen et al., 2004), which both indicated that the combination of *UBB* and *ACTB* genes had the most stable expression, therefore *UBB* and *ACTB* were used to normalize the expression of

IL-1β, LITAF and *iNOS*. The geometric mean of house-keeping gene values was used for expression normalization because it controls better for possible outliers and vast variations in expression levels between different genes than does the average (Vandesompele et al., 2002).

5.2.8 Quantitative polymerase chain reaction of target genes.

The qRT-PCR was performed using KAPA SYBR® FAST Master Mix (2X) (Kapa Biosystems Inc., MA, US) on StepOnePlus™ Real-Time PCR System (Applied Biosystems, CA, USA). The real-time PCR reaction contained 10 µl SYBR® Green Master Mix and 2 µl cDNA (1 ng/µl) and 0.5 µl (10 µm) of both forward and reverse primers for each reaction. All sample reactions were performed in duplicate using the following PCR program: an initial denaturation of 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 s and an annealing/extension at 60°C for 30 s. A melt (dissociation step), consisting of 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s, was performed at the end of each PCR reaction to ensure amplification of a single product. The amplification efficiency for each gene ranged from 83.9 to 100.1% (Table 5.2).

5.2.9. Normalizing RT-PCR data

After running in the qPCR machine, the Ct values relative quantities were obtained from the program. The Ct values and the quantities were plotted against the standard curve to validate the exponential amplification by the following equation:

$$\text{Exponential amplification} = 10^{(-1/\text{slope})}$$

The average amplified quantity of each sample was normalized with the geometric mean of the average quantity of the *ACTB* and *UBB* house-keeping genes of individual samples. This process was done by dividing the average quantity values of the genes of interest by the geometric averaging quantity values of *ACTB* and *UBB* house-keeping genes (Vandesompele et al., 2002).

5.2.10 Statistical analysis.

Log transformation was applied to the normalized gene expression data to obtain a normal data distribution, and then these values were analyzed with the PROC MIXED procedure of SAS (SAS Institute, 2002).

The RNA expression model is as follows:

$y = \mu$ + Early nutrition + 25-OH-D_3 + BMD antibiotic + LPS + 2-way interactions + 3-way interactions + e,

Where the fixed effect of early nutrition was the dietary level of early nutrition, 25-OH-D_3 and BMD were the supplementation of either with or without 25-OH-D_3 and BMD, respectively, LPS was the LPS injection or non-injected control, and e was the random error term. The random error term consisted of Pen (early nutrition x vitamin x antibiotic) and chick (early nutrition x vitamin x antibiotic). Chick was the experimental unit since LPS was administered for half of the randomly selected birds within pen. Because much of variation was caused by the injection of LPS, different variances between the non-injected LPS and injected LPS group was accounted by including the statement "repeated/group=lps" in the syntax. Differences between main effects, means,

and their interactions were classified by pairwise comparisons, and unless otherwise noted, differences were considered significant at $P< 0.05$.

5.3 RESULTS AND DISCUSSION

The influence of dietary treatments on the expression of house-keeping genes was examined using the geometric average of *ACTB* and *UBB* genes. Although the statistical analysis showed a significant interaction among nutrient density diets x 25-OH-D_3 x BMD on the geometric means of *ACTB* and *UBB* genes, there were no differences in the expression of geometric means among the different dietary treatments in the least square mean comparisons. In addition, there were no effects of main treatments on the geometric means. Thus geometric mean values of UBB and ACTB were appropriate to normalize the relative mRNA expression of the genes of *IL-1β, LITAF*, and *iNOS*.

Although Cheema et al. (2003) reported that high nutrient density in early life increases the immune response by supporting proliferation and maturation of phagocytic cells, nutrient density in the current experiment had no effect on the expression level of any of the genes tested. Cheema et al. (2003) assessed the innate immune response by evaluating the response of phagocytic cells to phytohaemagglutinin-P, whereas we measured the innate immune response by using non-pathogenic *E. coli in vivo.* The LPS injected birds displayed a significantly higher *IL-1β* mRNA level than the non-LPS birds and, in general, levels of *LITAF* and *iNOS* mRNA were higher in LPS-injected animals as compared to non-LPS (Table 5.3). LPS is a component of the outer membrane of gram negative bacteria, and is able to trigger an acute phase response and

inflammation (Mireles et al., 2005), subsequently changing the regulation of the endocrine and metabolic systems (Klasing et al., 1987), including a dramatic increase in pro-inflammatory cytokines (Kaiser et al., 2000). Kaiser et al. (2012) reported that *IL-1β*, *IL-6*, and the innate antimicrobial *iNOS* gene can be released by macrophages activated by the presence of LPS. Therefore, the increase in expression of *IL-1β, LITAF*, and *iNOS* mRNA after LPS injection in the current study was expected. Although initiation of these cytokines could be an essential mechanism for protection against invading pathogens, this reaction requires a high energy input, which decreases available resources for growth (Klasing et al., 1987). Korver et al. (1998) reported that *Salmonella typhimurium* LPS produced inflammatory responses correlating with a reduction in feed intake and decreased weight gain. In the current experiment, all LPS-injected broiler chicks were euthanized by cervical dislocation 3 h after injection for tissue collection. Therefore, the effects of the LPS-induced inflammatory response on broiler performance were not examined.

