Effects of dietary 25-hydroxycholecalciferol and vitamin D_3 on performance, meat yield, bone characteristics, innate immune response and gene expression of Ross 308 broilers grown on reused or fresh litter

by

Seyed Abolghasem Fatemi

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Animal Science

Department of Agricultural, Food and Nutritional Science

University of Alberta

ABSTRACT

The effects of dietary 25-hydroxycholecalciferol ($25OHD_3$) and vitamin D_3 (D_3) on performance, meat yield, bone characteristics, innate immune response and gene expression of broiler chickens grown on Reused and Fresh litter were investigated. Experimental diets were fed to Ross 308 chicks (n=512) for 41 d in a 4 x 2 factorial arrangement with 8 pens/treatment. Diets were: vitamin D_3 at 2,760 IU/kg feed (D; Control); $25OHD_3$ at 2,760 IU/kg feed (25OHD); D_3 at 2,760 IU/kg plus $25OHD_3$ at 2,760 IU/kg feed (D+25OHD); or D_3 at 5,520 IU/kg feed (Dx2); each diet was fed to broilers grown on either fresh (Fresh) or reused (Reused) pine shavings to increase bacterial exposure. Dietary $25OHD_3$ alone relative to vitamin D_3 at the same level of inclusion resulted in increased leg meat yield at processing (d 42), decreased broiler performance in the fresh litter at 12, and decreased breast meat yield at processing. Supplementation of $25OHD_3$ at 2,760 IU/kg feed relative to vitamin D_3 reduced acute phase proteins at d 41 and increased villus width, crypt depth and tended to increase villus surface area at the same day. However, deeper crypts could result in increased gut maintenance energy requirement, which could have been the reason for lower breast meat yield at processing. In addition, dietary supplementation of $25OHD_3$ as a complete or partial replacement for vitamin D_3 increased bone quality by increasing bone mineral density and tended to increase bone breaking strength throughout the 41 d experiment. Dietary $25OHD_3$ reduced inflammation in post-hatch and older birds as indicated by reduced serum concentration of α1 acid glycoprotein in broilers at d 41; haptoglobin tended to be decreased at d 12 relative to birds fed D. Moreover, $25OHD_3$ alone increased the expression of genes involved in muscle synthesis and adaptive immunity relative to vitamin D_3 at the same level of activity in broilers at d 12. However, $25OHD_3$ alone decreased the expression of genes involved in amino acid biosynthesis and transport as compared to vitamin D_3 at the same level. Reused as compared to Fresh litter resulted in reduced broiler

performance, breast meat yield, and bone mineralization and strength in post-hatch broilers. Reused litter resulted in decreased villus surface area and villus height to crypt depth ratio, and increased crypt depth in post-hatch broilers relative to Fresh litter. Plasma haptoglobin concentration increased at d 41 and tended to increase at d 12 in birds reared on Reused litter in comparison to those on Fresh litter. In addition, expression of genes involved in the inflammatory response increased when birds were reared on Reused relative to Fresh litter at d 12. Therefore, increased crypt depth, which is associated with increased gut maintenance requirements, and systemic inflammation in post-hatch broilers reared on Reused relative Fresh litter may explain the reduced broiler performance, and bone characteristics in this group. In this study, supplementation of $25OHD_3$ as a complete or partial replacement for vitamin D_3 relative to vitamin D_3 alone did not affect breast meat yield relative to vitamin D_3, but it increased bone mineralization and development and reduced inflammation through the 41 d experiment. In conclusion, dietary supplementation of $25OHD_3$ is more effective in broilers production and immune response when birds are reared in a challenging environment.

DEDICATION

I would like to dedicate this thesis to my parents, Mr. Mirjalal Fatemi and Ms. Felora Tarvij Efahani for their love, sacrifice, and tolerance. It is also dedicated to my sister, Solmaz Fatemi for her support.

ACKNOWLEDGMENTS

Dr. Douglas. R. Korver is not just my major supervisor, I also see him as a father. Not only has the completion of this study been made possible with his unrelenting help, he has immensely and significantly contributed to making me who I am today. I would like to express my immense gratitude to Dr. Douglas Korver for all his support during this fantastic journey through science, and also for having given me the opportunity to be here. I definitely do not have the exact words to quantify how much I am grateful to him. He was always available to help me out at any time I was in need of his expert opinion. I would also like to gratefully and sincerely thank to Dr. Carolyn Fitzsimmons, for her guidance, understanding, patience, support and most importantly, her sincere friendship. Thanks, for being always available for advices, assistances and contributions about my thesis. The completion of this study would have not been possible without her continuous supervision and helps. I want to express my enormous gratitude Dr. Martin Zuidhof as a supervisory committee member for his invaluable and constructive comments, suggestions, and criticisms and for the time he spent with me.

I am very appreciative of the provision of a commercially available premix form of 25-hydroxycholecalciferol by DSM Nutritional Products. My thanks go also to Dr. Francois Paradis for his endless support, encouragement and delicate attention to details. Heartiest thanks to Abiodun Bello for his invaluable friendship and help. I learned from him the skills necessary to conduct research in a professional manner. I also would like to extend my thanks to Koonphol Pongmanee (Kooney), Felipe Silva, Carlos Lozano have provided me with invaluable assistance, support, and inspiration. Special thanks go to Aidin Foroutannaddafi for his support in genomic lab and his help with the gene network analysis and interpretation. Special thanks to Dr. Arun

Kommadath for his support and help with RNA-seq data analysis. I will like to express gratitude to Kerry Nadeau for her help the Quantitative Computed Tomography scanning that was used for bone mineral density in this study. Also, I would like to thank to Sandeep Nain for his help in small intestine morphology measurement. Special thanks to the Poultry Research Centre (Edmonton, Alberta, Canada) and all the staff and students that enthusiastically supported my trial especially Dr. Irene Wenger, Chris Ouellette, Shawn Rankin, Nigel Davidson, Lyle Bouvier, Breanne Chmilar, Kerry Nadeau, Dr. Jennifer Saunders-Blades, Airell DesLaurier, Thania Moraes, Joshua Perryman, Misaki Cho, and Erin Backer. Special thanks to Jody Forslund for her quick and precise responses in terms of administrative works in AFNS.

I am more than blessed to have the undying support of my parents, Mr. Mirjalal Fatemi and Ms. Felora Tarvij Efahani. I am so thankful to you, mom and dad. Their consistent prayers and support in all aspects of my life throughout this study definitely helped in making it a successful one. The support I got from my wonderful and pretty sister, Solmaz Fatemi, could not be over-emphasized. I am so grateful to you. Finally, and most importantly, thanks to God for all that I have in my life.

Table of Contents

1. LITERATURE REVIEW 1
1.1 INTRODUCTION 1
1.2 VITAMIN D 2
1.2.1 Vitamin D and metabolites 3
1.2.2 Dietary supplementation of $25OHD_3$ in broiler chicken diets 5
1.3 MYOGENIC REGULATORY FACTORS GENES AND SKELETAL MUSCLE DEVELOPMENT IN CHICKEN 10
1.3.1 Role of vitamin D sources in post-hatch skeletal muscle development 11
1.4 NUTRIGENOMICS AND VITAMIN D RECEPTOR 13
1.4.1Next generation sequencing in chicken studies 14
1.5 FRESH AND REUSED LITTER 15
1.6 OBJECTIVES AND HYPOTHESES 19
1.7 REFERENCES 20
1.8 Tables 34
2. The effect of dietary 25-hydroxycholecalciferol on growth and carcass traits of broilers grown on reused litter 36
ABSTRACT 36
2.1 INTRODUCTION 37
2.2 MATERIALS AND METHODS 39
2.2.1 Experimental Diets 39
2.2.2 Experimental Conditions 39
2.2.3 Data Recorded 40
5.2.4. Statistical Analysis 40
2.3 RESULTS 41
2.3.1 Production Performance 41
2.3.2 Carcass Characteristics 42
2.4 DISCUSSION 43
2.5 REFERENCES 50
2.6 TABLES 59
3. EFFECTS OF DIETARY 25-HYDROXYCHOLECALCIFEROL ON GUT MORPHOLOGY, INNATE IMMUNE RESPONSE AND BONE MINERALIZATION OF BROILERS ON REUSED LITTER 69
ABSTRACT 69

3.1 INTRODUCTION 71
3.2 MATERIALS AND METHODS 73
3.2.1 Experimental Diets and Animal Housing 73
3.2.2 Measurement of Inflammatory Status 74
3.2.3 Intestinal Histology 74
3.2.4 Bone Characteristics 75
3.2.4.1 Femur Cross-sectional Area and Bone Mineral Density 75
3.2.4.2 Femur Breaking Strength 76
3.2.5 Statistical Analysis 76
3.3 RESULTS AND DISCUSSION 77
3.3.1 Innate Immune Response 77
3.3.2 Small Intestine Morphology 80
3.3.3 Bone characteristics 83
3.3.3.1 Bone Mineral Density 83
3.3.3.2 Cross Sectional Area 84
3.3.3.3 Bone Mineral Content 85
3.3.3.4 Bone Breaking Strength 86
3.4 REFERENCES 89
3.5 TABLES 101
4. EFFECTS OF DIETARY 25-HYDROXCHOLECALCIFEROL ON BREAST MUSCLE GENE EXPRESSION OF BROILER CHICKENS RAISED ON REUSED LITTER 116
ABSTRACT 116
4.1 INTRODUCTION 117
4.2 MATERIALS AND METHODS 119
4.2.1. Experimental Diets and Animal Housing 119
4.2.2. Tissue Collection, RNA Isolation, and Library Preparation 120
4.2.3. Whole-Transcriptome Sequencing 121
4.2.4. Analysis of Differential Gene expression 122
4.2.5. Reverse Transcription and Designing and Testing of Real-Time PCR Primers for Verification of DE Genes 125
4.2.6. Statistical Analysis 128
4.3 RESULTS 128

4.3.1. Differential Gene Expression 128
4.3.2. Results of DAVID Analysis 129
4.3.3. Results of IPA Analysis 129
4.3.4. Validation of RNA-seq Expression Data by Real-Time PCR 130
4.4 DISCUSSION 131
4.4.1 Growth Properties in Breast muscle 131
4.4.2 Immune Function Genes in the Breast Muscle 134
4.4.3 Amino Acid Metabolism and Inflammation in Breast Muscle 136
4.5 REFERENCES 139
4.6 TABLES 154
4.7 FIGURES 164
5. RESEARCH SYNTHESIS 176
5.1 SUMMARY 176
5.2 ANALYSIS AND IMPLICATIONS 178
5.3 RECOMMENDATION FOR FUTURE RESEARCH 182
5.4 STUDY LIMITATIONS 183
5.6 REFERENCES 184
Bibliography 188

List of Tables

Table 1.1 Examples of transcription-factor pathways mediating nutrient–gene interactions..... 34

Table 2.1 Basal composition of the experimental diets 59

Table 2.2 Effect of vitamin D source in Fresh and Reused litter on during starter (ST; 0 to 12d), grower (GW; 12 to 22 d) and finisher (FN; 23 to 41 d) periods. 61

Table 2.3 Total mortality and BW of sampled birds fed vitamin D source in Fresh and Reused litter at 12, 22 and 41 d. 62

Table 2.4 Effect of vitamin D source in Fresh and Reused litter on absolute weights of breast meat, Pectoralis major, Pectoralis minor and liver of sampled birds at 12, 22 and 41 d. 63

Table 2.5 Effect of vitamin D source in Fresh and Reused litter on weights relative to BW of breast, Pectoralis major, and Pectoralis minor of sampled birds at 12, 22 and 41 d. 64

Table 2.6 Effect of vitamin D source in Fresh and Reused litter on absolute weights of spleen, small intestine, liver and Abdominal fat of sampled birds at 12, 22 and 41 d. 65

Table 2.7 Effect of vitamin D source in Fresh and Reused litter on weights relative to BW of spleen, small intestine, liver and Abdominal fat of sampled birds at 12, 22 and 41 d. 66

Table 2.8 Effect of vitamin D source in Fresh and Reused litter on absolute weights relative to BW of carcass parts at processing (42 d)..... 67

Table 2.9 Effect of vitamin D source in Fresh and Reused litter on live BW, carcass, and weights relative to carcass of breast meat Pectoralis major and minor, leg , and wing yield at processing (42 d)..... 68

Table 3.1 Effect of supplementation vitamin D_3 and 25-hydroxyvitamin D_3 in Fresh and Reused litter on nitric oxide, chicken haptoglobin and α-1 acid glycoprotein concentration in the blood of broiler chickens..... 101

Table 3.2 Effect of supplementation vitamin D_3 and 25-hydroxyvitamin D_3 in Fresh and Reused litter on samll intestine morphology of broiler chickens at d12 103

Table 3. 3 Effect of supplementation vitamin D_3 and 25-hydroxyvitamin D_3 in fresh and reused litter on samll intestine morphology of broiler chickens at d22 105

Table 3. 4 Effect of supplementation vitamin D3 and 25-hydroxyvitamin D3 in Fresh and Reused litter on small intestine morphology of broiler chickens at d 41. 106

Table 3.5 Effect of treatments on femur bone mineral density as measured by quantitative computed tomography at 30% of femur length from the proximal epiphysis at 12, 22, and 41d. Dependent variable adjusted to a common BW with analysis of variance. 107

Table 3.6 Effect of treatments on femur bone mineral density as measured by quantitative computed tomography at 50% of femur length from the proximal epiphysis at 12, 22, and 41d. Dependent variable adjusted to a common BW with analysis of variance. 108

Table 3.7Significant and nearly significant interactions between diets and litter for femur bone mineral density, cross sectional area and bone mineral content as measured by quantitative computed tomography at 30% and 50% of femur length from the proximal epiphysis at 12, 22, and 41d of broilers. Dependent variables adjusted to a common BW with analysis of covariance. 109

Table 3.8 Effect of treatments on femur cross-sectional area as measured by quantitative computed tomography at 30% of femur length from the proximal epiphysis at 12, 22, and 41d. Dependent variable adjusted to a common BW with analysis of variance 111

Table 3.9 Effect of treatments on femur cross-sectional area as measured by quantitative computed tomography at 50% of femur length from the proximal epiphysis at 12, 22, and 41d. Dependent variable adjusted to a common BW with analysis of variance 112

Table 3.10 Effect of treatments on femur bone mineral content as measured by quantitative computed tomography at 30% of femur length from the proximal epiphysis at 12, 22, and 41d. Dependent variable adjusted to a common BW with analysis of variance 113

Table 3.11 Effect of treatments on femur bone mineral content as measured by quantitative computed tomography at 50% of femur length from the proximal epiphysis at 12, 22, and 41d. Dependent variable adjusted to a common BW with analysis of variance 114

Table 3.12 Effect of treatments on femur bone breaking strength at 12, 22, and 41d of age. Bone breaking strength adjusted to a common BW with analysis of covariance. 115

Table 4.1 Top biological functions of differentially-expressed genes at d 12in *pectoralis major* of broiler chickens fed either vitamin D_3[1] or 25-hydroxycholecalciferol[2]; obtained by Ingenuity Pathway Analysis (IPA). 154

Table 4.2 Top biological functions of differentially-expressed genes at d 12 in *pectoralis major* of broiler chickens rasied on Fresh[1] or Reused [2] litter; obtained by Ingenuity Pathway Analysis (IPA). 156

Table 4.3 Top biological functions of differentially-expressed genes at d 12 in *pectoralis major* of broiler chickens fed vitamin D_3[3] and raised on either Fresh[1] or Reused litter[2]; obtained by Ingenuity Pathway Analysis (IPA). 157

Table 4.4 Top biological functions of differentially-expressed genes at d 12 in *pectoralis major* of broiler chickens fed either vitamin D_3[1] or 25-hydroxyvitamin D_3[2] and raised on Reused[3] litter; obtained by Ingenuity Pathway Analysis (IPA). 158

Table 4.5 Top biological functions of all differentially-expressed genes at d 12 in *pectoralis major* of broiler chickens obtained by Ingenuity Pathway Analysis (IPA). 159

Table 4.6 List of the selected endogenous and target genes used for real-time PCR for *pectoralis major* of broiler chickens at d12. 161

Table 4.7 Number of differentially-expressed genes at d 12 in *pectoralis major* of broiler chickens obtained from the comparison between birds fed vitamin D_3 or 25-hydroxycholecalciferol and raised on either Fresh or Reused litter from 27 samples. 162

Table 4.8 Differentially-expressed genes at 12 d of age (p-value < 0.05) in *P. major* of broiler chickens fed various vitamin D treatments and raised on either Fresh or Reused litter. 163

Appendix 4. 1 The list of differentially-expressed genes obtained from the comparison between dietary inclusion vitamin D_3 or 25-hydroxycholecalciferol in either fresh or reused litter in pectoralis major of broiler chicken at d12 173

List of Figures

Figure 4. 1 Calcium signaling pathway. 164

Figure 4. 2 Gene interaction network between the 11 differentially expressed genes (bolded in this network) across treatment effects related to muscle development in pectoralis major of broiler chicken at d 12..... 166

Figure 4. 3 The gene interaction network between the 7 differentially expressed genes (bolded in this network) across treatment effects related to immune response and inflammation in pectoralis major of broiler chicken at d 12 168

Figure 4. 4 The gene interaction network between the 9 differentially expressed genes (bolded in this network) across treatment effects related to amino acid metabolism in pectoralis major of broiler chicken at d 12..... 170

Figure 4.5 The gene interaction network between the 11 differentially expressed genes (bolded in this network) across treatment effects related to growth and immune response in pectoralis major of broiler chicken at d 12 172