We also observed that BMD supplementation significantly decreased *IL-1β* expression (Table 5.3). Roura et al. (1992) reported that prophylactic antibiotic could minimize the necessity of the inflammatory response to bacteria. Engberg et al. (2000) reported that supplementation of zinc bacitracin suppressed the proliferation of lactobacilli, *L. salivarius* and *C. perfringens* in the gastrointestinal tract. In the current experiment, reduced the inflammatory response via the *IL-1β* mechanism could have been due to a suppression of the gastrointestinal bacteria populations.

There was a three-way interaction of LPS x 25-OH-D_3 x BMD on the expression of *LITAF* and *iNOS* genes (Table 5.3). All groups exhibited higher *LITAF* mRNA expression than the control (no LPS, 25-OH-D_3, or BMD), although the difference was not significant for the non-LPS group fed both 25-OH-D_3, and BMD. Broilers which were injected with LPS and consumed diets containing either 25-OH-D_3, or 25-OH-D_3 and BMD had higher expression of *LITAF* mRNA over all other groups, although it was only significantly different from the non-LPS injected group fed both BMD and 25-OH-D_3 and the control. Chou et al. (2009) reported that 25-OH-D_3 increased the immunoglobulin production when broilers were challenged with LPS. We postulated that the elevation of pro-inflammatory gene expression could benefit for health of broilers because it could prevent broilers from generating a vigorous inflammatory response to bacteria. The results suggested that 25-OH-D_3 could change the inflammatory response of broilers. Increased inflammatory response could compromise growth of broilers (Klasing et al., 1987), which was observed in this study (Chapter 2). However, an appropriate inflammatory response could ensure host survival and ability to recover from the challenge.

For the *iNOS* gene, non-LPS broilers supplemented with BMD and 25-OH-D_3 exhibited the same level of the expression as the control, as well as non-LPS with BMD and the LPS-injected group which received BMD. However, non-LPS birds that were supplemented with 25-OH-D_3 displayed higher *iNOS* mRNA expression than the control. The level of *iNOS* expression was similar in the LPS injected birds receiving both BMD and 25-OH-D_3 or neither. The LPS

injected broilers supplemented with 25-OH-D_3 only, exhibited a greater expression of *iNOS* than all other groups, although it was only significantly higher than the control, the non-LPS broilers fed both 25-OH-D_3 and BMD, and the LPS injected groups which received BMD (Table 5.3).

Nitric oxide (NO) is toxic to microbes and is elevated during LPS infection (Chapman and Wideman 2006; Bowen et al., 2007). The increase of NO level when birds are challenged with LPS plays a key role in their immune system because chicken heterophils produce ineffective oxidative bursts due to insufficient myeloperoxidase enzyme (Lam, 1997), and thus must rely on the NO mechanism. Heggen-Peay et al (2000) reported that NO level increased in response to poult eneritis ad mortality syndrome. *iNOS* is an essential enzyme in the generation of NO which converts the amino acid L-arginine to L-citruline (Qureshi, 1990); therefore, the up-regulation of *iNOS* mRNA may result in higher NO concentration. This corresponds to the general increase of *iNOS* mRNA in the LPS injected birds. The chick plasma NO concentration was measured from the same chicks before spleen tissues removed in the current study. LPS injected broilers had significant greater NO concentration than un-injected LPS broilers (results are shown in Table 4.2, CHAPTER 4). These observations were consistent, and occurred in all LPS injected birds.

Chicken *TNF-α* has a different structure from *TNF-α* in mammals, but they share similar functions such as inducing macrophage nitric oxide (NO) production and causing maturity in the MQ-NCSU macrophage cell line (Qureshi et al., 1990; Rautenschlein et al., 1999). Although *TNF-α* mRNA was not assessed in the

current study, a previous study demonstrated that *LITAF* has similar functions to *TNF-α* (Hong et al., 2006). Qureshi and Miller (1991) reported that TNF-like factor *α* production markedly increased by LPS. Together with *IL-1β, TNF-α* increased adhesive molecules on vascular endothelial cells, causing migration of phagocytic cells to the infected area (Kaiser et al., 2000). In the current study, a general increase in *LITAF* and *iNOS* mRNA in LPS-injected birds could be due to an increase in activated phagocytic cells, subsequently generating a strong innate immune response that could support broiler resistance to invading pathogens during the starter phase of life (Swaggerty et al., 2003).

The response of BMD and 25-OH-D_3 varied between the LPS-injected and the un-injected group. The combination of BMD and 25-OH-D_3 in the non-injected LPS broilers resulted in similar mRNA *LITAF* and *iNOS* expressions in the control group. However, the combination of BMD and 25-OH-D_3 in LPS-injected group had higher mRNA *LITAF* and *iNOS* expressions than the Control group. The mechanism by which this occurred is unknown in the current study. Chou et al. (2009) had observed that supplemental 25-OH-D_3 decreased IgA and IgG in uninfected broilers, but increased IgG in LPS-injected broilers at d 21 of age, suggesting 25-OH-D_3 may have varied effects, depending on the state of infection. The authors explained that broilers could have been able to use 25-OH-D_3 depending upon the demands for inflammatory response (Chou et al., 2009).

The effects of the dietary treatments in the current study on pro-inflammatory cytokine expression were dependent on whether or not there was an

inflammatory response. Prophylactic BMD decreased the expression of *IL-1β* mRNA, potentially increasing broiler performance by reducing the stress associated with the inflammatory response (Roura et al., 1992). The conserved energy then could be invested in growth for broilers. However, this hypothesis was not supported by the production data in the current trial. Supplemental 25-OH-D_3 significantly increased *LITAF* and *iNOS* cytokines, even in the non-LPS groups (but not exceeded the gene expression level in the injected-LPS group), suggesting that 25-OH-D_3 acted as an immunomodulator. The results suggested that the stress caused by inflammatory response could be somehow mitigated by the inclusion of 25-OH-D_3. An increased inflammatory response is usually an indicator of immunological stress, and preventing excessive inflammatory response would increase the ability recover of broilers after infections.

Table 5.1 Primers used in real-time PCR plus accession numbers of the genomic sequence used in their design.

Gene	Name	Forward (F) and Reverse (R) primers	Accession no.
ACTB	Beta Actin	F: CTGGCACCTAGCACAATGAA R: CATCGTACTCCTGCTTGCTG	NM_205518.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: GAGGGTAGTGAAGGCTGCTG R: CATCAAAGGTGGAGGAATGG	NM_204305.1
UBB	Ubiquitin B	F: TCCACTCATCTTGTCCTG R: GGTGTCACTGGGCTCGAC	XM_001233376.2
IL-1β	Interleukin 1-beta	F: GGGCATCAAGGGCTACAAG R: CAGGCGGTAGAAGATGAAGC	NM_204524.1
LITAF	LPS-induced TNF factor alpha	F: AGAACAGCACTACGGGTTGC R: TACTGCGTGTCCTTTCAACG	NC_006101.3
INOS	Nitric oxide synthase	F: AACTCCTTGCTCACCCTGAA R: GTGCGAAAGGCTTCTTCTTG	NM_204961.1

Table 5.2 PCR efficiencies of each amplified gene used for real-time quantitative PCR

Gene	Slope	PCR efficiency
ACTB[1]	-3.485	93.6%
GAPDH[2]	-3.387	97.3%
UBB[3]	-3.318	100.1%
IL-1β[4]	-3.336	99.4%
LITAF[5]	-3.778	83.9%
iNOS[6]	3.778	83.9%

[1]Beta Actin
[2]Glyceraldehyde-3-phosphate dehydrogenase
[3]Ubiquitin B
[4]Interleukin 1-beta
[5]LPS-induced TNF factor alpha
[6]Nitric oxide synthase

Table 5.3 Effect of LPS, starter nutrient density, 25-OH-D_3 and antibiotic on *IL-1β, LITAF* and *iNOS* expression levels in spleens of broiler chicks

LPS[1]	N [2]	D[3]	A[4]	*IL-1β*	*LITAF*	i*NOS*
					relative units	
Control				14.08[b]	2.97[b]	31.67[b]
Injected				93.03[a]	4.19[a]	47.72[a]
	LOW			39.87	3.87	43.73
	HIGH			32.83	3.23	34.55
		0		33.01	3.20	33.62[b]
		69		39.65	3.90	44.95[a]
			0	43.02[a]	3.55	42.39
			0.5	30.42[b]	3.51	35.64
SEM				1.11	1.09	1.10
Control		0	0	12.69	2.27[c]	24.32[c]
Control		0	0.5	13.64	3.48[ab]	25.06[c]
Control		69	0	18.51	3.76[ab]	40.16[ab]
Control		69	0.5	12.27	2.65[bc]	29.36[c]
Injected		0	0	123.05	3.81[ab]	54.45[ab]
Injected		0	0.5	55.80	3.50[ab]	27.51[c]
Injected		69	0	118.64	4.88[a]	60.74[a]
Injected		69	0.5	91.75	4.74[a]	56.99[ab]
SEM				1.23	1.15	1.31
Source of variation					Prob > F	
LPS (100 µg/ml)				0.0001	0.0005	0.0051
Nutrient density (N)				0.2024	0.1303	0.0861
25-OH-D_3 (D)				0.2281	0.1015	0.0375
Antibiotic (A)				0.0278	0.9349	0.1985
LPS x N				0.7146	0.0890	0.4739
LPS x D				0.7522	0.3528	0.5323
LPS x A				0.2429	0.5750	0.5750
N x D				0.1534	0.0987	0.7267
N x A				0.5995	0.3207	0.2515
D x A				0.9324	0.1357	0.2086
LPS x N x D				0.5240	0.5335	0.2133
LPS x N x A				0.7119	0.1652	0.2227
LPS x D x A				0.0986	0.0214	0.0218
N x D x A				0.8958	0.2105	0.5638