LIST OF ABBREVIATIONS AND SYMBOLS

$1,25\text{-}(OH)_2D_3$ – Calcitriol; 1,25 dihydroxycholecalciferol

18S – 18S ribosomal RNA

25OHD – Treatment providing 69 µg/kg 25-OH-D3 from d 0 to 40

$25OHD_3$ – Calcidiol; 25 hydroxycholecalciferol

α – Alpha

ACTB – Actin, beta

AGP – α1 acid glycoprotein

ALDH1L2 – Aldehyde dehydrogenase 1 family, member L2

ALDH18A1 – Aldehyde dehydrogenase 18 family, member A1

APP – Acute phase protein

ASNS – Asparagine synthetase

Arg – Arginine

ATF4 – Activating transcription factor 4

ß – Beta

BFIV21 – MHC class I alpha chain 2

BBS – Bone breaking strength

BMC – Bone mineral content

BMD – Bone mineral density

BLAST – Basic local alignment search tool

BW – Body weight

^oC – Centigrade

CaMKIIA – Calcium/calmodulin-dependent protein kinase II alpha

cAMP – Cyclic adenosine monophosphate

CCE – Capacitative calcium entry

CDK2AP1 – Cyclin-dependent kinase 2 associated protein 1

cDNA– Complementary DNA

CHRND – Cholinergic receptor, nicotinic, delta

cm – Centimeter

CPM – Counts per million

CREB – cAMP responsive element binding protein

CRHR2 – Corticotropin releasing hormone receptor 2

CSA – Cross-sectional area

CTH – Cystathionine gamma-lyase

CSA – Bone cross sectional area

Cys – Cysteine

d – Day

D – Vitamin D_3, dietary treatment with vitamin D_3 at 2,760 IU/kg feed

D+25OHD – Dietary treatment with D_3 at 2,760 IU/kg plus $25OHD_3$ at 2,760 IU/kg feed

DAVID – Database for Annotation, Visualization, and Integrated Discovery expression

DBP – Vitamin D_3 binding protein

DE – Differentially expressed

DFM – Direct-fed microbials

DNA – Deoxyribonucleic acid

Dx2 – Dietary treatment with D_3 at 5,520 IU/kg feed

ENPP2 – Ectonucleotide pyrophosphatase/phosphodiesterase 2

ERK1/2 – Extracellular signal-regulated kinases;

g – gram

GAA – Glucosidase, alpha; acid

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

Gly – Glycine

GO – Gene ontology

FCR – Feed conversion ratio

FDR – false discovery rate

FGFR2 – Fibroblast growth factor receptor 2

FI – Feed intake

Fresh – Experimental treatment group in which broilers were grown on fresh pine shavings litter

HERPUD1 – Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein

HLA-A – Human leukocyte antigen-A

Hp – Haptoglobin

IFNG – Interferon gamma

IFN-γ – Interferon-γ

IGF-1 – Insulin-like growth factor 1

IgA– Immunoglobulin A

IgJ – Immunoglobulin J

IKB– Ingenuity knowledge base

IL – Interleukin

IPA – Ingenuity pathway analysis

IP3 – Inositol trisphosphate

IU – International units

JCHAIN – Joining chain of multimeric IgA and IgM

Kg – Kilogram

LOC417083 – Class I histocompatibility antigen, F10 alpha chain-like, transcript variant X6

LOC769716 – Class I histocompatibility antigen, F10 alpha chain-like Mrna

LPS – Lipopolysaccharide

Lys – Lysine

m - Meter

m^2 – Square meter

MBD2 – Methyl-CpG binding domain protein 2

MDS – Multi-dimensional scaling

μ - Micro

μg – Micro gram

mg – Milligram

μg – Microgram

μl – Micro liter

μM – Micromoles

mm – Millimeter

ml – Milliliter

MRF4 – Myogenic regulatory factor 4

MRF – Myogenic regulatory factors

mTOR – Mechanistic target of rapamycin

MyoG – Myogenin

Myf5 – Myogenic factor 5

MyoD – Myogenic determination protein

n – Number of observations

NGS – next-generation sequencing

nM – Nano moral

Nnt – Nicotinamide nucleotide transhydrogenase

NO – Nitric oxide

NRC – National Research Council

OP – Optical density

π – Pi number

P – pectoralis

Pax7 – Null mutations in paired box 7

PHGDH – Phosphoglycerate dehydrogenase

PKA – Protein kinaseA

PLoS – Public Library of Science

P. major – Pectoralis major

P. minor – Pectoralis minor

QM7 – Quail myoblast

PTH – Parathyroid hormone

r^2 – Square radius

Reused – Experimental treatment group in which broilers were grown on recycled pine shavings litter

RIN – RNA integrity number

RNA – Ribonucleic acid

RNA-seq – RNA- sequencing

RPS6K – Phospho ribosomal P70 S6 kinase

RXR – Retinoid X receptor

s – Seconds

S6K – S6 kinase

SC – Saterlite cells

Ser – Serine

siRNA – Small interfering RNA

SPP1– Osteopontin

SLC1A4 – Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4

SLC38A3 – Solute carrier family 7 (cationic amino acid transporter, y+ system), member 3

SLC6A9 – Solute carrier family 6 (neurotransmitter transporter, glycine), member 9

SLC7A3 – Solute carrier family 7 (cationic amino acid transporter, y+ system), member 3

Th2 – T-helper 2

TD – Tibial dyschondroplasia

TNF – Tumor necrosis factor

TRPC – Transient receptor potential-canonical

TRPC3 – Certain mammalian TRP proteins

TRPV6 – Transient receptor potential vanilloid type 6

TMM – Trimmed mean of M-values

UBI – Ubiquitin extension protein

VCR – Villus height:crypt depth ratio

VDR – Vitamin D receptor

VDRE – Vitamin D response elements

VEGF – Vascular endothelial growth factor

Vitamin D_2 – Ergocalciferol

Vitamin D_3 – Cholecalciferol

VSA – Villus surface area

wk – Week

1. LITERATURE REVIEW

1.1 INTRODUCTION

Broiler chickens have been intensively selected for rapid growth, high meat yield and feed efficiency (Havenstein et al., 2003; Schmidt et al., 2009). With this pressure, birds have shown several undesirable traits, including skeletal disorders (Shim et al., 2012), impaired immune function (Cheema et al., 2003), and reproductive complications (De Beer and Coon, 2007). In addition, nutrition plays important roles in the expression of genes involved in biological process like immune response and growth (Wang et al., 2009). However, there is little information on the mechanisms by which nutrients can regulate gene expression related to growth, immune response and the reproductive systems of chickens. To achieve this goal, RNA-sequencing (RNA-seq) can provide invaluable information to help researchers investigate the relationship between nutrients and genes that may regulate canonical pathways or changes in phenotype in the chicken (Wang et al., 2009).

Vitamin D has crucial functions in calcium and phosphorus metabolism and prevention of skeletal development problems in birds (Fritts and Waldroup, 2003; Sun et al., 2013). Dietary vitamin D_3 and 25-hydroxycholecalciferol ($25OHD_3$) are commercially used in the poultry industry due to the lack of direct sun light for endogenous production vitamin D in the skin (Coffey et al., 2012). Dietary $25OHD_3$ can increase broiler performance (Yarger et al., 1995), breast meat yield (Vignale et al., 2015), adaptive immunity (Shojadoost et al., 2015), small intestine morphology (Chou et al., 2009), and bone ash (Świątkiewicz and Koreleski, 2005) relative to those fed vitamin D_3 at the same level of dietary inclusion.

Poultry litter is mixture of feces, feathers, feed and bedding material. This mixture may contain pathogenic bacteria which could increase enteric pathogens in birds grown on recycled litter in comparison to those reared on fresh litter (Torok et al., 2009; Shanmugasundaram et al., 2012). Additionally, the composition of gut microbiota can be different in birds are raised on reused relative to those in fresh litter. For example, intestinal origin (*Clostridiales*) bacteria are increased in birds are reared on reused litter, whereas, environmental bacteria are more prevent in birds grown on fresh litter (Lu et al., 2003; Lee et al., 2011; Cressman et al., 2010). Therefore, increased pathogenic bacteria in reused litter as compared to fresh may reduce growth performance (Torok et al., 2009), and increase inflammatory response of broiler chickens (Shanmugasundaram et al., 2012). However, poultry litter may also contain commensal bacteria from the previous flocks. These symbiotic microorganisms can colonize the small intestine of chickens to protect the gut from enteric pathogens. Commensal microbiota in the reused litter increased villus parameters, the expression of anti-inflammatory responses, and performance of broiler chickens (Lee et al., 2010; Lee et al., 2013; Kalita et al. 2012).

The objective of this review is to explore the mechanisms and effects of vitamin D sources and reused litter on broiler performance, carcass characteristics, immune response and gene expression.

1.2 VITAMIN D

The discovery of vitamin D and its roles goes back to the beginning of the 20th century (McCollum, 1925). Vitamin D_3 is a pro-hormone which requires two metabolic steps to be converted to the active hormone 1, 25-dihydroxyvitamin D_3 (1,25 $(OH)_2D_3$; Yarger et al., 1995). Vitamin D_3 is involved in biological processes, including calcium homeostasis, intestinal

absorption of calcium and phosphorus, and bone resorption (Soares et al., 1995; Atencio et al., 2005; Rama-Rao et al., 2006; 2009). In addition, vitamin D_3 has a wide range of metabolic effects including increasing bone mineralization and mobilization (Fritts et al., 2003), immune response (Morris et al., 2014) and muscle development in broiler chickens (Boland et al., 1995).

1.2.1 Vitamin D and metabolites

Vitamin D has antirachitic activity, and several vitamin D compounds have been discovered; however, the most important form in animal diets is cholecalciferol (vitamin D_3). In chickens, vitamin D_3 can be obtained from the diet or by the conversion of 7-dehydrocholesterol to vitamin D_3 in the skin by the action of ultra-violet light. On the other hand, as the bird's body is covered with feathers and there is little chance to access sunlight in commercial poultry houses, endogenous production of vitamin D_3 is limited (Soares et al., 1995; Atencio et al., 2005). Ergocalciferol (vitamin D_2) has low affinity for to vitamin D binding protein, leading to little metabolism of this form in chickens (Soares et al., 1995). Vitamin D_3 has been commercially used in poultry feed, but requires two hydroxylation steps to become the biologically active form in chickens (Soares et al., 1995). The National Research Council (NRC; 1994) recommended level of vitamin D_3 in broiler chicken diets is 200 IU/kg feed. Vitamin D_3 at 1,000 IU/kg feed or above increased bone mineralization (Rama-Rao et al., 2006), ash content (Fritts and Waldroup, 2003), growth performance (Fritts and Waldroup, 2003), breast muscle yield (Khan et al., 2010), walking ability and welfare status of chickens grown at high stocking density (Sun et al., 2013), and reduced tibial dyschondroplasia (TD) and rickets in broiler chickens relative to vitamin D_3 at 200 IU/Kg (Fritts and Waldroup, 2003; 2005). Dietary supplementation of vitamin D_3 is commonly used between 2,000 to 5,000 IU/kg feed in commercial poultry diets (Fritts and Waldroup, 2003).

Vitamin D_3 is hydroxylated to 25-hydroxylcholecalciferol ($25OHD_3$) by 25-hydroxylase in the hepatic cells of the liver (Atencio et al., 2005). Later, $25OHD_3$ is transported to the kidney by vitamin D_3 binding protein (DBP) and is hydroxylated to 1, 25-dihydroxylcholecalciferol [$1,25\text{-}(OH)_2D_3$] by 1 α-hydroxylase (; Shanmugasundaram and Selvaraj, 2012). In mammals and birds, 1 α-hydroxylase mRNA has been identified in kidney, skeletal muscle fibers, small intestine, macrophages and bone (Omdahl et al., 2002; Shanmugasundaram and Selvaraj, 2012). The $1,25\text{-}OH_2D_3$ is the most metabolically active form of vitamin D, having 4 to 13 times more activity than vitamin D itself (Yarger et al., 1995). However, this metabolite of vitamin D_3 is toxic for birds at three times the recommended level of 200 IU /kg feed in the broiler industry (Pesti and Shivaprasad, 2010; Soares et al., 1995). Additionally, $25OHD_3$ is more physiologically active than vitamin D_3 (Soares et al., 1995), and is the natural circulating and storage form of vitamin D_3 in birds (Yarger et al., 1995). Dietary $25OHD_3$ is less toxic than $1,25\text{-}OH_2D_3$, also, $25OHD_3$ has a higher absorption rate in the small intestine (Bar et al., 1980), and higher bio-potency at low levels of inclusion relative to vitamin D_3 (Fritts and Waldroup, 2003). In addition after absorption, vitamin D_3 is highly converted to $25OHD_3$ (Ehrhardt et al., 2016). Therefore, $25OHD_3$ is the most appropriate marker to determine vitamin D_3 status as compared to other metabolites (Soares et al., 1995; Bar et al., 2003). In renal cells, $25OHD_3$ is also converted to $24,25(OH)_2D_3$ the excretory form of vitamin D_3, by 24 hydroxylase (Norman, 1978). This metabolite also participates in bone development (Seo et al., 1997). The serum level of $1,25\text{-}(OH)_2D_3$ is highly regulated by parathyroid hormone (PTH), Ca serum level , $1,25\text{-}(OH)_2D_3$ itself, estrogens, prolactin, and growth hormone. Hypocalcemia increases $1,25\text{-}(OH)_2D_3$ production in response to increased PTH. However, production of $24, 25(OH)_2D_3$ for excretion

increases in response to decreased 1 α-hydroxylase activity when serum concentration of calcium is normal or high (De Matos, 2008).

In the broiler chicken, dietary $25OHD_3$ has higher absorption rate and lower excretion rate relative to vitamin D_3 at same level of dietary inclusion (Chou et al., 2009; Bar et al, 1980). The absorption of vitamin D_3, as fat-soluble vitamin, is facilitated by the presence of fat and bile salts (Garrett and Young, 1975). More polar molecule have greater absorption rate from the digestive tract due to less dependency on bile salts (Borel, 2003). Additionally $25OHD_3$ is a more polar molecule than vitamin D_3, and its absorption is less dependent on bile salts relative to vitamin D_3. Therefore, the 1.5 times higher intestinal absorption of $25OHD_3$ relative to vitamin D_3 at same level of dietary inclusion (2,760 IU/kg) is likely related to the greater polarity of $25OHD_3$ (Bar et al., 1980). During the first two weeks of life the absorption of vitamin D_3 by the chick is low due to immaturity of the digestive tract and low activity of enzymes involved in lipid absorption (Noy and Sklan, 1995). During stressful conditions, such as mycotoxicosis (Yarru et al., 2009), or bacterial infection (Peighambari et al., 2000) hydroxylation is reduced in liver of chickens (Thaxton and Puvadolpirod, 2000). In this situation, vitamin D status can still be supported by dietary supplementation of $25OHD_3$ since the first hydroxylation step of vitamin D_3 in liver can be bypassed (Käppeli et al., 2011). Commercially-available $25OHD_3$ is manufactured from cholecalciferol by DSM Nutritional Products, Basel, Switzerland under the commercial name of HyD® (Michalczuk et al., 2010).

1.2.2 Dietary supplementation of $25OHD_3$ in broiler chicken diets

The dietary supplementation of $25OHD_3$ from 50 to 69 μg/kg to broiler chickens increased BW, BW gain, feed intake and enhanced feed efficiency (Bar et al., 2003; Yarger et al., 1995), particularly when included throughout the growing period in comparison to vitamin

D_3 at the same levels of inclusion (; Fritts and Waldroup, 2003). At processing, BW from 46 to 52 d increased by an average of 42 g for birds fed $25OHD_3$ at 2,760 IU/kg feed relative to vitamin D_3 (Yarger et al., 1995). Dietary inclusion of $25OHD_3$, as a partial replacement of dietary vitamin D_3 activity, increased BW, BW gain (Michalczuk et al., 2010), and decreased feed conversion ratio (FCR) by 0.026 kg/kg (Yarger et al., 1995; Michalczuk et al., 2010). The increased performance of broiler chickens fed the combination of $25OHD_3$ and vitamin D_3 might have been due to more efficient absorption of $25OHD_3$ in the upper small intestine, and the ability to bypass the first hydroxylation step in the liver for the endocytosis of $25OHD_3$ (Broulík, 2002; Papešová et al 2008). However, there were no differences in broiler performance when they were fed $25OHD_3$ at a high level of inclusion (5,520 IU/kg feed) relative to those fed vitamin D_3 at the same level (Vignale et al., 2015). Therefore, supplementation of $25OHD_3$ at a commercial level of inclusion is more effective in broiler performance relative to vitamin D_3, possibly due to the close inhibition of conversion of $25OHD_3$ to 1, $25(OH)_2D_3$ by serum Ca level.

The mucosa has a crucial role in protection of the mucus membrane against infection and enteric pathogens and other foreign materials (Sun et al., 2010). In chickens, vitamin D affects the morphological and functional development of the intestinal mucosa by mediating putrescine (Shinki et al., 1991). Putrescine, spermidine, and spermine are three main polyamines that modulate proliferation and differentiation in the small intestine (Tabor and Tabor, 1984). Ornithine decarboxylase and spermidine N-acetyltransferase are linked to putrescine production; $1,25\text{-}(OH)_2D_3$ induces production of both enzymes (Shinki et al., 1981 and 1985). Therefore, $1,25\text{-}(OH)_2D_3$ can change the morphological and functional development of small intestine by regulating putrescine production.

The serum concentration of $25OHD_3$ was higher in broilers fed dietary of $25OHD_3$ (at 2,760 IU/kg feed) relative to those vitamin D_3 at the same level of activity (Yarger et al., 1995; Hutton et al., 2014; Vignale et al., 2015). However, serum concentration of $1,25\text{-}(OH)_2D_3$ did not differ between $25OHD_3$-fed birds and D_3-fed birds (Yarger et al., 1995). This could be due to the tight regulation of $1,25\text{-}(OH)_2D_3$ by VDR and $1,25\text{-}(OH)_2D_3$ itself (De Matos, 2008; Russell et al., 1993). On the other hand, 1 α-hydroxylase, which converts $25OHD_3$ to $1,25\text{-}(OH)_2D_3$ has been identified in the intestine of mammals and chickens (Omdahl et al., 2002; Shanmugasundaram and Selvaraj, 2012). $25OHD_3$ can be converted to $1,25\text{-}(OH)_2D_3$ in the small intestine by the action of 1 α-hydroxylase to stimulate putrescine production, leading to an increase in small intestine development, however, this enzyme cannot convert vitamin D_3 to $1,25(OH)_2D_3$, Therefore the increase in intestinal morphological parameters in $25OHD_3$-fed birds relative to D_3-fed birds could be due to increased expression of 1 α-hydroxylase in small intestine of $25OHD_3$-fed chickens.