[a, b, c] Treatment means within the same column within effect with no common superscripts are significantly different ($P < 0.05$).

[1]Lipopolysaccharide (LPS) was injected individually to half of the birds (3 ml of a 100 µg/ml solution of *Salmonella typhimurium* LPS, the other half were non-injected control at d 14 of age.

[2]Starter nutrient density: HIGH (3,025 kcal/kg; 23.9% CP), or LOW (2,858 kcal/kg; 22.3% CP) fed from 0 to 14 d.

[3]Broilers fed a diet supplemented with 0 or 69 µg of 25-OH-D_3 (all basal diets contained an additional 4,000 IU of vitamin D_3 per kg of diet).

[4]Bacitracin Methylene Disalicylate (BMD® 110): 0 or 0.5 g/kg, containing 55 mg of BMD per kg of diet.

5.4 REFERENCES

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Andersen, C. L., J. L. Jensen, and T. F. Ørntoft. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64:5245-5250.

Bar, A., M. Sharvit, D. Noff, S. Edelstein, and S. Hurwitz. 1980. Absorption and excretion of cholecalciferol and of 25-hydroxycholecalciferol and metabolites in birds. J. Nutr. 110:1930-1934.

Blanchette, J., M. Jaramillo, and M. Olivier. 2003. Signaling events involved in interferon-gamma-inducible macrophage nitric oxide generation. Immunology. 108:513-522.

Bowen, O. T., G. F. Erf, M. E. Chapman, and J. R. F. Wideman. 2007. Plasma nitric oxide concentrations in broilers after intravenous injections of lipopolysaccharide or microparticles. Poult. Sci. 86:2550-2554.

Canadian Council on Animal Care. 1993. A guide to the care and use of experimental animals. 2nd ed. CCAC, Ottawa, ON, Canada.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52:159-161.

Chapman, H., and Z. Johnson. 2002. Use of antibiotics and roxarsone in broiler chickens in the USA: analysis for the years 1995 to 2000. Poult. Sci. 81:356-364.

Chapman, M., and R. Wideman. 2006. Evaluation of total plasma nitric oxide concentrations in broilers infused intravenously with sodium nitrite, lipopolysaccharide, aminoguanidine, and sodium nitroprusside. Poult. Sci. 85:312-320.

Cheema, M. A., M. A. Qureshi, and G. B. Havenstein. 2003. A comparison of the immune profile of commercial broiler strains when raised on marginal and high protein diets. Int. J. Poult. Sci. 2:300-312.

Chou, S. H., T. K. Chung, and B. Yu. 2009. Effects of supplemental 25-hydroxycholecalciferol on growth performance, small intestinal morphology, and immune response of broiler chickens. Poult. Sci. 88:2333-2341.

Edwards, H. M. 1993. Dietary 1,25-dihydroxycholecalciferol supplementation increases natural phytate phosphorus utilization in chickens. J. Nutr. 123:567-577.

Engberg, R., M. Hedemann, T. Leser, and B. Jensen. 2000. Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. Poult. Sci. 79:1311-1319.

Engster, H., D. Marvil, and B. Stewart-Brown. 2002. The effect of withdrawing growth promoting antibiotics from broiler chickens: a long term comercial industry study. J. App. Poult. Res. 11:431-436.

Gómez-Verduzco, G., R. Morales-López, and E. Avila-Gozàlez. Use of 25-hydroxycholecalciferol in diets of broiler chickens: Effects on growth performance, immunity and bone calcification. J. Poult. Sci. 50:60-64.

Heggen-Peay, C. L., M. A Qureshi, F. W. Edens, and H. J. Barnes. 2000. Alterations in macrophage-produced cytokine and nitrite associated with poult enteritis and mortality syndrome. Avian Dis. 44:69-65.

Hofacre, C. L., T. Beacorn, S. Collet, and G. Mathis. 2003. Using competitive exclusion, mannan-oligosaccharide and other intestinal products to control necrotic enteritis. J. Appl. Poult. Res. 12:60-64.

Hong, Y. H., H. S. Lillehoj, S. H. Lee, D. W. Park, and E. P. Lillehoj. 2006. Molecular cloning and characterization of chicken lipopolysaccharide-induced TNF-alpha factor (*LITAF*). Dev. Comp. Immunol. 30:919-929.

Kaiser, M. G., S. S. Block, C. Ciraci, W. Fang, M. Sifri, and S. J. Lamont. 2012. Effects of dietary vitamin E type and level on lipopolysaccharide-induced cytokine mRNA expression in broiler chicks. Poult. Sci. 91:1893-1898.

Kaiser, P., L. Rothwell, E. E. Galyov. 2000. Differential cytokine expression in avian cells reponse to invasion by *Salmonella typhymurium*, *Salmonella enteritidis*, and *Salmonella gallinarium*. Microbiology. 146:3217-3226.

Kent, W. J., C. W. Sugnet, T. S. Furey, K. M. Roskin, T. H. Pringle, A. M. Zahler, and D. Haussler. 2002. The human genome browser at UCSC. Genome Res.12:996-1006.

Klasing, K. C., D. E. Laurin, R. K. Peng, and D. M. Fry. 1987. Immunologically mediated growth depression in chicks: influence of feed intake, corticosterone and interleukin-1. J. Nutr. 117:1629-1637.

Kogut, M. H., M. Iqbal, H. He, V. Philbin, P. Kaiser, and A. Smith. 2005. Expression and function of Toll-like receptors in chicken heterophils. Dev. Comp. Immunol. 29:791-807.

Kogut, M. H. 2009. Impact of nutrition on the innate immune response to infection in poultry. J. Appl. Poult. Res. 18:111-124.

Korver, D. R., and K. C. Klasing. 1997. Dietary fish oil alters specific and inflammatory immune responses in chicks. J. Nutr. 127:2039-2046.

Korver, D. R., E. Roura, and K. C. Klasing. 1998. Effect of dietary energy level and oil source on broiler performance and response to an inflammatory challenge. Poult. Sci. 77:1217-1227.

Koutsos, E. E., J. C. G. López, and K. C. Klasing. 2006. Carotenoids from in ovo or dietary sources blunt systemic indices of the inflammatory response in growing chicks (*Gallus gallus domesticus).* J, Nutri. 136:1027-1031.

Kumar, S., C. Ciraci, S. B. Redmond, P. Chuammitri, C. B. Andreasen, D. Palić, and S. J. Lamont. 2011. Immune response gene expression in spleens of diverse chicken lines fed dietary immunomodulators. Poult. Sci. 90:1009-1013.

Lam, K. M. 1997. Myeloperoxidase activity in chicken heterophils and adherent cells. Vet. Immunol. Immunopathol. 57:327-335.

Manolagas, S. C., D. M. Prowedini, and C. D. Tsoukas. 1985. Interactions of 1, 25-dihydroxyvitamin D_3 and the immune system. Mol. Cell Endocrinol. 43:113-122.

Mireles, A. J., S. M. Kim, and K. C. Klasing. 2005. An acute inflammatory response alters bone homeostasis, body composition, and the humoral immune response of broiler chickens. Poult. Sci. 84:553-560.

Mountzouris, K. C., P. Tsistsikos, E. Kalamara, S. Nitsh, G. Schatzmayr, and K. Fegeros. 2007. Evaluation of the efficacy of a probiotic containing *Lactobacillus, Bifidobacterium, Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poult. Sci. 86:309-317.

Owczarzy, R., A. V. Tataurov, Y. Wu, J. A. Manthey, K. A. McQuisten, H. G. Almabrazi, K. F. Pedersen, Y. Lin, J. Garretson, N. O. McEntaggart, C. A. Sailor, R. B. Dawson, and A. S. Peek. 2008. IDT SciTools: a suite for analysis and design of nucleic acid oligomers. Nucleic Acids Res. 36:163−169.

Qureshi, M. A., and L. Miller. 1991. Signal requirements for the acquisition of tumoricidal competence by chicken peritoneal macrophages. Poult. Sci. 70:530–538.

Qureshi, M. A., L. Miller, H. S. Lillehoj, and M. D. Ficken. 1990. Establishment and characterization of a chicken mononuclear cell line. Vet. Immunol Imunnopathol. 26:237-250.

Rautenschlein, S., A. Subramanian, and J. M. Sharma. 1999. Bioactivities of a tumour necrosis-like factor released by chicken macrophages. Dev. Comp. Immunol. 23:629-640.

Roura, E., J. Homedes, and K. C. Klasing. 1992. Prevention of immunologic stress contributes to the growth-permitting ability of dietary antibiotics in chicks. J. Nutri. 122:2382-2390.

SAS Institute. 2002. SAS/STAT User's Guide Release 9.02. SAS Institute Inc., Cary, NC.

Skinner, J. T., S. Bauer, V. Young, G. Pauling and J. Wilson. 2010. An economic analysis of the impact subclinical (mild) necrotic enteritis in broiler chickens. Avian Dis. 54:1237:1240.