Ding et al. (2011) reported that dietary inclusion of $25OHD_3$ at 4,000 IU/kg feed in the maternal diet increased villus length in the jejunum and duodenum and crypt depth in all small intestine sections of post-hatch broiler chickens at d 7 of age relative to those from hens fed vitamin D_3 at 3,000 IU/kg feed and $25OHD_3$ at 1,000 IU/kg feed. Additionally, supplementation of $25OHD_3$ at 2,760 IU/kg feed resulted in shallower crypts, greater villus height, thicker intestinal muscular layer, and higher villus length to crypt depth ratio in the duodenum and jejunum in comparison to vitamin D_3 at the same level inclusion of broiler chickens at d28 (Chou et al., 2009). Longer villus length is linked to increased nutrient absorption in response to the associated increase in mucosal surface area (Onderci et al., 2006), shallower crypts are associated with a decrease in gut maintenance energy due to lower turnover rate of the intestinal

epithelium (Yang et al., 2008). Therefore, dietary $25OHD_3$ could result in increased nutrient absorption in the gut relative to vitamin D_3.

Supplementation of $25OHD_3$ relative to vitamin D_3 increased bone characteristics in broiler chickens. Dietary supplementation of $25OHD_3$ as a complete or partial replacement for vitamin D_3 at the same level of inclusion increased bone ash (Fritts and Waldroup, 2003; Świątkiewicz and Koreleski, 2005; 2006), bone calcification (Gómez-Verduzco et al., 2013), bone strength (Świątkiewicz and Koreleski, 2005; 2006), and reduced incidence and severity of tibial dyschondroplasia in broiler chickens (Rennie and Whithead, 1996). Also, male chicks from egg injected with $25OHD_3$ at 100 μL had increased bone breaking strength in comparison to male chicks from non-injected eggs at d 21 and d 28 (Bello et al., 2014). In mice, increased bone mineralization was associated with increased expression of 1 α-hydroxylase in bone tissue, but was not linked to increased circulating $1,25(OH)_2D_3$ levels (Anderson et al., 2005). Therefore, increased in expression of 1 α-hydroxylase, which converts $25OHD_3$ to the active metabolite $1,25(OH)_2D_3$, and an increased rate of absorption of $25OHD_3$ in the small intestine could allow for subsequent conditions that increase bone formation in $25OHD_3$-treated birds.

Dietary $25OHD_3$ has also shown effects on immune response of broiler chickens. Dietary $25OHD_3$ relative to vitamin D_3 reduced inflammatory response and increased humoral immunity in broiler chickens when birds were challenged with pathogens (Morris et al., 2014; Chou et al., 2009). In lipopolysaccharide-injected birds, the expression of 1 α-hydroxylase in the liver increased in $25OHD_3$-fed birds relative to vitamin D_3-fed birds. This may result in increased $1,25(OH)_2D_3$ in the liver and therefore suppresses inflammatory response in $25OHD_3$-fed birds (Christakos et al., 2010). 1 α-hydroxylase and VDR are expressed in chicken macrophages and liver (Shanmugasundaram and Selvaraj, 2012; Shojadoost et al., 2015) and increased expression

of 1 α-hydroxylase resulted in increased expression of VDR in response to increased biosynthesis of $1,25(OH)_2D_3$ from $25OHD_3$ (Shojadoost et al., 2015). The $1,25(OH)_2D_3$ also stimulates macrophages to produce NO and up-regulates pro-inflammatory response genes. During the inflammation, $1,25(OH)_2D_3$ induced NO production in macrophages to inhibit the growth of pathogens and secrete inflammatory mediators and cytokines. Contrary to *in vivo* results, when the chicken macrophage cell line HD11 was treated with $25OHD_3$ and stimulated with LPS, the cells produced more nitric oxide (NO) and IL-1β, possibly due to increased expression of biosynthesis of $1,25(OH)_2D_3$ from $25OHD_3$ and subsequent expression of VDR in response to increased 1 α-hydroxylase (Morris and Selvaraj, 2014; Shojadoost et al., 2015). However, in live birds 1 α-hydroxylase seems to be more expressed in liver than macrophages which can result in reduced inflammatory response and increase anti-inflammatory response (Morris and Selvaraj, 2014).

Dietary inclusion of $25OHD_3$ increased breast muscle yield as compared to vitamin D_3 at the same level of inclusion (Yarger et al., 1995; Vignale et al., 2015). The increased in breast muscle yield might have been occurred through activation of the mechanistic target of rapamycin (mTOR)/ phospho ribosomal P70 S6 kinase (RPS6K) signaling pathway (Vignale et al., 2015). The mTOR pathway is most likely linked to growth and cell proliferation (Sabatini and Laplanteand, 2012) and S6 kinase (S6K) is associated with an increase in protein synthesis (Ma and Blenis, 2009). Satellite cells (SC) are present in post-hatch broiler skeletal muscle and play an important role in hypertrophic skeletal muscle growth (Moss, 1968). An increased number of SC is associated with increased myofibrillar protein synthesis in broiler chickens (Moss and Leblond, 1971). Supplementation of $25OHD_3$ increased the activity of satellite cells in the chicken pectoralis major at d 42 relative to vitamin D_3 at the same level of inclusion (Hutton et

al. 2014). Therefore, $25OHD_3$ not only up-regulated genes involved in protein synthesis and muscle hypertrophy growth, but also triggered the activity of SC leading to the hypertrophic growth of muscle.

1.3 MYOGENIC REGULATORY FACTORS GENES AND SKELETAL MUSCLE DEVELOPMENT IN CHICKEN

Skeletal muscle development begins in embryogenesis and continues throughout adulthood in the chicken. Progenitor cells induce myoblasts to proliferate and fuse into multinucleated fibers resulting in enlargement of myofibers. This process occurs during embryogenesis and slows as the animal grows (Yablonka-Reuveni, 1995a). However, in the adult chicken during injury, exercise or stretch, fusion of myoblasts into myofibers can still take place (Yablonka-Reuveni, 1995b). Additionally, satellite cells are the primary source of postnatal growth of skeletal muscle. Satellite cells are located below the myofiber basement membrane and are characterized by myogenic precursors in postnatal and adult muscle. (Yablonka-Reuveni, 1995b; Yablonka–Reuveni and Paterson, 2001). Chicken myogenic populations involved in muscle development are broadly characterized in three categories, including early in embryogenesis (embryonic myoblasts); late in embryogenesis (fetal myoblasts); and satellite cells which are present in postnatal and adult muscle (adult myoblasts). The development of embryonic myoblasts takes place on embryonic day 5, the development of fetal myoblasts occurs between embryonic days 8 and 12, and the adult myoblasts are most abundant at the final stages of embryogenesis (Yablonka–Reuveni 1995a; b).

Satellite cells also are the dominant myogenic precursors in immature and adult avian muscles (Armand et al., 1983). The skeletal muscle lineage is determined by expression patterns

of the chicken myogenic regulatory factors (MRF), including myogenic factor 5 (Myf5), myogenic determination protein (MyoD), myogenin (MyoG) and myogenic regulatory factor 4 (MRF4; Liu et al., 2012; Yablonka-Reuveni and Paterson, 2001). These three nuclear regulatory factors are involved in skeletal muscle hypertrophy (Rudnick and Perry, 2000). Myf5 is the first MRF expressed during avian muscle development (Mok et al., 2015). Expression of Myf5 promotes cell proliferation, leading to an increase in the number of mononuclear myoblasts (Mok et al., 2015; Biressi et al., 2013). Myf5 and MyoD together are associated with maintenance of myoblasts (Haldar et al., 2008; Mok et al., 2015). The expression of MyoG occurs after Myf5 and MyoD and is involved in engagement of muscle differentiation process. MyoG also is the differentiation factor in the myogenic process, for example it aids in the specification of muscle lineage during the formation of myoblasts in mice (Moncaut et al., 2013). Interestingly, MyoG and Myf5 regulate their own expression and interact with MyoD. In the chickens, MyoD and MyoG play a role in proliferation to differentiation of fetal and adult myoblasts (Yablonka–Reuveni and Paterson, 2001). In chickens, expression of MyoG and Myf5 are linked to increased breast and leg muscle yield in early post-hatch and adult chickens (Genxi et al., 2014). Additionally, increased expression of MyoG is associated with increased SC proliferation (Clark et al., 2016). Therefore, increased expression of MRF may result in increased meat production in both mammals and chickens (Singh and Dilworth, 2013; Genxi et al., 2014).

1.3.1 Role of vitamin D sources in post-hatch skeletal muscle development

Postnatal skeletal muscle growth occurs through hypertrophic growth of myofibers and increased protein synthesis (Armand, 1983). An increase in the number of satellite cells can increase the potential for hypertrophic skeletal muscle growth and increase myofibrillar protein synthesis in chickens (Armand, 1983; Moss and Leblond, 1971). The active metabolite

$1,25(OH)_2D_3$ stimulates the proliferation and differentiation of myofibers into myotubes in broiler chickens (Giuliani and Boland, 1984). The connection of vitamin D with skeletal muscle development may be determined by the expression of vitamin D receptor (VDR; Boland, 2011). In mice, the inhibition of VDR resulted in decreased skeletal muscle fiber size in response to down-regulation of myogenic regulatory factors (Endo et al., 2003). Increased serum level of $1,25\text{-}(OH)_2D_3$ stimulates expression of VDR in both mammals and chickens (Russell et al., 1993). Additionally, the 1 α-hydroxylase responsible for conversion of $25OHD_3$ to $1,25\text{-}(OH)_2D_3$ has been identified in high amount in the leg and breast muscles of chickens (Shanmugasundaram and Selvaraj, 2012). Therefore, $25OHD_3$ may increase the $1,25\text{-}(OH)_2D_3$ and VDR as a result of an increase in expression of 1 α-hydroxylase in chicken muscle.

Starkey et al. (2014) suggested that vitamin D plays a role in the maturation and hypertrophic growth of postnatal skeletal muscle, leading to an increase in muscle protein deposition in both chickens and mammals. *In vitro* $25OHD_3$, as a partial replacement for vitamin D_3 increased satellite cell activity in chickens (Hutton et al., 2014; Starkey et al., 2014) and swine (Hines et al., 2014; Starkey et al., 2014). The paired box 7 (Pax7) is associated with SC activity in skeletal muscle (Von Maltzahn et al., 2013). The supplementation of $25OHD_3$ increased nuclear density and muscle fiber cross-sectional area, tended to increase Pax7+satellite cells in pectoralis major of broiler chickens at d 35 (Hutton et al., 2014) and increased Pax7+ satellite cells in longissimus muscles of piglets relative to vitamin D_3 (Hines et al., 2014). Taking this data in to account, dietary $25OHD_3$ is most likely to increase skeletal muscle hypertrophy growth through increased myofibrillar protein synthesis and satellite cell activity in both chickens and mammals (Hutton et al., 2014; Hines et al., 2014; Starkey et al., 2014).

1.4 NUTRIGENOMICS AND VITAMIN D RECEPTOR

Nutrigenomics as a multidisciplinary research field in nutritional science has emerged to explain how nutrients can affect gene expression to regulate animal health and production (Neibergs and Johnson, 2012; Ghormade et al., 2012; Ashwell and Angel, 2011). Bioactive food compounds are physiologically active ingredients in nutritional regimes or animal diets which play a role in health and production (Kris-Etherton et al., 2002). A transcription factor is a protein that binds to specific DNA to initiate and regulate the transcription of genes (Szpirer et al., 1991). Nutrigenomics provides invaluable information to understand how diets or particular bioactive food compounds regulate the activation of genes, transcription factors, proteins, and metabolites (Ghormade et al., 2012). The purpose of nutrigenomics can be summarized in four aspects: 1) identification of transcription factors (as nutrient targets); 2) identification of signaling pathways involved at the cellular level and characterization of the main dietary signals; 3) measurement of particular nutrients associated with functional genes; 4) identification of interactions between nutrients linked to metabolic canonical pathways. (Loor et al.., 2006; Loor et al., 2013; Loor et al., 2015; Müller and Kersten, 2003; Fenech et al., 2011). Nutrients or nutritional regimes are associated with transcription factors involved in regulation of gene, protein and metabolite signatures (Müller and Kersten, 2003). The transcription factor of vitamin D, VDR is heterodimerized with 9-*cis*-retinoic acid receptor (RXR) in the nucleus. Specific target gene promoter regions known as vitamin D response elements (VDRE) via the VDR-RXR heterodimer complex promote gene expression (Haussler et al., 1995; Deeb et al., 2007). Binding of nuclear VDR to VDRE is facilitated by 1,25-$(OH)_2D_3$ in order to regulate gene expression. Additionally, 1,25-$(OH)_2D_3$ ligand alters the conformation of the hormone-binding domain of VDR to reach strong dimerization with RXR in order to link to the VDRE (Haussler et al., 1995).

In mammals, 1,25-$(OH)_2D_3$-VDR triggers ossification by activation of bone osteopontin (SPP1) gene in osteoblasts (Weissen-Plenz et al., 2008); and stimulates intestinal calcium uptake by activation of the transient receptor potential vanilloid type 6 (TRPV6; Barthel et al., 2007). The TRPV6 is the key calcium channel gene which promotes osteoblastogenesis and facilitates dietary calcium for bone mineralization and thereby prevents rickets in the mouse (Barthel et al., 2007). The 1,25-$(OH)_2D_3$ via VDR stimulates an increase in Ca^{2+} in skeletal muscle of chickens and rat osteoblastic-like cells through the involvement of transient receptor potential-canonical (TRPC) proteins in capacitative calcium entry (CCE; Santillan et al., 2004). The CCE is identified in chicken muscle and is involved in Ca^{2+} mobilization (Baldi et al., 2002). The expression of certain mammalian TRP proteins (TRPC3) induce the CCE in response to an increment in 1,25-$(OH)_2D_3$ (Santillan et al., 2004). This indicates the regulation of gene expression through VDR and 1,25-$(OH)_2D_3$.

1.4.1Next generation sequencing in chicken studies

High-throughput technologies, including microarrays and RNA-sequencing (RNA-seq) as next-generation sequencing (NGS) have been developed to investigate functional biological networks between genes linked to dietary nutrients (Bordbar and Palsson, 2012). RNA-seq as a high-throughput NGS technology has recently emerged in gene expression studies, and provides analysis of gene transcription through cDNA sequencing on a massive scale (Mortazavi et al., 2008; Voelkerding et al., 2009). With this revolutionary technology, a number of challenges posed in the microarray method are eliminated, for example RNA-seq has higher dynamic range of detection which allows detection of greater differentially expressed genes with higher fold-changes relative to traditional microarray and Affymetrix arrays (Marioni et al., 2008; Zhao et al., 2014). Additionally, RNA-seq is able to provide more detailed information about the coding

RNA portion of the total RNA, detection of genes with low levels of expression, splice variants and novel transcripts, as compared to the microarray technique (Marioni et al., 2008; Mortazavi et al., 2008; Cloonan et al., 2008; Wang et al., 2009; O'Brien et al., 2014; Zhao et al., 2014). With these advantages, RNA-seq is becoming a very reliable method for studying nutrigenomics in chickens (Loor et al., 2015). For example, in a recent genome wide RNA-seq analysis, 11,560 transcripts and 9,824 genes per sample were identified in breast muscle of broiler chickens (Piórkowska et al. 2016). RNA-seq data revealed that birds with more tender meat had increased expression of genes linked to meat tenderness and muscle development. In another study between high and low efficient broiler chickens, 1,059 genes identified were differentially expressed; feed efficiency was linked to inflammatory response, oxidative stress and breast muscle growth in broiler chickens (Zhou et al., 2015). Taking this data in to account, the information regarding functional genes identified using RNA-seq contribute to the understanding of the relationship between the whole genome and particular dietary or environmental conditions in chickens.

1.5 FRESH AND REUSED LITTER

The placement of day-old broilers on recycled litter is widely used in modern intensive commercial broiler production in many countries (Volkova et al., 2009). Recycled litter contains the bedding materials, feathers, feed, and microbiota. The majority of microbiota in poultry litter are gram positive and can surpass 10^{10} cells per g of litter (Bolan et al., 2010). Poultry litter may contain 30% crude protein and high levels of minerals (Martin and McCann, 1998), and contain B-complex vitamins (Halbrook et al. 1950). Reuse of litter can be cost-efficient due to reduced cost of removal, disposal, and purchase of new litter (Coufal et al., 2006). In addition, the microbiota in poultry litter used by previous flocks can function as a competitive exclusion

culture for newly-placed chickens, in which beneficial bacteria colonize the gastro-intestinal tract and protect gut from enteric pathogens (Nurmi and Rantala, 1973; Nakamura et al., 2002; Zhang et al., 2007). These bacteria are able to delay intestinal colonization by pathogens such as *C. perfringens* during the early post-hatch period (Wei et al., 2013). The use of recycled litter resulted in increased broiler BW as compared to those reared on fresh litter (Kalita et al,. 2012). Birds reared on reused litter had lower expression of pro-inflammatory mediators including interferon-γ (IFN-γ), and interleukin 1 beta (IL-1β) as compared to those reared on fresh litter at d 28. At 42, birds reared on reused litter had lower serum antibodies against *Eimeria spp*. and *C. perfringens* than fresh litter (Lee et al., 2013). This increased in performance and innate immune response in chicken raised in reused litter may involve the concept of competitive exclusion.

Chickens fed direct-fed microbials (DFM) have longer villi, deeper crypts, and a greater ratio of villus to crypt as compared to non-DFM-fed birds (Lee et al., 2010). This may indicate that DFM could enhance nutrient absorption in broiler chickens. DFM also have immunomodulatory effects in broiler chickens challenged with enteric pathogens, including increased antibody production and epithelial cell turnover, decreased epithelial cell apoptosis, and development of T-cells (Erickson and Hubbard, 2000; Corthesy et al., 2007; Galdeano et al., 2007; Ng et al., 2009). Additionally, DFM reduce the acute phase inflammation biomarker α-1 acid glycoprotein at d 35 and 41, reduce expression of the inflammatory response (such as interleukin -6), and up-regulate interleukin 2 (IL-2) and interlukin-4 (IL-4; Waititu et al., 2014; Lee et al., 2010). These cytokines are produced in response to antigenic stimulation from T-helper 2 (Th2) cells. The increased expression of IL-2 is associated with increased T-cell production (Park et al., 2008).

In spite of these potential advantages, reused litter may also contain many pathogenic bacteria, including *Salmonella* spp., *Campylobacter* spp., *Escherichia coli*, *Clostridium perfringens*, and *Staphylococcus aureus* (Lu et al., 2003; Cressman et al., 2010; Roll et al., 2011). With those accumulated pathogens, there is a health concern for flocks raised on reused litter. In addition, the composition of gut microbiota changes when birds are raised on reused litter in comparison to fresh litter. Using 16S rRNA gene sequences, birds raised on reused litter were found to have increased bacteria of intestinal origin (such as *Clostridiales*), whereas birds reared on fresh litter had more environmental bacteria (such as *Lactobacillus*; Cressman et al., 2010). This alteration in the predominant microbial populations in the gut may result in changes in immune response of broiler chickens. Birds raised on fresh litter have increased anti-inflammatory response, whereas birds reared on reused litter tended to have increased inflammatory response (Lee et al., 2011; Cressman et al., 2010). For example, recycling litter increased expression of the pro-inflammatory cytokine IL-1 at 10 and 35 d of age; and also resulted in higher expression of IL-4 in the gut of broiler chickens reared on reused litter. IL-4 is produced by Th2 cells and is involved in IgA production in order to protect birds from pathogenic bacteria (Shanmugasundaram et al., 2012). Additionally, birds reared on fresh litter had higher expression of CD4 and CD25 relative to reused litter at 10 and 35 d of age. Both CD4 and CD25 are regulatory T cell markers, which are present in mucosal tissues, particularly the cecal tonsils of chickens (Shanmugasundaram and Selvaraj, 2011). Moreover, regulatory T cells contribute to colonization by commensal bacteria in the small intestine. Regulatory T cells numbers were decreased by the change in the gut microbiota and the lipopolysaccharide content of the diet (Round and Mazmanian, 2010). Also, reused litter may change regulatory T cell properties in response to changes in gut microbial population. The discrepancies in the effects of

fresh vs reused litter on inflammatory gene expression in broilers in different studies could be due to the age and different genetic backgrounds of the chickens, and differences in the gut microbiota. For example, IL-1β increased in birds at d 35 (Shanmugasundaram et al. 2012), but it decreased at d 28 in broiler chickens grown on reused relative to fresh litter (Lee et al., 2013). Digesta passage rate, and rearing environment can change intestinal microbiome composition in chickens (Pan and Yu, 2014). Additionally, subsequent flocks could be exposed to different microbes depending on the microbial composition of the reused litter (Lu et al., 2003; Torok et al., 2009). This could change the immune response of chickens reared on reused litter.

Recycling litter can influence broiler performance results. Broiler chickens reared on reused litter had reduced BW and feed intake at d 14 relative to those reared on fresh litter, but there were no differences in performance of broiler chickens between fresh or reused litter at d 28 (Torok et al., 2009). The reduction in early broiler chicken performance was due to increased pathogenic bacteria in birds reared on reused as compared to fresh litter (Lee et al., 2011). Also, the composition of microbiota in the gut changes with age, and the variability of microbiota in the gut for chickens regardless of litter type was higher for birds at d 14 relative to d 28 (Torok et al., 2009; Lu et al., 2003). Increased bacteria exposure caused by reused litter in early post-hatch broilers may delay small intestine development as a result of a delay in colonization of the gut by symbiotic microorganisms (Pan and Yu, 2014). This may indicate that post-hatch broilers reared on reused litter as compared to fresh litter are more susceptible to bacterial exposure, leading to decreased growth performance. However, the more stable composition of commensal populations in older chickens may provide the chance for the symbiotic microorganisms to colonize in gut to protect the gut from enteric pathogens (Nakamura et al., 2002; Zhang et al., 2007).

1.6 OBJECTIVES AND HYPOTHESES

It was hypothesized that the dietary $25OHD_3$ would increase growth performance and meat yield throughout the growing period; and the same parameters would decrease for birds reared on reused litter due to an increase in bacteria exposure and subsequent inflammatory responses. Additionally, $25OHD_3$ would increase growth performance of broilers due to reduced inflammatory response caused by pathogens in reused litter. This hypothesis was addressed in Chapter 2, in which the objectives of the study were to examine the effect of dietary vitamin D_3 and 25-hydroxycholecalciferol on growth and carcass traits, including BW, weight gain, feed intake, FCR, and carcass portion weights and yields in fresh and reused litter.

It was hypothesized that dietary $25OHD_3$ would decrease indicators of the acute phase inflammatory response; enhance small intestine morphology, and increases bone mineralization. Additionally reused litter would increase acute phase inflammatory response, damage gut morphology, and decrease bone mineralization and strength due to increase in bacterial exposure. This hypothesis was addressed in Chapter 3, where the objective of the research was to evaluate the effects of dietary $25OHD_3$ on innate immune function, duodenum, jejunum and ileum villus and crypt morphology; and bone characteristics measured as bone mineral density and bone cross-sectional areas (using quantitative computed tomography) of broilers were evaluated.

It was hypothesized that the dietary $25OHD_3$ would increase the expression of genes linked to muscle development and inflammation; and reused litter would induce inflammatory response genes in broiler chickens. This hypothesis was addressed in Chapter 4, where the objective of the study was to determine the effects of dietary $25OHD_3$ on breast muscle gene expression of broiler chickens raised on reused litter using RNA-seq.

Several studies have indicated the effects of dietary vitamin D sources on growth performance, meat yield production, small intestine morphology and immunity of broiler chickens, however, few studies have associated the effects of dietary supplementation vitamin D_3 and $25OHD_3$ on fresh *vs* reused litter on growth performance and carcass characteristics (Chapter 2), as well as small intestine morphology, innate immune function and bone development (Chapter 3). The current study also investigated the molecular mechanisms and physiological factors involved in the breast meat yield and immunity when either vitamin D_3 vs $25OHD_3$ was fed to broiler chickens, or birds are reared on fresh vs reused litter (Chapter 4).

1.7 REFERENCES

Anderson, P. H., P. D. O'Loughlin, B. K. May, and H. A. Morris. 2005. Modulation of CYP27B1 and CYP24 mRNA expression in bone is independent of circulating $1,25(OH)_2D_3$ levels. Bone. 36:654– 662.

Armand, O., A. M. Boutineau, A. Mauger, M. P. Pautou, and M. Kieny. 1983. Origin of satellite cells in avian skeletal muscles. Arch. Anat. Microsc. Morphol. Exp. 72:163-181.

Atencio, A., G. M. Pesti, and H. M. Edwards. 2005. Twenty-five hydroxycholecalciferol as a cholecalciferol substitute in broiler breeder hen diets and its effect on the performance and general health of the progeny. Poult. Sci. 84:1277-1285.

Baldi, C., G. Vazquez, and R. Boland. 2002. Characterization of a $1,25(OH)_2$-vitamin D_3-responsive capacitative Ca^{2+} entry pathway in rat osteoblast-like cells. J. Cell. Biochem. 86:678-687.

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, A., V. Razaphkovsky, E. Vax, and I. Plavnik. 2003. Performance and bone development in broiler chickens given 25-hydroxycholecalciferol. Br. Poult. Sci. 44:224-233.

Barthel, T. K., D. R. Mathern, G. K Whitfield. C. A. Haussler, H. A. Hopper, J. C. Hsieh, S. A. Slater, G. Hsieh, M. Kaczmarska, P. W. Jurutka, O. I. Kolek, F. K. Ghishan, and M. R. Haussler. 2007. 1,25-dihydroxyvitamin D_3/VDR-mediated induction of FGF23 as well as transcriptional control of other bone anabolic and catabolic genes that orchestrate the regulation of phosphate and calcium mineral metabolism. J. Steroid. Biochem. Mol. Biol. 103:381-388.

Beal, R. K., P. Wigley, C. Powers, S. D. Hulme, P. A. Barrow, and A. L. Smith. 2004. Age at primary infection with *Salmonella enterica* serovar Typhimurium in the chicken influences persistence of infection and subsequent immunity to re-challenge. Vet. Immunol. Immunopathol. 100:151–164.

Bello, A., P. Y. Hester, P. D. Gerard, W. Zhai, and E. D. Peebles. 2014. Effects of commercial in ovo injection of 25-hydroxycholecalciferol on bone development and mineralization in male and female broilers. Poult. Sci. 93:2734-2739.

Biressi, S., C. R. Bjornson, P. M. Carlig, K. Nishijo, C. Keller, and T. A. Rando. 2013. Myf5 expression during fetal myogenesis defines the developmental progenitors of adult satellite cells. Dev. Biol. 379:195-207.

Bolan, N. S., A. A. Szogi, T. Chuasavathi, B. Seshadri, M. J. Rothrock, and P. Panneerselvam. 2010. Uses and management of poultry litter. World's Poult. Sci. J. 66:673-698.

Boland, R., A. R. de Boland, M. J. Marinissen, G. Santillan, G. Vazquez, and S. Zanello. 1995. Avian muscle cells as targets for the secosteroid hormone 1,25-dihydroxy-vitamin D_3. Mol. Cell. Endocrinol. 114:1-8.

Boland, R. L. 2011. VDR activation of intracellular signaling pathways in skeletal muscle. Mol. Cell. Endocrinol. 347:11-6.

Borel, P., P. Grolier, M. Armand, A. Partier, H. Lafont, D. Lairon, and V. Azais-Braesco. 1996. Carotenoids in biological emulsions: solubility, surface-to-core distribution, and release from lipid droplets. J. Lipid. Res. 37:250-261.

Bordbar, A., and B. O. Palsson. 2012. Using the reconstructed genome-scale human metabolic network to study physiology and pathology. J. Intern. Med. 271:131–141.

Cheema, M. A., Qureshi, M. A., and G. B. Havenstein. 2003. A comparison of the immune response of a 2001 commercial broiler with a 1957 random bred broiler strain when fed representative 1957 and 2001 broiler diets. Poult. Sci. 82:1519-1529.

Christakos, S., D. V. Ajibade, P. Dhawan, A. J. Fechner, and L. J. Mady. 2010. Vitamin D: metabolism. Endocrinol. Metab. Clin. North. Am. 39:243–253.

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.

Coffey, J. D., E. A. Hines, J. D. Starkey, C. W. Starkey, and T. K. Chung. 2012. Feeding 25-hydroxycholecalciferol improves gilt reproductive performance and fetal vitamin D status. J. Anim. Sci. 11:3783-3788.

Corthesy, B., H. R. Gaskins, and A. Mercenier. 2007. Cross-talk between probiotic bacteria and the host immune system. J. Nutr. 137:781S-790S.

Clark, D. L., C. S. Coy, G. M. Strasburg, K. M. Reed, and S. G. Velleman. 2016. Temperature effect on proliferation and differentiation of satellite cells from turkeys with different growth rates. Pout. Sci. 95:934–947.

Cloonan, N., N. Cloonan, A. R. R. Forrest, G. Kolle, B. B. A. Gardiner, G. J. Faulkner, M. K. Brown, D. F. Taylor, A. L. Steptoe, S. Wani, G. Bethel, A. J. Robertson, A. C. Perkins, S. J. Bruce, C. C. Lee, S. S. Ranade, H. E. Peckham, J. M. Manning, K. J. McKernan, and S. M Grimmond. 2008. Stem cell transcriptome profiling via massive-scale mRNA sequencing. Nat. Methods. 5:613-619.

Coufal, C. D., C. Chavez, P. R. Niemeyer, and J. B. Carey. 2006. Effects of top-dressing recycled broiler litter on litter production, litter characteristics, and nitrogen mass balance. Poult. Sci. 85:392-397.

Cressman, M. D., Z. Yu, M. C. Nelson, S. J. Moeller, M. S. Lilburn, and H. N. Zerby. 2010. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. Appl. Environ. Microbiol.76:6572-6582.

De Matos, R. 2008. Calcium metabolism in birds. Vet. Clin. North. Am. Exot. Anim. Pract. 11:59-82.

De Beer, M., and C. N. Coon. 2007. The effect of different feed restriction programs on reproductive performance, efficiency, frame size, and uniformity in broiler breeder hens. Poult. Sci. 86:1927-1939.

Deeb, K. K., D. L. Trump, and C. S. Johnson. 2007. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat. Rev. Cancer. 7:684-700.

Fenech, M., A. El-Sohemy, L. Cahill, L. R. Ferguson, T. A. French, E. S. Tai, J. Milner, W. P. Koh, L. Xie, M. Zucker, M. Buckley, L. Cosgrove, T. Lockett, K. Y. Fung, and R. Head.2011. Nutrigenetics and nutrigenomics: viewpoints on the current status and applications in nutrition research and practice. J. Nutrigenet. Nutrigenomics. 4:69-89.

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.

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.

Fritts, C. A., and P. W. Waldroup. 2005. Comparison of cholecalciferol and 25-hydroxycholecalciferol in broiler diets designed to minimize phosphorus excretion. J. Appl. Poult. Res. 14:156-166.

Ehrhardt, M., A. Gerber, F. Hannemann, and R .Bernhardt. 2016. Expression of human CYP27A1 in B. megaterium for the efficient hydroxylation of cholesterol, vitamin D_3 and 7-dehydrocholesterol. J. Biotechnol. 218:34-40.

Erickson, K., and N. E. Hubbard. 2000. Probiotic immunomodulation in health and disease. J. Nutr. 130:403S-409S.

Galdeano, M. C., A. de Moreno de LeBlanc, G. Vinderola, M. E. Bibas Bonet, and G. Perdigón. 2007. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. Clin. Vaccine. Immunol. 14:485-492.

Garrett, R. L., and R. J. Young. 1975. Effect of micelle formation on the absorption of neutral fat and fatty acids by the chicken. J. Nutr. 105:827-838.

Genxi Z., T. Ying, Z. Tao, W. Jinyu, and W. Yongjuan. 2014. Expression profiles and association analysis with growth traits of the MyoG and Myf5 genes in the Jinghai yellow chicken. Mol. Biol. Rep. 11:7331-7338.

Girgis, C. M., R. J. Clifton-Bligh, M. W. Hamrick, M. F. Holick, and J. E. Gunton. 2013. The roles of vitamin D in skeletal muscle: Form, function, and metabolism. Endocr. Rev. 34:33-83.

Halbrook, E. R., A. R. Winter, and T. S. Sutton. 1950. The effect of management on the vitamin B_{12} content of poultry house litter: I. as measured by chick growth. Poult. Sci. 29:672-678.

Haldar, M., G. Karan, P. Tvrdik, and M. R. Capecchi. 2008. Two cell lineages, Myf5 and Myf5-independent, participate in mouse skeletall myogenesis. Dev. Cell. 14:437-445.

Havenstein, G. B., Ferket, P. R., and M. A. Qureshi. 2003. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult. Sci. 82:1500-1508.

Haussler, M. R., P. W. Jurutka, J. C. Hsieh, P. D. Thompson, S. H. Selznick, C. A. Haussler, and G. K. Whitfield. 1995. New understanding of the molecular mechanism of receptor-mediated genomic actions of the vitamin D hormone. Bone. 17:33S-38S.

Hutton, K. C., M. A. Vaughn, G. Litta, B. J. Turner, and J. D. Starkey. 2014. Effect of vitamin D status improvement with 25-hydroxycholecalciferol on skeletal muscle growth characteristics and satellite cell activity in broiler chickens. J. Anim. Sci. 92:3291-3299.

Kalita, K. P., R. Saikia, and J. D. Mahanta. 2012. Performance of commercial broilers raised on reused and mixed type of litters. Indian. J. Hill. Farm. 25:33-36.

Käppeli, S., E. Fröhlich, S. G. Gebhardt-Henrich, A. Pfulg, H. Schäublin, R. Zweifel, H. Wiedmer, and M. H. Stoffel. 2011. Effects of dietary supplementation with synthetic vitamin D_3 and 25-hydroxycholecalciferol on blood calcium and phosphate levels and performance in laying hens. Arch. Geflügelk. 75:175-184.

Khan, S. H., R. Shahid, A. A. Mian, R. Sardar, and M. A. Anjum. 2010. Effect of the level of cholecalciferol supplementation of broiler diets on the performance and tibial dyschondroplasia. J. Anim. Physiol. Anim. Nutr. 94:584-593.

Kris-Etherton P. M., K. D. Hecker, A. Bonanome, S. M. Coval, A. E. Binkoski, K. F. Hilpert, A. E. Griel, and T. D. Etherton. 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am. J. Med. 113:71S-88S.

Laplante, M, and D. M. Sabatini. 2012. mTOR signaling in growth control and disease. Cell. 149:274-293.

Lee, K. W., S. H. Lee, H. S. Lillehoj, G. X. Li, S. I. Jang, U. S. Babu, M. S. Park, D. K. Kim, E. P. Lillehoj, A. P. Neumann, T. G. Rehberger, and G. R. Siragusa. 2010. Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. Poult. Sci. 89:203-216.

Lee, K. W., H. S. Lillehoi, S. H. Lee, S. I. Jang, G. D. Ritter, D. A. Bautista, and E. P. Lillehoj. 2011. Impact of fresh or used litter on the posthatch immune system of commercial broilers. Avian. Dis. 55:539-544.

Lee, K. W., H. S. Lillehoi, S. H. Lee, S. I. Jang, D. A. Bautista, and G. R. Siragusa. 2013. Effect of bacillus subtilis-based direct-fed microbials on immune status in broiler chickens raised on fresh or used litter. Asian-Australas. J. Anim. Sci. 26: 1592-1597.