Soares, J. H., J. M. Kerr, and R. W. Gray. 1995. 25-hydroxycholecalciferol in poultry nutrition. Poult. Sci. 74:1919-1934.

Sterneck, E., C. Blattner, T. Graf, and A. Leutz. 1992. Structure of the chicken myelomonocytic growth factor gene and specific activation of its promoter in avian myelomonocytic cells by protein kinases. Mol. Cell Biol. 12:1728-1735.

Swaggerty, C. L., I. Y. Pevzner, V. K. Lowry, M. B. Farnell, and M. H. Kogut. 2003. Functional comparison of heterophils isolated from commercial broiler chickens. Avian Pathol. 32:95-102.

Thomke, S., and K. Elwinger. 1998. Growth promotants in feeding pigs and poultry. II. Mode of action of antibiotic growth promotants. Ann. Zootech. 47, 153–167.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:1-11.

World Health Organization. 2002. The medical impact of the use of antimicrobials in food animals: report of a WHO meeting, Berlin, Germany. http:// whqlibdoc.who.int/hq/1997/WHO_EMC_ZOO_97.4.pdf. Accessed August 2013.

CHAPTER 6: Research synthesis

6.1 SUMMARY

The overall goal of the current research was to find a nutritional mitigation strategy for antibiotic-free broiler production. This objective was pursued in the thesis research by testing these following hypotheses:

1. We hypothesized that early nutrient density diets, 25-hydroxy vitamin D_3, and bacitracin methylene disalicylate would influence broiler performance and meat yield.

This hypothesis was addressed in CHAPTER 2 of the thesis. We investigated the effect on broiler performance and meat yield at d 40 of two diets differing in nutrient density, administered during the starter phase and two levels of 25-OH-D_3 and BMD administered during all dietary phases. Our hypothesis was supported partially by the results in CHAPTER 2, which indicated that the LOW diet reduced BW and meat yield. BMD reduced mortality and increased feed efficiency. Supplementation of 25-OH-D_3 (69 µg/kg; 2,760 IU/kg) decreased breast yield compared with broilers fed without 25-OH-D_3. However, the higher mortality rate in broilers to which BMD was not initially administered might have been partially due to an outbreak of necrotic enteritis at d 28. Also, the necrotic enteritis outbreak could have interfered with the effect of 25-OH-D_3 on breast yield.

2. We hypothesized that early nutrient density diets, 25-hydroxy vitamin D_3, and bacitracin methylene disalicylate would change ileal morphology.

This hypothesis was addressed in CHAPTER 3 of the thesis. The villus height and

crypt depth were measured to assess the effect of the nutrient density diets, the 25-hydroxy vitamin D_3 and BMD on ileal morphology. Feeding either 25-OH-D_3 or BMD increased the ratio of villus height to crypt depth in broilers fed the HIGH nutrient density diet. However, the combination of both 25-OH-D_3 and BMD in the low nutrient density diet decreased the ratio of villus height to crypt depth. Average villus height was increased when birds were fed the HIGH nutrient density diet. The changes of ileal morphology were transient, although it did not have negative impact on broiler performance.

3. We hypothesized that early nutrient density, 25-hydroxy vitamin D_3, and bacitracin methylene disalicylate would influence bacterial killing capacity, phagocytosis and NO concentration.

This hypothesis was addressed in CHAPTER 4 of the thesis. We examined the responses of innate immunity indices including bacterial killing capacity, phagocytosis and NO level when broiler chicks were fed two levels of nutrient density, two levels of 25-OH-D_3, and two levels of BMD from d 0 to d 14. The high nutrient density diet numerically increased bacterial killing capacity of broilers (P= 0.058). NO level increased when broilers were challenged with *Salmonella typhimurium* lipopolysaccharide (LPS). There was no effect of either 25-OH-D_3 or BMD on bacterial killing capacity, phagocytosis or NO level.

4. We hypothesized that early nutrient density, 25-hydroxy vitamin D_3, and bacitracin methylene disalicylate would alter the expression of pro-inflammatory cytokine mRNA and iNOS mRNA.

This hypothesis was addressed in CHAPTER 5 of the thesis. RT-PCR was used to

evaluate the effect of the two levels of nutrient density, the two levels of 25-OH-D_3 and the two levels of BMD on the expression of selected genes from d 0 to d 14. The effect of treatments was assessed in a control group and a group injected with *Salmonella typhimurium* LPS on d 14. Feeding either 25-OH-D_3 or BMD increased the expression of *LITAF* and *iNOS* mRNA when birds were mounting an inflammatory response. Overall, broilers fed BMD had lower expression of *IL-1β* than those which did not receive BMD.