Liu, Q. C., X. H. Zha, H. Faralli, H. Yin, C. Louis-Jeune, E. Perdiguero, P. Muñoz-Cànoves, M. A. Rudnicki, M. Brand, C. Perez-Iratxeta, and F. J. Dilworth. 2012. Comparative expression profiling identifies differential roles for MyoGenin and p38a MAPK signaling in myogenesis. J. Mol. Cell. Biol. 4:386-397.

Loor, J. J., H. M. Dann, N. A. Guretzky, R. E. Everts, R. Oliveira, C. A. Green, N. B. Litherland, S. L. Rodriguez-Zas, H. A. Lewin, and J. K. Drackley. 2006. Plane of nutrition prepartum alters hepatic gene expression and function in dairy cows as assessed by longitudinal transcript and metabolic profiling. Physiol. Genomics 27:29-41.

Loor, J. J., K. M. Moyes, and M. Bionaz. 2011. Functional adaptations of the transcriptome to mastitis-causing pathogens: The mammary gland and beyond. J. Mammary Gland Biol. Neoplasia. 16:305-322.

Loor, J. J., M. Bionaz, and J. K. Drackley. 2013. Systems physiology in dairy cattle: Nutritional genomics and beyond. Annu. Rev. Anim. Biosci. 1:365-392.

Lorri, J. J., M. Vailati-Riboni, J. C. McCann, Z. Zhou, and M. Bionaz. 2015. Nutrigenomics in livestock: Systems biology meets nutrition. J. Anim. Sci. 93:5554-5574.

Lu, J., S. Sanchez., C. Hofacre, J. J. Maurer, B. G. Harmon, and M. D. Lee. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. Appl. Environ. Microbiol. 69:901-908.

Ma, X. M., and J. Blenis. 2009. Molecular mechanisms of mTOR-mediated translational control. Nat. Rev. Mol. Cell. Biol. 10:307-318.

Martin, S. A., and M. A. McCann. 1998. Microbiological survey of Georgia poultry litter. J. Appl. Poult. Res. 7:90–98.

Marioni, J. C., C. E. Mason, S. M. Mane, M. Stephens and Y. Gilad. 2008. RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. Genome. Res. 18:1509-1517.

McCollum. M.V. 1925. Studies on experimental rickets. J. Biol. Chem. 65:97.100.

Michalczuk, M., D. Pietrzak, J. Niemiec, 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.

Mok, G. F., R. H. Mohammed, and D Sweetman. 2015. Expression of myogenic regulatory factors in chicken embryos during somite and limb development. J. Anat. 227:352-60.

Moncaut, N., P. W. Rigby, and J. J. Carvajal. 2013. Dial M(RF) for myogenesis. FEBS. J. 280:3980-3990.

Morris, A., R. Shanmugasundaram. M. S. Lilburn, and R. K. Selvaraj. 2014. 25-Hydroxycholecalciferol supplementation improves growth performance and decreases inflammation during an experimental lipopolysaccharide injection. Poult. Sci. 93:1951-1956.

Morris, A., and R. K. Selvaraj. 2014. In vitro 25-hydroxycholecalciferol treatment of lipopolysaccharide-stimulated chicken macrophages increases nitric oxide production and mRNA of interleukin-1 beta and 10.

Moss, F. P. 1968. The relationship between the dimensions of the fibers and the number of nuclei during normal growth of skeletal muscle in the domestic fowl. Am. J. Anat. 122:555–563.

Moss, F. P., and C. P. Leblond. 1971. Satellite cells as the source of nuclei in muscles of growing rats. Anat. Rec. 170:421–435.

Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer, and B. Wold. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods. 5:621-628.

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.

Nakamura, A., Y. Ota, A. Mizukami, T. Ito, Y. B. Ngwai, and Y. Adachi. 2002. Evaluation of aviguard, a commercial competitive exclusion product for efficacy and after-effect on the antibody response of chicks to *Salmonella*. Poult Sci. 8:1653-1660.

Neibergs, H. L., and K. A. Johnson. 2012. Nutrition and the genome. J. Anim. Sci. 90:2308-2316.

Ng, S. C, A. L. Hart, M. A. Kamm, A. J. Stagg, and S. C. Knight. 2009. Mechanisms of action of probiotics: Recent advances. Inflamm. Bowel. Dis. 15:300-310.

Norman, A. W. 1987. Studies of the vitamin D endocrine system in the avian. J. Nutr. 117:797–807.

Noy, Y., and D. Sklan, D. 1995. Digestion and absorption in the young chick. Poult. Sci. 74:366-373.

Nurmi, E., and M. Rantala. 1973. New aspects of *Salmonella* infection in broiler production. Nature. 241:210-211.

Omdahl, J. L., H. A. Morris, and B. K. May. 2002. Hydroxylase enzymes of the vitamin D pathway: expression, function, and regulation. Annu. Rev. Nutr. 22:139-166.

Pan, D., and Z. Yu. 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut. Microbes. 5:108-119.

Papešová, L., A. Fučíková, M. Pípalová, P. Tupý. 2008. The synergic effect of vitamin D_3 and 25-hydroxycholecalciferol/calcidiol in broiler diet. SAB. 39: 273-277.

Peighambari, S. M., R. J. Julian, and C. L. Gyles. 2000. Experimental *Escherichia coli* respiratory infection in broilers. Avian. Dis. 44:759-769.

Perry, R. L., and M. A Rudnick. 2000. Molecular mechanisms regulating myogenic determination and differentiation. Front. Biosci. 5:750-767.

Piórkowska, K., K. Żukowski, J. Nowak, K. Połtowicz, K. Ropka-Molik, and A .Gurgul. 2016. Genome-wide RNA-Seq analysis of breast muscles of two broiler chicken groups differing in shear force. Anim. Genet. 47:68-80.

Rama-Rao, S. V., M.V. L. N. Raju, A. K. Panda, A. K., G. Shyam Sunder, and R. P. Sharma. 2006. Effect of high concentrations of cholecalciferol on growth, bone mineralization, and

mineral retention in broiler chicks fed suboptimal concentrations of calcium and nonphytate phosphorus. J. Appl. Poult. Res. 15:493-501.

Rama-Rao, S. V., M.V. L. N. Raju, A. K. Panda, A. K., G. Shyam Sunder, and R. P. Sharma. 2009. Performance and bone mineralization in broiler chicks fed on diets with different concentrations of cholecalciferol at a constant ratio of calcium to non-phytate phosphorus. Br. Poult. Sci. 50:528-535.

Rennie, J. S., and C. C. Whitehead. 1996. Effectiveness of dietary 25 and 1 hydroxycholecalciferol in combating tibial dyschondroplasia in broiler chickens. Br. Poult. Sci. 37:413-421.

Roll, V. F. B., M. A. Dai Prá, and A. P. Roll. 2011. Research on *Salmonella* in broiler litter reused for up to 14 consecutive flocks. Poult. Sci. 90:2257-2262.

Round, J. L., and S. K. Mazmanian. 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc. Natl. Acad. Sci. 107:12204-12209.

Russell, J., A. Bar, L. M. Sherwood, and S. Hurwitz. 1993. Interaction between calcium and 1,25-dihydroxyvitamin D_3 in the regulation of preproparathyroid hormone and vitamin D receptor messenger ribonucleic acid in avian parathyroids. Endocrinology. 132:2639-2644.

Santillan, G., S. Katz, C. Buitrago, and R. Boland. 2004. $1\alpha,25(OH)_2D_3$ induces capacitative calcium entry involving a trpc3 protein in skeletal muscle and osteoblastic cells. Biol. Res. 37: 647-651.

Schmidt, C. J., M. E. Persia, E. Feierstein, B. Kingham, and W. W. Saylor. 2009. Comparison of a modern broiler line and a heritage line unselected since the 1950s. Poult. Sci. 88:2610-2619.

Shanmugasundaram, R., and R. K. Selvaraj. 2011. Regulatory T cell properties of chicken $CD4^+$ $CD25^+$ cells. J. Immunol. 186:1997-2002.

Shanmugasundaram, R., M. S. Lilburn, and R. K. Selvaraj. 2012. Effect of recycled litter on immune cells in the cecal tonsils of chickens. Poult. Sci. 91:95-100.

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

Shim, M. Y., A. B. Karnuah, A.D. Mitchell, N. B. Anthony, G. M. Pesti, and S. E. Aggrey. 2012. The effects of growth rate on leg morphology and tibia breaking strength, mineral density, mineral content, and bone ash in broilers. Poult. Sci. 91:1790-1795.

Singh, K., and F. J. Dilworth. 2013. Differential modulation of cell cycle progression distinguishes members of the myogenic regulatory factors. FEBS. J. 280:3991-4003.

Shinki, T., N. Takahashi, T. Kadofuku, T. Sato, and T. Suda. 1985. Induction of spermidine N1-acetyltransferase by 1α, 25-dihydroxyvitamin D_3 as an early common event in the target tissues of vitamin D. J. Biol. Chem. 260:2185–2190.

Shinki, T., N. Takahashi, C. Miyaura, K. Samejima, Y. Nishii, and T. Suda. 1981. Ornithine decarboxylase activity in chick duodenum induced by 1α,25-dihydroxycholecalciferol. Biochem. J. 195:685–690.

Shinki, T., H. Tanaka, J. Takito, A. Yamaguchi, Y. Nakamura, S. Yoshiki, and T. Suda. 1991. Putrescine is involved in the vitamin D action in chick intestine. Gastroenterology. 100:113–122.

Shojadoost, B., S. Behboudi, A. I. Villanueva, J. T. Brisbin, A. A. Ashkar, and S. Sharif. 2015. Vitamin D_3 modulates the function of chicken macrophages. Res. Vet. Sci.100:45-51.

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

Sun, Z. W., L. Yan, Y. Y. G, J. P. Zhao, H. Lin, and Y. M. Guo. 2013. Increasing dietary vitamin D_3 improves the walking ability and welfare status of broiler chickens reared at high stocking densities. Poult. Sci. 92:3071-3079.

Świątkiewicz, S, and J. Koreleski. 2005. The effect of particulate limestone and 25-hydroxy-cholecalciferol in broiler chicken diets on mechanical properties of bones. J. Anim. Food. Sci. 14:495-498.

Świątkiewicz, S., J. Koreleski, and J. Kopowski. 2006. Effect of phytase and 25-hydroxycholecalciferol on the performance and bone quality in broiler chickens. Med. Weter. 62:81-84.

Szpirer, J., C. Szpirer, M. Riviere, G. Levan, P. Marynen, J. J. Cassiman, R. Wiese, and H. F. De Luca. 1991. The Sp1 transcription factor gene (SP1) and the 1,25-dihydroxyvitamin D_3 receptor gene (VDR) are colocalized on human chromosome arm 12q and rat chromosome 7. Genomics. 11:168-73.

Thaxton, J. P., and S. Puvadolpirod. 2000. Model of physiological stress in chickens. 5. Qualitative evaluation. Poult. Sci. 79:391-395.

Torok, V. A., R. J. Hughes, K. Ophel-Keller, M. Ali, and R. MacAlpine. 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. Poult. Sci. 88:2474–2481.

Von Maltzahn, J., A. E. Jones, R. J. Parks, and M. A. Rudnicki. 2013. Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. Proc. Natl. Acad. Sci. 110:16474-16479.

Vignale, K., E. S. Greene, J. V. Caldas, J. England, N. Boonsinchai, P. Sodsee, E. D. Pollock, S. Dridi, and C. N. Coon. 2015. 25-Hydroxycholecalciferol enhances male broiler breast meat yield through the mTOR pathway. J. Nutr. 145:855-863.

Volkova, V. V., R. H. Bailey, and R. W. Wills. 2009. *Salmonella* in broiler litter and properties of soil at farm location. Plos. One. 4:e6403-e6409.

Von Essen M.R., M. Kongsbak, P Schjerling, , Klaus Olgaard, N. Ødum , C. Geisler. 2010. Vitamin D controls T cell antigen receptor signalingand activation of human T cells. Nat. Immunol. 11:344-349.

Waititu, S. M., A. Yitbarek, E. Matini, H. Echeverry, E. Kiarie, J. C. Rodriguez-Lecompte, and C. M. Nyachoti. 2014. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poult. Sci. 93:625-635.

Wang, Z., M. Gerstein, and M. Snyder. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. 10:57-63.

Wang T. T., B. Dabbas, D. Laperriere, A. J. Bitton, H. Soualhine, L. E. Tavera-Mendoza, S. Dionne, M.J. Servant , A. Bitton, E. G. Seidman, S. Mader, M. A. Behr, J. H.White. 2010. Direct and indirect induction by 1,25-dihydroxyvitamin D_3 of the NOD2/CARD15-defensin beta2 innate immune pathway defective in Crohn disease. J. Biol. Chem. 4:2227-2231.

Weissen-Plenz, G., Nitschke, Y. and Rutsch F. 2008. Mechanisms of arterial calcification: spotlight on the inhibitors. Adv. Clin. Chem. 46:263-293.

Wei, S., A. Gutek, M. Lilburn, and Yu Z. 2013. Abundance of pathogens in the gut and litter of broiler chickens as affected by bacitracin and litter management. Vet. Microbiol. 166:595-601.

Yablonka-Reuveni, Z. 1995a. Myogenesis in the chicken: the onset of differentiation of adult myoblasts is influenced by tissue factors. Basic. Appl. Myol. 5:33-41.

Yablonka-Reuveni, Z. 1995b. Development and postnatal regulation of adult myoblasts. Microsc. Res. Tech. 30:366-380.

Yablonka-Reuveni Z., and B. M. Paterson. 2001. MyoD and myogenin expression patterns in cultures of fetal and adult chicken myoblasts. J. Mol. Histol. 49:455-462.

Yarger, J.G., C.A., Sunders, 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.

Yarru, L. P., R. S. Settivari, E. Antoniou, D. R. Ledoux, and G. E. Rottinghaus. 2009. Toxicological and gene expression analysis of the impact of aflatoxin B_1 on hepatic function of male broiler chicks. Poult. Sci. 88:360-371.

Zhang, G., L. Ma, and M.P. Doyle. 2007. Potential competitive exclusion bacteria from poultry inhibitory to *Campylobacter jejuni* and *Salmonella*. J. Food. Prot.70:867-873.

Zhao, S., W. P. Fung-Leung, A. Bittner, K. Ngo, and X. Liu. 2014. Comparison of RNA-seq and microarray in transcriptome profiling of activated T cells. PLoS One. 9:e78644-e78657.

Zhou, N., W. R. Lee, and B. Abasht. 2015. Messenger RNA sequencing and pathway analysis provide novel insights into the biological basis of chickens` feed efficiency. BMC Genomics. 16:195-215.

1.8 Tables

Table 1.1 Examples of transcription-factor pathways mediating nutrient–gene interactions

Nutrient	Compound	Transcription factor
Macronutrients		
Fats	Fatty acids	PPAR, SREBP, LXR, HNF4, ChREBP

	Cholesterol	SREBP, LXR, FXR
Carbohydrates	Glucose	USF, SREBP, ChREBP
Proteins	Amino acids	C/EBP
Micronutrients		
Vitamins	Vitamin A	RAR, RXR
	Vitamin D	VDR
	Vitamin E	PXR
Minerals		
	Calcium	Calcineurin/NF-AT
	Iron	IRP1, IRP2
	Zinc	MTF1
Other food components		
	Flavonoids	ER, NFκB, AP1
	Xenobiotics	CAR, PXR

AP1, activating protein1; CAR, constitutively active receptor; C/EBP, CAAT/enhancer binding protein; ChREBP, carbohydrate responsive element binding protein; ER, oestrogen receptor; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; IRP, iron regulatory protein; LXR, liver X receptor; MTF1, meta lresponsive transcription factors; NFκB, nuclear factor κB; NF-AT, nuclear factor of activated T cells; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SREBP, sterol-responsive-element binding protein; USF, upstream stimulatory factor; VDR, vitamin D receptor

2. The effect of dietary 25-hydroxycholecalciferol on growth and carcass traits of broilers grown on reused litter

ABSTRACT

Dietary 25-hydroxycholecalciferol ($25OHD_3$) increases broiler breast yield, and vitamin D metabolites influence adaptive immunity. The objective was to study the effects of dietary vitamin D_3 and $25OHD_3$ on performance of broilers raised on fresh or reused litter. Day-old chicks (n=512) were allocated (4.18 birds/m^2) into a 4x2 factorial arrangement with 4 pens/treatment. Dietary treatments were: vitamin D_3 at 2,760 IU/kg feed (D; Control); 25OH D_3 at 2,760 IU/kg feed (25OHD); vitamin D_3 at 2,760 IU/kg plus $25OHD_3$ at 2,760 IU/kg feed (D+25OHD); or D_3 at 5,520 IU/kg feed (Dx2); each diet was fed to broilers on either Fresh or Reused litter. At 12, 22, and 41 d of age, bird performance was measured on a pen basis, and 3 birds from each pen were weighed, and pectoralis (P) major and minor, liver and spleen weights were measured. At d 42, carcass and portion (P. major and minor, legs and wings) weights and yields (portion weight as a % of carcass weight) were determined. Differences were considered significant at $P \leq 0.05$. Breast yield at d 41 was not affected by diet. At d 12 in the Fresh pens, BW of D (331±5.87g) and Dx2 (339±5.87g) birds were higher than 25OHD (300.7±5.87g) and D+25OHD (305±5.87g) birds, and in the Reused pens, Dx2 (316.4±5.87g) birds had higher BW than D and D+25OHD birds. P. major and P. minor yields were higher by 9% and 7%, respectively in the Fresh than in the Reused litter. At 42 d, P. minor yield was higher for D-fed birds by 6, 3 and 4% as compared to 25OHD, D+25OHD and Dx2, respectively. In addition, breast meat yield was higher for D-fed birds as compared to 25OHD or Dx2 birds. Leg yield was 3% greater in 25OHD birds than in D birds. Contrary to expectations, 25OHD increased leg

rather than breast yield. The Reused litter reduced performance in young birds in spite of a lack of a measurable febrile response at any age.

Keywords: 25-hydroxycholecalciferol, vitamin D_3, breast yield, reused litter, inflammation

2.1 INTRODUCTION

Recycling litter from previous flocks is commonly used in modern poultry production (Torok et al., 2009; Coufal et al., 2006) due to reduced cost of removal, disposal, and the cost associated with obtaining new litter. Also, poultry litter is able to provide microbes which will allow the earlier establishment of a more stable gut microflora in young broiler chickens (Torok et al., 2009; Cressman et al., 2010). Direct-fed microbials (DFM) can increase mucus production (Mattar et al., 2002), and epithelial cell turnover leading to increased nutrient absorption (Savage et al., 1981), and reduced inflammation (Lee et al., 2010). However, the gut microbial population changes qualitatively and quantitatively when chickens are reared on reused litter (Lee et al., 2011; Cressman et al., 2010). In addition, more pathogenic bacteria are observed in reused litter relative to fresh. For example, environmental bacteria (*Lactobacillus*) are the predominant microbes in the small intestine of birds reared on fresh litter, while bacteria of intestinal origin (*Clostridiales)* are predominant in birds reared on reused litter (Cressman et al., 2010; Lu et al., 2003). This alteration in composition of intestinal microbiota of birds grown on reused litter may result in reduced growth performance, and initiate humoral and cell-mediated immune responses of broiler chickens (Lee et al., 2011; Torok et al., 2009; Lu et al., 2003).

In commercial chickens, vitamin D_3 is most commonly obtained from the diet. In the liver, vitamin D_3 is converted by the action of 25-hydroxylase to 25-hydroxycholecalciferol ($25OHD_3$). Then, $25OHD_3$ is transported to the kidney and is hydroxylated to 1,25-

dihydroxylcholecalciferol [1,25-$(OH)_2D_3$] by 1α-hydroxylase (Shanmugasundaram and Selvaraj, 2012). The serum level of 1,25-$(OH)_2D_3$ is highly regulated by 1,25-$(OH)_2D_3$ itself, vitamin D_3 receptor (VDR) and serum concentration of Ca (De Matos, 2008). In comparison to vitamin D_3, $25OHD_3$ is almost twice as biologically active (Bar et al., 1980), has a longer half-life (Yarger et al, 1995), and also has higher intestinal absorption and lower excretion (Chou et al., 2009). Chickens at early ages are not able to absorb vitamin D_3 efficiently due to an immature digestive tract and low activity of lipase and therefore lipid digestion and absorption (Noy and Sklan, 1995). With bacterial infection, liver function could be reduced and this may reduce endogenous production of $25OHD_3$ from vitamin D (Peighambari et al., 2000). This circumstance can be avoided by providing dietary $25OHD_3$, which by-passes the liver in the bioconversion process (Soares et al., 1995). Dietary $25OHD_3$ increased broiler chicken performance (Yarger et al., 1995; Fritts and Waldroup, 2003), breast muscle yield (Yarger et al., 1995; Vignale et al., 2015), and decreased pro-inflammatory response (Morris et al., 2014) when compared to vitamin D_3 at the same level of inclusion. In addition, dietary $25OHD_3$ increased expression of VDR in breast muscle by three-fold relative to vitamin D_3 in broiler chickens (Vignale et al., 2015). Vitamin D_3 receptor, in cooperation with 1,25-$(OH)_2D_3$, plays important roles in skeletal muscle development and adaptive and innate immunity (Boland et al, 1995; Shojadoost et al., 2015). Poultry litter contains pathogenic bacteria (Lu et al., 2003; Cressman et al., 2010), which can cause inflammation in chickens, however supplementation of $25OHD_3$ triggers anti-inflammatory responses to reduce inflammation in chickens (Morris et al., 2014; Shojadoost et al., 2015). At the same level of dietary inclusion, $25OHD_3$ increased the performance of lipopolysaccharide (LPS)-injected birds as compared vitamin D, likely due to a decrease in pro-inflammatory response (IL-1β; Morris et al., 2014). We hypothesized that dietary

supplementation of $25OHD_3$ would increase broiler growth performance in an unsanitary environment relative to vitamin D_3. Therefore, the objective of this experiment was to investigate the effect of dietary inclusion $25OHD_3$ and vitamin D_3 on growth performance of male broiler chickens raised on fresh or reused litter.

2.2 MATERIALS AND METHODS

2.2.1 Experimental Diets

Wheat-soybean meal-based diets were formulated according to primary breeder nutrition recommendations. A three phase feeding program with starter (0 to 12 d), grower (13 to 22 d) and finisher (23 to 41 d) phases was used (Table 2.1). Dietary treatments were: vitamin D_3 at 2,760 IU/kg feed (D; control); $25OHD_3$ at 2,760 IU/kg feed (25OHD); D_3 at 2,760 IU/kg plus $25OHD_3$ at 2,760 IU/kg feed (D+25OHD); or D_3 at 5,520 IU/kg feed (Dx2). Each of the 4 dietary treatments was randomly assigned to 8 pens (1.9 x 2.2 m^2), with 16 birds per pen and 8 replicates per treatment.

2.2.2 Experimental Conditions

All experimental procedures were approved by the University of Alberta Animal Care and Use Committee in accordance with the Canadian Council of Animal Care (2009) guidelines. The experiment was a completely randomized design with a factorial arrangement of treatments (4 diet and 2 litter treatments). A total of 512 Ross 308 broiler chicks were obtained from a commercial hatchery and randomly assigned to 32 pens at a rate of 16 chicks per pen (4.18 birds/m^2) and grown for 41 d. Broilers were reared in an environmentally-controlled room on either fresh (Fresh) or reused (Reused) pine shavings. The reused litter had been used by one previous flock and was left in place for approximately 4 weeks before it was moved to the

experimental barn. Environmental temperature and light were maintained as specified by the primary breeder (Aviagen, 2009). Chicks had ad libitum access to feed and fresh water. Diets were fed as crumble from 0 to 22 d of age and then as pellets from 23 to 41 d of age.

2.2.3 Data Recorded

At 12, 22, and 41 d of age, BW was measured on a pen basis, and feed intake (FI) determined for the starter, grower and finisher phases, respectively. Feed conversion ratio (FCR; g feed/g gain) was calculated and adjusted for mortality and culled birds. At the same ages, three birds per pen were randomly selected, euthanized, dissected and individual weights of pectoralis major (P. major) and pectoralis minor (P. minor), liver and small intestine (duodenum, jejunum and ileum) were obtained. The three segments of small intestine were excised as described by Wu et al. (2013). Breast weight was the sum of the P. major and minor; yield was calculated as a proportion of live BW.

The remaining birds (from 22 to 26 birds/treatment) were processed at d 42 d of age. Prior to slaughter, birds did not have access to feed or water for 12 h. The birds were electrically stunned, and then bled for 90 seconds (Schneider et al., 2012). Carcasses were mechanically defeathered, manually eviscerated, and carcass traits assessed. Carcass and portion (P. major and minor, legs and wing) weights and yields (portion as a % of carcass weight) were determined. Carcass yield was calculated as the percent of live BW [(carcass weight / live BW) × 100 %].

5.2.4. Statistical Analysis

The pen was the experimental unit and diet and litter treatments were considered fixed effects for production and processing data; for sampling data, bird was the replicate. All data were analyzed as a two-way ANOVA with 4 dietary treatments and 2 litter treatments using the procedure for

linear mixed models (PROC MIXED) of SAS (SAS 9.3© for Windows; SAS Institute Inc., Cary, NC). Differences were considered as significant at $P < 0.05$. For production performance and carcass traits, the following model was used:

$$Yij = \mu + D_i + L_j + (DL)_{ij} + E_{ij},$$

Where μ was the population mean; D_i was the effect of each dietary treatments ($i = 1$ to 4); L_j was the effect of litter ($j = 1$ to 2); $(DL)_{ij}$ was the interactions of each dietary treatment with litter treatment; and E_{ij} was the residual error.

2.3 RESULTS

2.3.1 Production Performance

Birds in the 25OHD group had lower d 0 BW as compared to other dietary treatments (Table 2.2). There were interactions between diet and litter at d 12. In the Fresh litter; diets containing only vitamin D_3 resulted in higher BW and BWG than any diet containing $25OHD_3$. However, D and D+25OHD resulted in lower BW and BWG from d 0 to d 12 than Dx2 in the Reused litter. At d 22, any diet containing $25OHD_3$ resulted in lower BW as compared to the D and Dx2 treatments. In addition, birds fed D+25OHD had lower BWG from d 13 to d 22 relative to other dietary treatments. Birds reared on reused litter tended to have increased BWG ($p= 0.088$) from d 23 to d 41. At d 12, bird reared on Fresh litter had 0.055% greater FI relative to Reused litter, however, birds reared on reused litter tended to have increased FI ($p= 0.077$) at d 41 . There were no significant differences in FCR among treatments at any age, however, birds fed 25OHD tended to have decreased ($P= 0.067$) FCR in comparison to D in the Reused litter at d 12 (Table 2.2). There were no significant differences for total mortality between treatments, however, there was a nearly significant interaction in total mortality (Table 2.3). Birds fed D

tended to have increased (P= 0.072) mortality relative to those fed 25OHD or Dx2 in the Fresh litter; also, birds fed $25OHD_3$ tended to have lower mortality as compared to those fed any diet containing vitamin D_3 in the Reused litter.

2.3.2 Carcass Characteristics

There were interactions between diet and litter in sample birds BW at d 12 and d 41. In the Fresh litter, birds fed any diet containing vitamin D_3 alone had greater BW relative to those fed D+25OHD, however, birds fed 25OHD had lower BW as compared to those fed D or D+25OHD in the Reused litter at d 12. At d 41, birds fed D had lower sample bird BW relative to other dietary treatments on the Reused litter (Table 2.3). At d 12, birds reared on Fresh litter had greater P. minor and major, and breast meat yields and weights relative to those in Reused litter (Tables 2.4 and 2.5). Additionally, birds fed $25OHD_3$ had lower P. minor weight in comparison to those fed Dx2 at the same day. There were no differences in P. minor, P. major, or breast meat yields or weights at 22 or d 41 among treatments. However, birds fed 25OHD tended to have decreased P. minor (p=0.079) and breast meat weights (p=0.053) relative to birds fed any diet containing vitamin D_3 alone, and lower P. major weight than those fed D at d 22. At d 41, there was a nearly significant interaction between dietary treatments in breast meat (p=0.090), P. major (p=0.057), and P. minor weight (p=0.087). In the Fresh litter, birds fed 25OHD tended to have decreased breast muscle relative to Dx2; also, 25OHD-fed birds relative to fed-Dx2 tended to have decreased P. major and minor weight in the Reused litter (Table 2.4). There were no differences in liver yields and weights at d 12 and 22 among the treatments, but chickens fed D+25OHD had greater liver yields and weights by 10% relative to other dietary treatments at d 41 (Tables 2.4 and 2.5). Birds fed 25OHD had lower abdominal fat weight in comparison to other treatments in the Fresh litter (Table 2.6). Also, birds fed 25OHD tended to have decreased

(p=0.060) abdominal fat yield relative to those birds fed D and D+25OHD in the Fresh litter at d 41 (Table 2.7). Birds reared on Reused litter had lower spleen yields and weights relative to those in Fresh litter at d 41 (Tables 2.6 and 2.7). Birds reared on Reused litter had greater small intestine yields and weights as compared to those in Fresh litter at d 12. At d 22, birds fed $25OHD_3$ had greater small intestine yields relative to other dietary treatments (Table 2.6). Additionally, birds fed Dx2 had higher small intestine weight relative to those fed D and D+25OHD in Fresh litter at d 22 (Table 2.7). At processing, there were no differences in BW or carcass weight (Table 2.8). However, the 25OHD diet tended to increased BW (p=0.064) relative to D+25OHD in the fresh litter; also, DX2 tended to have increased BW as compared to D+25OHD in the reused litter. Birds fed D+25OHD tended to have decreased (p=0.054) carcass weight relative to 25OHD and DX2 in the fresh litter. There were no differences in wing yield or weight among treatments (Tables 2.8 and 2.9), but birds fed D+25OHD tended to have increased (p=0.053) wing weight in Reused vs Fresh litter. Chickens fed vitamin D_3 alone had greater breast meat yield as compared to those fed 25OHD or Dx2 (Table 2.9). Also, chickens fed had greater P. minor yields relative to other dietary treatments (Table 2.9). Birds fed D tended to have increased (p=0.0548) P. major yields as compared 25OHD and Dx2 (Table 2.9). Dietary $25OHD_3$ at 2760 IU/kg feed increased leg meat yields relative to vitamin D_3 at the same level of inclusion (Table 2.9).

2.4 DISCUSSION

Microorganisms play a major role in gut development, and the composition of the microbiota is modulated by antibiotics, housing conditions, pathogen exposure, and dietary nutrients (Pan and Yu, 2014). A change in the composition of the gut microbiota can result in alteration of morphological development of gut and immunological status of the bird (Pan and

Yu, 2014). Antibiotic growth promoters (AGP) inhibit the growth of pathogenic bacteria and promote the digestion and absorption function in chickens, leading to lower mortality and the incidence of diseases (Gunal et al., 2006). However, the usage of AGP is banned in the European Union due to widespread concerns regarding antibiotic resistance and the Canadian poultry industry has started to eliminate the use of AGP in poultry diets (Diarra and Malouin, 2014). Bacitracin, used in this study, is approved as a feed additive for poultry (Diarra and Malouin, 2014). In this study, broiler performance and breast meat yield were reduced in post-hatch broilers grown on Reused litter relative to Fresh litter, but there were no significant differences at 41 d of age. Poultry litter is a reservoir for pathogens such as *Eimeria* spp., *Salmonella* spp., *C. perfringens* (Chinivasagam et al., 2010). This group of pathogens can increase inflammation in broiler chickens reared on Reused litter (Shanmugasundaram et al., 2012). Peripheral lymphoid organs, including the spleen have important roles in development and maturation of B and T cells (Shafey et al., 2006). The expression of genes involved in B cell development was associated with increased chicken spleen size (Schneider et al., 2004). Higher expression of B cells is likely linked to higher expression of anti-inflammatory responses, including production of IL-10 in the spleen, which triggers production of B cells and suppresses pro-inflammatory responses (Pestka et al., 2004; Goth et al., 2012). Birds reared on reused litter had lower expression of IL-10 as compared to those reared on fresh litter (Shanmugasundaram et al., 2012). In addition, the expression of genes linked to inflammatory cytokines increased with decreased spleen size in mammals (Seifert et al., 2013). Therefore, the decreased spleen weight observed in birds reared on Reused, relative to Fresh litter, may have been linked to increased inflammation that may have been caused by increased bacteria exposure. Reused litter resulted in higher small intestine weight in post-hatch chicks relative to Fresh litter. A heavier small intestine has been

linked to more gut maintenance energy requirement in chickens (Spratt et al., 1990) and lower expression of genes involved in nutrient transport (Hu et al., 2010). Also, the composition of the small intestine microbiota of newly-hatched broiler chickens is not stable, especially when reared on reused litter (Torok et al., 2009; Lu et al., 2003). Additionally, the composition of the gut microbiota alters with age and broilers at d 14 had higher variability in intestinal microbiota as compared to birds at d 28. A rapidly developing small intestine in early post-hatch broilers contributes to microbial colonization (Uni et al., 1999). In addition, the intestinal bacteria population plays a crucial role in intestinal morphology development. Enteric pathogens such as *C. perfringens* resulted in delayed small intestine development in newly post hatched broilers (Golder et al., 2011).

Similar to this study, Torok et al. (2009), reported BW and FI of broiler chickens were reduced at d 14 in reused in comparison to fresh hardwood shavings. The authors suggested that an increase in bacterial exposure and the alteration in microbiota associated with reused litter could have reduced performance during the post-hatch period. Thus, reduced growth performance and meat yield of chickens on Reused litter from 0 to 12 d of age could be due to an increase of bacteria exposure and changing gut microbiota composition in this group as compared to the Fresh litter group (Cressman et al., 2010; Torok et al., 2009; Lee et al., 2011). Birds raised on reused litter were able to overcome the negative effects on BW and FI in at d 28 and d 42 (Torok et al., 2009; Kalita et al., 2012). A nearly significant increase in performance of broiler chickens reared on Reused relative to those on Fresh litter at d 41 may be explained by two possibilities. One possibility is via competitive exclusion, in which adult-type microbiota in the small intestine protects the gut from pathogens (Torok et al 2009; Zhang et al., 2007). The stable microbial composition in the small intestine of broilers contributed to colonization by

commensal microbiota rather than pathogens in birds at 28 as compared to those reared on reused litter (Torok et al., 2009). Another possibility is an enhancement in small intestine morphology. Longer villus length and higher villus length to crypt depth (VCR) ratio is associated with greater nutrient absorption (Onderci et al., 2006; Montagne, 2003). Shallower crypts indicate slower tissue turnover, which is associated with lower energy demand for gut maintenance (Yason et al., 1987). Crypt depth and VCR was lower at d 12; and VCR was greater when birds were reared on Reused litter as compared to Fresh in older birds; and villus length also was greater in the jejunum for this group of birds at d 41 (Chapter 3). As birds in the Reused relative to Fresh litter aged, they were more efficient due to increase in villus parameters and may have been able to allocate more nutrients towards the production rather than gut maintenance. Therefore, as birds in the Reused litter aged, they became more efficient relative to post hatched broilers due to increase villus parameters and may have been able to allocate more nutrients towards the production than gut maintenance

Stressful environments, such as birds challenged by pathogens can result in a higher rate of mortality relative to non-challenged broilers (Shojadoost et al., 2012) and increased inflammatory mediators such as α1-acid glycoprotein (Chen et al., 2015) due to increased systemic inflammation. Dietary supplementation of $25OHD_3$ relative to vitamin D_3 at the same level of activity reduced pro-inflammatory response (IL-1β) and increased anti-inflammatory response (IL-10) in broiler chickens (Morris et al., 2014). Therefore, the reason that birds fed $25OHD_3$ alone had similar BW and BWG to d 12 on either fresh or reused litter could be due to lower inflammation relative to birds fed vitamin D_3 alone at d 12. Also, the tendency (P=0.072) towards lower total mortality in birds fed $25OHD_3$ alone relative to those fed vitamin D_3 at same

level of activity could have been due to reduced systemic inflammation in birds fed $25OHD_3$, particularly when they were reared on reused litter.