6.2 SYNTHESIS

Necrotic enteritis disease is not a new disease, but remains a threat to the poultry industry because of the withdrawal of prophylactic antibiotics (Skinner et al., 2010). The main mechanism of prophylactic antibiotics is to limit the proliferation of intestinal microflora, subsequently reduce the nutrient competition between the host and microflora populations (Knarreborg et al., 2008). Prophylactic antibiotics could also reduce the immunologic stress (Roura et al., 1992). The main objective of this project was to investigate potential nutritional strategies which could mitigate the removal or ban of prophylactic antibiotics in the poultry industry. The effect of early nutrient density diets (HIGH: 24% CP and 3,025 kcal/kg, LOW: 22% CP and 2,858 kcal/kg), 25-OH-D_3 (0 or 69 µg/kg), and bacitracin methylene disalicylate (BMD: 0 or 0.5 g/kg) and their potential interactions were investigated by observing broiler performance, meat yield, innate immunity, gut morphology and pro-inflammatory cytokine mRNA expression.

Production is the most important concern in the broiler industry, therefore determining supplementations that allow broilers to optimize innate immune

response without compromising growth potential is imperative. Several options can be considered in regard to increased broiler performance, including enhancing the efficiency of nutrient utilization (Kidd et al., 2005; Dozier et al., 2009), providing probiotic or prebiotic to aid nutrient absorption and reduce risks of infections (Knap et al., 2011), supplementing exogenous enzymes that increase the digestive capacity of the chickens (Engberg et al, 2004), and suppressing pathogenic bacteria with non-antibiotic supplementation such as herbs, spices, or essential oils (Stringfellow et al., 2009).

A growing number of studies have demonstrated the benefits of supplementing probiotics and prebiotics in poultry industry. Falaki et al. (2011) reported that probiotics increased the performance of broilers, although meat yield was not affected by probiotic supplementation. Also, probiotics was found to increase the innate immune response of broiler chickens. Knap et al. (2011) reported that probiotics maintained a beneficial microbial populations by competitive exclusion and immune modulation mechanisms. However, it is still a challenge to determine which bacterial groups are the most relevant to the small intestine in regards to increase digestive ability of chickens (Mountozouris et al., 2007). Moreover, the effect of current alternatives to prophylactic antibiotics on performance is not consistent throughout experiments, and depends greatly on rearing environment. For example, lactobacilli probiotic increased BW of female broilers by 12%, but decreased feed efficiency and elevated mortality when broilers were raised under heat stress condition (Zulkifli et al., 2000), suggesting that more research is needed to apply effectively probiotics on a large scale poultry industry.

In commercial poultry industry, feed cost per kg is the most critical factor that affects the profits of chicken producers. Least feed cost per kg would allow chicken producers to maintain their competitive business. In the current study, broilers fed the HIGH diet had greater body weight than broilers in the LOW diet. The increased in body weight gain could be attributable to the changed in ilea of broilers. Ileal villi crypt depth was reduced in broiler fed the HIGH nutrient density diet. A reduction in the crypt depth of villi would decrease the intestinal regular maintenance cost in broilers (Chou et al., 2009). Broiler performance and meat yield results revealed that the reduced villus height and crypt depth due to the combination of early HIGH nutrient density diet and 25-OH-D_3 had no negative impact on performance parameters of broilers.

There was an extra cost of 1 cent per kg of BW gain when feeding broilers with the LOW nutrient diet, compared to broilers fed the HIGH nutrient diet. Although the two nutrient density diets were only fed until d 14 of age, the feed to gain ratio was lower for broilers fed with the HIGH nutrient diet compared to broilers fed with the LOW nutrient diet at d 40 (1.69 vs. 1.76). In addition, the breast yield was higher by 2.2% in broilers that were fed the HIGH nutrient diet compared to those fed the LOW nutrient diet (CHAPTER 2). The goal of poultry management is to minimize production costs. However, Alberta chicken producers might have lost approximately 1,333,779 kg of breast meat yield if the LOW diets were provided to Ross 708 broilers (data were extrapolated from Statistics Canada, 2012). BMD did not favor body weight gain or feed efficiency of broilers in the current study, although BMD may increase gross income by increasing the

number of birds at the processing. A brief economic analysis using data from the current study suggested that removing prophylactic BMD resulted in a higher cost of production by $0.10/kg.

By feeding the early HIGH nutrient density diet, *ex vivo* bacterial killing capacity numerically increased by 10% compared to broilers fed with the early LOW nutrient density diet (P=0.058). If this holds true in the commercial flocks, the early HIGH nutrient diet could help the Ross 708 broilers become more resistant to invading pathogens. In the current experiment, broilers fed early HIGH nutrient diet and 25-OH-D_3 had greater nitric oxide synthase and lipopolysaccharide induced tumor necrosis factor alpha gene expression. The increased in gene expression level of those pro-inflammatory cytokine genes could initiate the inflammatory response of broiler chickens, which was evaluated by measuring nitric oxide level when broiler were challenged with the LPS. Although a stronger innate immune response could take away nutrients for growth (Swaggerty et al., 2005), an appropriate inflammatory response would ensure host survival. In the current study, broiler fed no 25-OH-D_3 had greater breast yield than broilers fed 25-OH-D_3. It is likely that the inclusion level of 25-OH-D_3 (69 µg/kg added to 4,000 IU of vitamin D_3) did not favor breast yield while increased the innate immune response. In the current experiment, BMD decreased the expression of interleukin 1 beta. Roura et al. (1992) reported that prophylactic antibiotics increased broiler performance by decreasing immunologic stress.