Similar to this study, the combination $25OHD_3$ and vitamin D_3 had almost the same result on broiler performance and breast meat yield in birds at d 42 as vitamin D_3 at 2,760 IU/kg feed (Striplin et al., 2015; Fritts et al., 2003), but vitamin D_3 at 5,520 IU/kg feed resulted in lower breast meat yield relative to vitamin D_3 at 2,760 IU/kg feed. This might indicate a physiological limit in the liver that does not allow it to convert vitamin D_3 to $25OHD_3$ when birds are being fed higher amounts of vitamin D_3 (Vignale et al., 2013). Similar to this study, dietary $25OHD_3$ at 2,760 IU/kg of feed had no effects on BW, BWG and FI at d 41 relative to vitamin D_3 at the same level of activity (Vignale et al., 2015; Angel et al., 2006; Fritts and Waldroup, 2005; Roberson et al., 2005; and Bar et al., 2003). Dissimilarly, dietary $25OHD_3$ increased BW (Yarger et al, 1995; Fritts and Waldroup, 2003) and decreased FCR relative to vitamin D_3 at 2,760 IU/kg of feed (Yarger et al, 1995). The reason for this dissimilarity in growth performance among studies is not clear and it could be due to experimental conditions, for example wheat vs corn based diets, environmental conditions and management factors. Also, $25OHD_3$ was more effective relative to vitamin D_3 particularly when $25OHD_3$ was fed at 2,000 IU/kg and Ca and P were restricted (Bar et al., 2003). Moreover, $25OHD_3$ at 2,760 IU/kg feed relative to vitamin D_3 at the same level of activity increased crypt depth and villus width and tended to increase ($P<0.1$) villus surface area (VSA) and villus width at d 41. However VSA did not increase at d 12 and d 22, and VCR decreased for 25OHD-fed birds as compared to D-fed birds at d 22 (Chapter 3). Increments in VCR or VSA can increase nutrient absorption (Onderci et al., 2006; Perić et al., 2010). An increase in nutrient absorption could provide more nutrients for bone mineralization or strength (Chapter 3), leg meat yield growth, and once maintenance energy requirement is met.

Therefore, potential to increase nutrient absorption in older birds fed 25OHD$_3$ relative to those fed vitamin D$_3$ could explain the lack of vitamin D source on growth performance with bird age in this study.

Dietary 25OHD relative to vitamin D$_3$ reduced BW in birds reared on Fresh litter at d 12, also, BW did not differ between vitamin D sources at d 41 (Table 2.2). However, BW of sampled birds fed 25OHD was lower at d 12 and d 41 as compared to those fed D (Table 2.3). We can extrapolate that the tendency for lower meat weight in birds fed 25OHD relative to those birds fed vitamin D$_3$ at d 12 and d 41 could be due to the lower BW of sample birds relative to the entire population (Tables 2.3 and 2.4). Dissimilarly, previous studies showed that 25OHD$_3$ increased breast meat yield throughout the rearing period relative to vitamin D$_3$ at the same level of inclusion (2,760 IU/Kg feed; Yarger et al., 1995) and at processing (d42) at twice the level of inclusion in this study (5,520 IU/Kg feed; Vignale et al., 2015). Vignale et al. (2015), suggested that the increment in breast muscle yield with dietary 25OHD$_3$ was due to an increase in protein synthesis, expression of VDR in breast muscle, and activation of the mechanistic target of rapamycin (mTOR) pathway in birds fed a high level of 25OHD$_3$. In addition, supplementation of 25OHD$_3$ as a complete or partial replacement for vitamin D$_3$ relative to vitamin D$_3$ at the same level of inclusion did not increase breast muscle yield in Ross 308 or 708 broilers (Papešová et al., 2008; Hutton et al., 2014) or with wheat-based diets (Papešová et al., 2008). However, supplementation of 25OHD$_3$ increased breast meat yield in Arbor Acres or Cobb 500 broilers fed corn-based diets (Yarger et al., 1995; Vignale et al., 2015). Contrary to expectations, 25OHD$_3$ did not enhance breast meat yield in either young or older birds. There might be two possibilities for this discrepancy. One possibility is the increased expression of genes related to amino acid metabolism in birds fed vitamin D$_3$ relative to birds fed 25OHD$_3$ at d 12 (Chapter 4). The

increased expression of genes related to amino acid transport may lead to increased performance and higher serum amino acid concentrations in pigs (Morales et al., 2014). Another possibility is related to the increased small intestine weight and crypt depth in older birds in birds fed $25OHD_3$ alone relative those bird fed vitamin D_3 at the same level of inclusion (Chapter 3). Deeper crypts and heavier small intestines are associated with more rapid intestinal epithelial cell turnover leading to more energy and protein demand for gut maintenance (Yason et al., 1987; Yang et al. 2008). Gut maintenance energy requirement is about 20% of body energy expenditure (Choct et al, 2009). Therefore, dietary $25OHD_3$ at 2,760 IU/kg feed seems to have increased the gut maintenance energy which may have caused decreased nutrient availability for breast muscle growth.

In this study, leg meat yield increased in birds fed $25OHD_3$ relative to those fed vitamin D_3 at the same level of activity. In previous studies increased breast meat yield was reported in $25OHD_3$-fed birds relative to vitamin D_3-fed birds, although leg muscle yield was not measured (Yarger et al., 1995; Vignale et al., 2015). The 1α-hydroxylase converts $25OHD_3$ to 1,25-$(OH)_2D_3$ and is expressed at high levels in both breast and thigh muscle (Shanmugasundaram and Selvaraj, 2012). Additionally, dietary $25OHD_3$ did not affect breast meat yield in Ross 308 or 708 (Papešová et al., 2008; Hutton et al., 2014). Genetic selection of broilers has resulted in faster allometric growth of breast muscle relative to thigh muscle in modern chickens and turkeys (Zuidhof et al., 2014; Peng., et al 1984). Also, breast muscle mass exhibits positive allometry and increases proportionally faster than body mass in Ross 308 broiler chickens from post-hatch until 6 weeks of age (Tickle et al., 2014). This indicates more nutrients being used support deposition of breast relative to leg muscle in fast-growing type chickens. Therefore,

dietary supplementation of $25OHD_3$ seemed to be more effective in leg muscle deposition rather than breast muscle deposition in birds in the current study.

In conclusion, Reused litter reduced broiler performance and breast meat yield at d 12 relative to Fresh litter. This could be due to increased inflammation, variability of gut microbial composition and gut maintenance energy requirement in birds reared on reused litter. However, birds reared on Reused litter were able to overcome negative effects in growth performance and meat yield production at d 41, which could be due to maturation of the immune system and stability in gut microbial composition. Vitamin D_3 at 2,760 IU/kg feed tended to increase total mortality relative to $25OHD_3$ at the same level of inclusion, which could be due to a tendency towards increased inflammation in D-fed birds relative to 25OHD-fed birds. The reason for increased growth performance at d 12 and breast meat yield at processing of birds fed vitamin D_3 at 2,760 IU/kg feed relative to 25OHD-fed birds is not clear, but it may be linked to changes in gut morphology. However, increased leg meat yield at processing may be linked to increased expression of 1α-hydroxylase in thigh muscle in 25OHD-fed birds as compared to D-fed birds.

2.5 REFERENCES

Angel, R., W. W. Saylor, A. D. Mitchell, W. Powers, and T. J. Applegate. 2006. Effect of dietary phosphorus, phytase, and 25-hydroxycholecalciferol on broiler chicken bone mineralization, litter phosphorus, and processing yields. Poult. Sci. 85:1200-1211.

Atencio, A., G. M. Pesti, and H. M. Edwards. 2005. Twenty-five hydroxycholecalciferol as a cholecalciferol substitute in broiler breeder hen diets and its effect on the performance and general health of the progeny. Poult. Sci. 84:1277-1285.

Aviagen. 2009. Ross Broiler Management Manual. Aviagen Ltd., Newbridge, UK.

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, A., V. Razaphkovsky, E. Vax, and I. Plavnik. 2003. Performance and bone development in broiler chickens given 25-hydroxycholecalciferol. Br. Poult. Sci. 44:224-233.

Boland, R., A. R. de Boland, M. J. Marinissen, G. Santillan, G. Vazquez, and S. Zanello 1995. Avian muscle cells as targets for the secosteroid hormone 1,25-dihydroxy-vitamin D_3. Mol. Cell. Endocrinol. 114:1-8.

Bolan, N. S., A. A. Szogi, T. Chuasavathi, B. Seshadri, M. J. Rothrock, and P. Panneerselvam. 2010. Uses and management of poultry litter. World's Poult. Sci. J. 66:673-698.

Canadian Council on Animal Care. 2009. A guide to the care and the use of experimental animals. CCAC, Ottawa, ON, Canada.

Chen, J., G. Tellez, J. D. Richards, and J. Escobar. 2015. Identification of potential biomarkers for gut Barrier Failure in Broiler Chickens. Front. Vet. Sci. 2:14.

Chinivasagam, H. N., M. Redding, G. Runge, and P. J. Blackall. 2010. Presence and incidence of food-borne pathogens in Australian chicken litter. Br. Poult. Sci. 51:311-318.

Choct, M. 2009. Managing gut health through nutrition. Br. Poult. Sci. 50:9–15.

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.

Coufal, C.D., C. Chavez, P.R. Niemeyer, and J.B. Carey. 2006. Effects of top-dressing recycled broiler litter on litter production, litter characteristics, and nitrogen mass balance. Poult. Sci. 85:392-397.

Cressman, M. D., Z. Yu, M. C. Nelson, S. J. Moeller, M. S. Lilburn, and H. N. Zerby. 2010. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. Appl. Environ. Microbiol.76:6572–6582.

Crhanova, M., H. Hradecka, M. Faldynova, M. Matulova, H. Havlickova, F. Sisak, and I. Rychlik. 2011. Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella enterica serovar enteritidis* infection. Infect. Immun. 79:2755-2763.

Deng, B., M. Wehling-Henricks, S. A. Villalta, Y. Wang, and J. G. Tidball. 2012. IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. J. Immunol. 189:3669-3680.

De Matos, R. 2008. Calcium metabolism in birds. Vet. Clin. North Am. Exot. Anim. Pract. 11:59-82.

Diarra, M. S. and F. Malouin. 2014. Antibiotics in Canadian poultry productions and anticipated alternatives. Front. Microbiol. 5:282-297.

Dibner, J. J., J. D. Richards, and C. D. 2008. Microbial imprinting in gut development and health. J. Appl. Poult. Res. 17:174–188.

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.

Fritts, C. A., and P. W. Waldroup. 2005. Comparison of cholecalciferol and 25-hydroxycholecalciferol in broiler diets designed to minimize phosphorus excretion. J. Appl. Poult. Res. 14:156-166.

Gao, H., G. Wu, T. E. Spencer, G. A. Johnson, F. W. Bazer. 2009. Select nutrients in the ovine uterine lumen. IV. Expression of neutral and acidic amino acid transporters in ovine uteri and peri-implantation conceptuses. Biol. Reprod. 80:1196-1208.

Genxi Z., T. Ying, Z. Tao, W. Jinyu, and W. Yongjuan. 1990. Expression profiles and association analysis with growth traits of the MyoG and Myf5 genes in the Jinghai yellow chicken. Mol. Biol. Rep. 11:7331-7338.

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

Gotoh, K., M. Inoue, T. Masaki, S. Chiba, T. Shimasaki, H. Ando, K. Fujiwara, I. Katsuragi, T. Kakuma, M. Seike, T. Sakata, and H. Yoshimatsu. 2012. A novel anti-inflammatory role for spleen-derived interleukin-10 in obesity-induced inflammation in white adipose tissue and liver. Diabetes. 61:1994-2003.

Gunal, M., G. Yayli, O. Kaya, N. Karahan, O. Sulak. 2006. The effects of antibiotic growth promoter, probiotic or organic acid supplementation on performance, intestinal microflora and tissue of broilers. Int. J. Poult. Sci. 5:149-55.

Hosokawa, H., T. Sawamura, S. Kobayashi, H. Ninomiya, S. Miwa, and T. Masaki. 1997. Cloning and characterization of a brain-specific cationic amino acid transporter. J. Biol. Chem. 272:8717-8722.

Hu, X. F., Y. M. Guo, B. Y. Huang, S. Bun, L. B. Zhang, J. H. Li, D. Liu, F. Y. Long, X. Yang, and P. Jiao. 2010. The effect of glucagon-like peptide 2 injection on performance, small intestinal morphology, and nutrient transporter expression of stressed broiler chickens. Poult Sci. 89:1967-1974.

Lee, K. W., S. H. Lee, H. S. Lillehoj, G. X. Li, S. I. Jang, U. S. Babu, M. S. Park, D. K. Kim, E. P. Lillehoj, A. P. Neumann, T. G. Rehberger, and G. R. Siragusa. 2010. Effects of direct-fed

microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. Poult. Sci. 89:203-216.

Lee, K. W, H. S. Lillehoj, S. H. Lee, S. I. Jang, G. Donald Ritter, D. A. Bautista, and E. P. Lillehoj. 2011. Impact of fresh or used litter on the posthatch immune system of commercial broilers. Avian Dis. 55:539–544.

Lee, K. W., H. S. Lillehoj, S. I. Jang, S. H. Lee, D. A. Bautista1, and G. R. Siragusa. 2013. Effect of bacillus subtilis-based direct-fed microbials on immune status in broiler chickens raised on fresh or used litter. Asian–Australas. J. Anim. Sci. 26:1592-1597.

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

Lim, C. H., W. Jeong, W. Lim, J. Kim, G. Song, F. W. Bazer. 2012. Differential expression of select members of the SLC family of genes and regulation of expression by microRNAs in the chicken oviduct. Biol. Reprod. 87:145-166.

Lu, J., S. Sanchez., C. Hofacre, J. J. Maurer, B. G. Harmon, and M. D. Lee. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. Appl. Environ. Microbiol. 69.901–908.

Lysne, V., B. Bjørndal, R. Vik, J. E. Nordrehaug, J. Skorve, O. Nygård, and R. K. Berge. 2015. A protein extract from chicken reduces plasma homocysteine in rats. Nutrients. 7:4498-4511.

Mattar, A. F., D. H. Teitelbaum., R. A. Drongowski., F. Yongi., C. M. Harmon, and A. G. Coran. 2002. Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model. Pediatr. Surg. Int. 18:586–590.

Montagne, L., J. R. Pluske, and D. J. Hampson. 2003. A review of interactions between dietary fiber and the intestinal mucosa, and their consequences on digestive health in young nonruminant animals. Anim. Feed Sci. Technol. 108:95–117.

Morris, A., R. Shanmugasundaram. M. S. Lilburn, and R. K. Selvaraj. 2014. 25-Hydroxycholecalciferol supplementation improves growth per-formance and decreases inflammation during an experimental lipopolysaccharide injection. Poult. Sci. 93:1951-1956.

Noy, Y., and D. Sklan, D. 1995. Digestion and absorption in the young chick. Poult. Sci. 74:366-373.

Onderci, M., N. Sahin, K. Sahin, G. Cikim, A. Aydìn, I. Ozercan, and S. Aydìn. 2006. Efficacy of supplementation of α-amylaseproducing bacterial culture on the performance, nutrient use, and gut morphology of broiler chickens fed a corn-based diet. Poult. Sci. 85:505–510.

Papešová, L., A. Fučíková, M. Pípalová, P. Tupý. 2008. The synergic effect of vitamin D3 and 25-hydroxycholecalciferol/calcidiol in broiler diet. SAB. 39: 273-277.

Peighambari, S. M., R. J. Julian, and C. L. Gyles. 2000. Experimental *Escherichia coli* respiratory infection in broilers. Avian Dis. 44: 759-769.

Pan, D., and Z. Yu. 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut. Microbes. 5:108-119.

Peng, I. C., R. L. Adams, E. J. Furumoto, P. Y. Hester, J. E. Larsen, O. A. Pike, and W. J. Stadelman. 1985. Allometric growth patterns and meat yields of carcass parts of turkey toms as influenced by lighting programs and age. Poult. Sci. 64:871-876.

Pestka, S., C. D. Krause, D. Sarkar, M. R. Walter, Y. Shi, and P. B. Fisher. 2004. Interleukin-10 and related cytokines and receptors. Annu. Rev. Immunol. 22:929–979.

Perić, L., N. Milosevic, D. Zikic, S. Bjecov, D. Cvetkovic, S. Markov, M. Mohnl, and T. Steiner. 2010. Effects of probiotic and phytogenic products on performance, gut morphology and cecal microflora of broiler chickens. Archiv. Anim. Breed. 53:350-359.

Roberson, K. D., M. F. Ledwaba, and R. A. Charbeneau. 2005. Studies on the efficacy of twenty-five-hydroxycholecalciferol to prevent tibial dyschondroplasia in Ross broilers fed marginal calcium to market age. Int. J. Poult. Sci. 4:85-90.

Savage, D. C., J. E. Siegel, J. E. Snellen, and D. D. Whitt. 1981. Transit time of epithelial cells in the small intestines of germfree mice and ex-germfree mice associated with indigenous microorganisms. Appl. Environ. Microbiol. 42:996–1001.

Schneider, K., S. Kothlow, P. Schneider, A. Tardivel, T. Göbel, B. Kaspers, and P. Staeheli. 2004. Chicken BAFF--a highly conserved cytokine that mediates B cell survival. Int. Immunol. 16:139-48.

Schneider, B. L., R. A. Renema, M. Betti, V. L. Carney , and M. J. Zuidhof. 2012. Effect of holding temperature, shackling, sex, and age on broiler breast meat quality. Poult. Sci. 91:468-477.