Under the conditions of this study, the LOW diet reduced performance and meat yield of broilers. Providing maximum growth for broilers at the lowest feed cost is

crucial in the poultry industry. However, a lower cost diet may cost more than just a reduction in broiler performance. A diet formula may need to be re-formulated when feed cost changes, but it is critical not to reduce nutrient levels in the early phase of broilers. The results suggested that the LOW diet is not a great strategy for cost reduction and is not recommended to the Ross 708 broilers. The current study also suggests that 25-OH-D_3 was involved in mRNA expression of pro-inflammatory cytokines. The necrotic enteritis outbreak occurred at d 28 in this trial was a good test for the effect of 25-OH-D_3 on the innate immune response of broiler chicks, although the initiation of inflammatory response reduced breast yield of broilers. This project provides further information that the supplementation of 25-OH-D_3 and early nutrient density diet could not prevent the broilers from the colonization of *Clostridium perfringens*, even in the broilers fed BMD group.

6.3 REFERENCES

Chou, S. H., T. K. Chung, and B. Yu. 2009. Effects of supplemental 25-hydroxycholecalciferol on growth performance, small intestinal morphology, and immune response of broiler chickens. Poult. Sci. 88:2333-2341.

Dozier, W. A. III, A. Corzo, M. T. Kidd, P. B. Tillman, and S. L. Branton. 2009. Digestible lysine requirements of male and female broilers from fourteen to twenty-eight days of age. Poult. Sci. 88:1676-1682.

Engberg, R. M., M. S. Hedemann, S. Steenfeldt, and B. B. Jensen. 2004. Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poult. Sci. 84:925:938.

Falaki, M., M. S. Shargh, B. Dastat, and S. Zerehdaran. 2011. Effects of different levels of probiotic on performance and carcass characteristics of broiler chickens. J. Anim. Vet. Adv. 10:378-384.

Kidd, M., A. Corzo, D. Hoehler, E. Miller, and W. Dozier. 2005. Broiler responsiveness (Ross x 708) to diets varying in amino acid density. Poult. Sci. 84:1389-1396.

Knap I., A. B. Kehlet, M. Bennedsen, G. F. Mathis, C. L. Hofacre, B. S. Lumpkins, M. M. Jensen, M. Raun, and A. Lay. 2011. Bacillus subtilis (DSM17299) significantly reduces Salmonella in broilers. Poult. Sci. 90:1690-1694.

Knarreborg, A., E. Brockmann, K. Høybye, I. Knap, B. Lund, N. Milora, and T. D. Leser. 2008. *Bacillus subtilis* (DSM17299) modulates the ileal microbial communities and improves growth performance in broilers. Int. J. Prebiotic Probiotic. 3:83–88.

Mountzouris, K. C., P. Tsistsikos, E. Kalamara, S. Nitsh, G. Schatzmayr, and K. Fegeros. 2007. Evaluation of the efficacy of a probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poult. Sci. 86:309-317.

Roura, E., J. Homedes, and K. C. Klasing. 1992. Prevention of immunologic stress contributes to the growth-permitting ability of dietary antibiotics in chicks. J. Nutri. 122:2382-2390.

Skinner, J. T., S. Bauer, V. Young, G. Pauling and J. Wilson. 2010. An economic analysis of the impact subclinical (mild) necrotic enteritis in broiler chickens. Avian Dis. 54:1237:1240.

Statistics Canada. 2012. Poultry and egg statistics – April to June 2012. Statistic Canada, Ottawa, Ontario, Canada. http://www.statcan.gc.ca/pub/23-015-x/2012002/t002-eng.pdf. Accessed Mar. 2013.

Stringfellow, K., J. McReynolds, J. Lee, J. Byrd, D. Nisbet, and M. Farnell. 2009. Effect of bismuth citrate, lactose, and organic acid on necrotic enteritis in broilers. Poult. Sci. 2280-2284.

Swaggerty, C. L., P. J. Ferro, I. Y. Pevzner, M. H. Kogut. 2005. Heterophils are associated with resistance to systemic Salmonella enteritidis infection in genetically distinct lines of chickens. FEMS Immunol. Med. Microbiol. 43:149:154.

Zulkifli, I., N. Abdullah, N. M. Azrin, and Y. W. Ho. 2000. Growth performance and immune response of two commercial broiler strains fed diets containing *Lactobacillus* cultures and oxytetracycline under heat stress conditions. Bri. Poult. Sci. 41: 593–597.