Seifert, H. A., A. A. Hall, C. B. Chapman, L. A. Collier, A. E. Willing, and K. R. Pennypacker. 2012. A transient decrease in spleen size following stroke corresponds to splenocyte release into systemic circulation. J. Neuroimmune. Pharmacol. 7:1017-1024.

Shafey, T. M., S. Al-Mufarej and, H. A. Al-Batshan. 2006. Effect of electric field during incubation of eggs on the immune responses of hatched chickens. Electromagn. Biol. Med. 25:163-75.

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

Shanmugasundaram, R., M. S. Lilburn, and R. K. Selvaraj. 2012. Effect of recycled litter on immune cells in the cecal tonsils of chickens. Poult. Sci. 91:95-100.

Shojadoost, B., A. R. Vince, and J. F Prescott. 2012. The successful experimental induction of necrotic enteritis in chickens by *Clostridium perfringens*: a critical review. Vet Res. 43:74-86.

Shirkey, T. W., R. H. Siggers, B. G. Goldade, J. K. Marshall, M. D. Drew, B. Laarveld, and Van A. G. Kessel. 2006. Effects of commensal bacteria on intestinal morphology and expression of proinflammatory cytokines in the gnotobiotic pig. Exp. Biol. Med. 231:1333-1345.

Shojadoost, B., S. Behboudi, A. I. Villanueva, J. T. Brisbin, A. A. Ashkar, and S. Sharif. 2015. Vitamin D_3 modulates the function of chicken macrophages. Res. Vet. Sci. 100:45-51.

Spratt, R. S., B. W. McBride, H. S. Bayley, and S. Leeson. 1990. Energy metabolism of broiler breeder hens. 2. Contribution of tissues to total heat production in fed and fasted hens. Poult. Sci. 69:1348–1356.

Tickle, P. G., H. Paxton, J. W. Rankin, J. R. Hutchinson, and J. R. Codd. 2014. Anatomical and biomechanical traits of broiler chickens across ontogeny. Part I. Anatomy of the musculoskeletal respiratory apparatus and changes in organ size. Peer. J. 2:e432-e449.

Torok, V. A., R. J. Hughes, K. Ophel-Keller, M. Ali, and R. MacAlpine. 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. Poult. Sci. 88:2474–2481.

Uni, Z., Y. Noy, and D. Sklan. 1999. Posthatch development of small intestinal function in the poult. Poult. Sci. 78:215-22.

Vignale, K., J. V. Caldas, J. England, N. Boonsinchai, P. Sodsee, E. D. Pollock, and C. N. Coon. 2013. The effect of HyD on broiler breast meat yield. Poult. Sci. 92(Suppl. 1):120. (Abstr.).

Vignale, K., E. S. Greene, J. V. Caldas, J. England, N. Boonsinchai, P. Sodsee, E. D. Pollock, S. Dridi, and C. N. Coon. 2015. 25-Hydroxycholecalciferol enhances male broiler breast meat yield through the mTOR pathway. J. Nutr. 145:855-863.

Wu, Q. J., Y. M. Zhou, Y. N. Wu, and T. Wang. 2013. Intestinal development and function of broiler chickens on diets supplemented with clinoptilolite. Asian-Australas. J. Anim. Sci. 26:987-994.

Yang, Y., P. A. Iji, A. Kocher, L. L. Mikkelsen, and M. Choct. 2008. Effects of dietary mannanoligosaccharide on growth performance, nutrient digestibility, and gut development of broilers given different cereal-based diets. J. Anim. Physiol. Anim. Nutr. Berl. 92:650–659.

Yason, C. V., B. A. Summers, and K. A. Schat. 1987. Pathogenesis of rotavirus infection in various age-groups of chickens and turkeys: pathology. Am. J. Vet. Res. 48:927–938.

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.

Zhang, G., L. Ma and, M.P. Doyle. 2007. Potential competitive exclusion bacteria from poultry inhibitory to *Campylobacter jejuni* and *Salmonella*. J. Food. Prot.70:867-73.

Zuidhof, M. J., B. L. Schneider, V. L. Carney, D. R. Korver, and F. E. Robinson. 2014. Growth, efficiency, and yield of commercial broilers from 1957, 1978, and 2005. Poult. Sci. 93:2970–2982.

2.6 TABLES

Table 2.1 Basal composition of the experimental diets

	Starter	Grower	Finisher
Item	0 to 12 d	12 to 22 d	23 to 41 d
Ingredient (%)			
Wheat, Hard	57.49	61.15	64.32
Soybean Meal	26.11	21.71	18.50
Canola Meal	7.50	7.50	7.50
Canola Oil	3.61	4.96	5.33
Calcium Carbonate	1.23	0.98	0.94
Dicalcium Phosphate	1.16	0.92	0.77
Salt	0.19	0.19	0.19
L-Lysine	0.18	0.11	0.04
DL - Methionine	0.31	0.25	0.20
L-Threonine	0.06	0.03	
Vitamin D_3 permix[1]	———	0.05	———
HyD premix[1]	———	0.05	———
Vitamin & mineral premix[2]	1	1	1
Enzyme[3]	0.50	0.50	0.50
Choline Chloride premix[4]	0.50	0.50	0.50
Vitamin E premix[5]	0.50	0.50	0.50
Antibiotic growth promoter[6]	0.05	0.05	0.05
Total	100	100	100
Calculated nutrients			
CP (%)	24.47	22.64	21.35
ME (kcal/kg)	3,025	3,150	3,200
Ca (%)	0.65	0.9	0.85
Available P (%)	0.50	0.45	0.42
Total methionine + cysteine (%)	1.11	1.01	0.93
Total methionine (%)	0.69	0.61	0.54
Total Lysine (%)	1.35	1.17	1.04

[1] Vitamin D_3 containing 2,670 IU/kg feed and added to D, 25OHD+D and Dx2.

[1] Dietary supplementation of 25-hydroxycholecalciferol containing 2,670 IU/kg feed and added to 25OHD and 25OHD+D.

[2] The broiler premix provided per kilogram of diet: vitamin A (retinyl acetate), 10,000 IU; cholecalciferol, 4,000 IU; vitamin E (DL-α-tocopheryl acetate), 50 IU; vitamin K, 4.0 mg; thiamine mononitrate (B_1), 4.0 mg; riboflavin (B_2), 10 mg; pyridoxine HCL (B_6), 5.0 mg; vitamin B_{12} (cobalamin), 0.02 mg; D-pantothenic acid, 15 mg; folic acid, 0.2 mg; niacin, 65 mg;

biotin, 1.65 mg; iodine (ethylene diamine dihydroiodide), 1.65 mg; Mn ($MnSO_4H_2O$) , 120 mg; Cu, 20 mg; Zn, 100 mg, Se, 0.3 mg; Fe ($FeSO_47H_2O$), 800 mg.

[3] Phyzyme XP, Phytase enzyme, Danisco Animal Nutrition, Marlborough, Wiltshire, UK.

[4] Provided 100 mg choline per kg of diet

[5] Provided 15 IU vitamin E per kg of diet

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

Table 2.2 Effect of vitamin D source in Fresh and Reused litter on during starter (ST; 0 to 12d), grower (GW; 12 to 22 d) and finisher (FN; 23 to 41 d) periods.

			BW				Gain			Feed Intake			FCR		
Treatment		n[1]	d0	d12	d22	d41	0-12d	13-22d	23-41d	0-12d	13-22d	23-41d	0-12d	13-22d	23-41d
				g				g/d					Feed/gain		
Fresh[2]															
	D[4]	4	43.64^{ab}	331^{ab}	1011^{a}	3068	24.02^{a}	65.46^{a}	118.9	31.77^{a}	99.2	197	1.32	1.51	1.65
	25OHD[5]	4	43.03^{b}	301^{cd}	945^{b}	3146	21.58^{bc}	62.50^{ab}	124.3	30.29^{a}	95.6	204	1.40	1.53	1.64
	D+25OHD[6]	4	43.86^{a}	305^{cd}	902^{c}	2987	21.83^{bc}	59.11^{b}	118.3	31.25^{a}	97.2	199	1.43	1.64	1.68
	Dx2[7]	4	43.80^{a}	339^{a}	1016^{a}	3139	24.60^{a}	65.51^{a}	120.2	32.55^{a}	103.3	206	1.32	1.57	1.71
Reused[3]															
	D	4	43.16^{ab}	298^{d}	969^{a}	3209	21.20^{c}	65.25^{a}	126.8	30.80^{b}	100.8	220	1.45	1.54	1.74
	25OHD	4	43.33^{b}	301^{cd}	956^{b}	3110	21.45^{bc}	63.89^{ab}	125.6	29.94^{b}	103.2	213	1.39	1.61	1.70
	D+25OHD	4	44.03^{a}	298^{d}	948^{c}	3136	21.16^{c}	63.55^{b}	121.5	28.90^{b}	102.2	195	1.36	1.60	1.60
	Dx2	4	44.04^{a}	316^{bc}	1000^{a}	3230	22.78^{b}	66.25^{a}	128.7	29.22^{b}	102.1	234	1.28	1.54	1.82
	Pooled SEM		0.281	5.87	17.42	77.95	0.461	1.38	4.19	0.748	3.69	10.79	0.04	0.046	0.078
								P-values							
Source of variance		DF													
Diet		3	0.015	0.001	0.001	0.482	0.001	0.012	0.631	0.310	0.800	0.220	0.067	0.245	0.414
Litter		1	0.766	0.001	0.994	0.129	0.001	0.115	0.088	0.003	0.220	0.077	0.814	0.723	0.466
Diet*Litter		3	0.479	0.037	0.099	0.623	0.035	0.392	0.789	0.213	0.650	0.461	0.085	0.522	0.669

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

[1] Pen was the experimental unit.

[2] Fresh pine shavings litter

[3] Reused pine shaving litter; litter was used one previous flock

[4] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[7] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

Table 2.3 Total mortality and BW of sampled birds fed vitamin D source in Fresh and Reused litter at 12, 22 and 41 d.

Treatment		n[2]	BW			Total Mortality
			d12	d22	d41	%
			g			
Fresh[2]						
	D[4]	4	370[a]	1,026	3,180[ab]	8.23
	25OHD[5]	4	338[abc]	928	2,929[bc]	4.62
	D+25OHD[6]	4	307[cd]	1,005	3,084[abc]	7.69
	Dx2[7]	4	352[ab]	1,059	3,071[abc]	3.88
Reused[3]						
	D	4	332[abc]	1,029	2,857[c]	6.15
	25OHD	4	275[d]	963	3,179[ab]	4.62
	D+25OHD	4	347[abc]	1,030	3,195[ab]	3.88
	Dx2	4	312[cd]	1,029	3,272[a]	6.15
	Pooled SEM		14.23	50.10	137.6	0.10
			P-values			
Source of variance		DF				
Diet		3	0.018	0.101	0.452	0.127
Litter		1	0.038	0.509	0.424	0.237
Diet*Litter		3	0.005	0.989	0.036	0.071

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

[1] Pen was the experimental unit.

[2] Fresh pine shavings litter

[3] Reused pine shaving litter; litter was used one previous flock

[4] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[7] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

Table 2.4 Effect of vitamin D source in Fresh and Reused litter on absolute weights of breast meat, Pectoralis major, Pectoralis minor and liver of sampled birds at 12, 22 and 41 d.

			Breast[1]			**Pectoralis major**			**Pectoralis minor**		
		n[2]	d12	d22	d41	d12	d22	d41	d12	d22	d41
Treatment							g				
Fresh[3]											
	D[5]	4	41.54[a]	162.2	608.5	33.89[a]	133.5	502.1	7.65[ab]	28.66	106.3
	25OHD[6]	4	38.66[a]	144.2	548.6	31.76[a]	118.8	435.1	6.89[b]	25.46	95.7
	D+25OHD[7]	4	40.59[a]	148.8	585.2	33.45[a]	121.1	481.7	7.14[ab]	27.72	103.5
	Dx2[8]	4	44.73[a]	164.6	555.9	36.35[a]	135.9	457.9	8.37[a]	28.74	97.9
Reused[4]											
	D	4	34.24[b]	158.6	550.8	27.71[b]	131.5	449.7	6.52[cd]	27.04	95.7
	25OHD	4	33.21[b]	142.3	606.1	27.21[b]	117.1	501.6	6.00[d]	24.61	104.5
	D+25OHD	4	38.00[b]	148.1	596.7	31.07[b]	121.1	493.8	6.92[cd]	26.96	102.9
	Dx2	4	38.06[b]	159.8	643.0	31.09[b]	132.1	533.0	6.96[c]	27.71	110.0
	Pooled SEM		2.057	8.309	29.38	1.693	6.890	25.79	0.289	1.323	4.725
Source of variance		DF					p-value				
Diet		3	0.065	0.057	0.245	0.079	0.053	0.725	0.036	0.079	0.835
Litter		1	0.001	0.632	0.868	0.001	0.731	0.169	0.002	0.262	0.477
Diet*Litter		3	0.674	0.995	0.090	0.712	0.994	0.057	0.516	0.987	0.087

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

[1] Breast: Pectoralis major and Pectoralis minor, bone and skin not included. Pen was the experimental unit.

[2] Pen was the experimental unit.

[3] Fresh pine shavings litter.

[3] Reused pine shaving litter; litter was used one previous flock.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[7] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[8] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

Table 2.5 Effect of vitamin D source in Fresh and Reused litter on weights relative to BW of breast, Pectoralis major, and Pectoralis minor of sampled birds at 12, 22 and 41 d.

			Breast[1]			**Pectoralis major**			**Pectoralis minor**		
		n[2]	D12	d22	d41	d12	d22	d41	d12	d22	D41
Treatment			% of live weight								
Fresh[3]											
	D[5]	4	10.58[a]	15.51	19.20	9.75[a]	12.96	15.83	2.20[a]	2.80	3.93
	25OHD[6]	4	11.28[a]	15.36	18.28	9.63[a]	12.60	14.68	2.09[a]	2.73	3.69
	D+25OHD[7]	4	11.29[a]	15.49	18.98	10.26[a]	12.59	15.62	2.20[a]	2.89	3.71
	Dx2[8]	4	11.51[a]	15.44	18.04	9.92[a]	12.74	14.85	2.28[a]	2.69	3.80
Reused[4]											
	D	4	11.96[b]	15.95	19.14	8.56[b]	13.21	15.65	2.01[b]	2.73	3.91
	25OHD	4	11.73[b]	15.12	18.98	9.22[b]	12.49	15.69	2.05[b]	2.63	4.02
	D+25OHD	4	12.47[b]	14.66	18.51	9.19[b]	11.98	15.31	2.04[b]	2.67	3.78
	Dx2	4	12.21[b]	15.60	19.55	9.41[b]	12.89	16.21	2.10[b]	2.70	3.52
	Pooled SEM		0.369	0.270	0.499	0.290	0.327	0.454	0.089	0.081	0.145
Source of variance		DF	p-value								
Diet		3	0.315	0.190	0.737	0.226	0.093	0.677	0.603	0.532	0.586
Litter		1	0.001	0.523	0.247	0.001	0.731	0.148	0.028	0.106	0.871
Diet*Litter		3	0.568	0.516	0.218	0.481	0.552	0.169	0.826	0.585	0.501

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

[1] Breast: Pectoralis major and Pectoralis minor, bone and skin not included. Pen was the experimental unit.

[2] Pen was the experimental unit.

[3] Fresh pine shavings litter.

[3] Reused pine shaving litter; litter was used one previous flock.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[7] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[8] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

Table 2.6 Effect of vitamin D source in Fresh and Reused litter on absolute weights of spleen, small intestine, liver and Abdominal fat of sampled birds at 12, 22 and 41 d.

Treatment			**Spleen**			**Small intestine**			**Liver**			**Abdominal fat**
Fresh[2]		n[1]	d12	d22	d41	d12	d22	d41	d12	d22	d41	d41
								g				
	D[4]	4	0.256	0.075	3.00^{a}	17.95^{b}	37.04^{b}	67.49	13.74	30.71	66.95^{b}	59.90^{a}
	25OHD[5]	4	0.245	0.082	2.52^{a}	17.15^{b}	41.20^{ab}	65.48	12.35	30.61	63.56^{b}	46.39^{b}
	D+25OHD[6]	4	0.262	0.093	2.48^{a}	16.93^{b}	35.98^{b}	69.4	12.2	31.51	73.65^{a}	59.09^{a}
	Dx2[7]	4	0.253	0.086	2.91^{a}	17.70^{b}	43.36^{a}	69.33	13.00	35.51	66.82^{b}	49.98^{ab}
Reused3												
	D	4	0.283	0.091	2.18^{b}	22.24^{a}	38.01^{ab}	60.99	12.58	32.24	58.50^{b}	52.92^{ab}
	25OHD	4	0.242	0.089	2.48^{b}	29.29^{a}	41.31^{ab}	64.07	11.66	32.09	67.38^{b}	58.93^{a}
	D+25OHD	4	0.253	0.083	2.40^{b}	20.95^{a}	40.09^{ab}	63.20	12.72	32.84	77.33^{a}	52.97^{ab}
	Dx2	4	0.249	0.080	2.43^{b}	21.40^{a}	36.50^{b}	69.70	11.46	32.09	67.47^{b}	60.48^{a}
	Pooled SEM		0.021	0.005	0.1965	1.693	1.950	3.199	0.848	1.940	3.320	4.061
Source of variance		DF						p-value				
Diet		3	0.670	0.567	0.658	0.111	0.204	0.353	0.587	0.522	0.002	0.793
Litter		1	0.809	0.928	0.013	<0.001	0.765	0.133	0.110	0.903	0.974	0.390
Diet*Litter		3	0.839	0.322	0.153	0.533	0.044	0.630	0.350	0.457	0.217	0.023

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

[1] Pen was the experimental unit.

[2] Fresh pine shavings litter

[3] Reused pine shaving litter; litter was used one previous flock

[4] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[7] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

Table 2.7 Effect of vitamin D source in Fresh and Reused litter on weights relative to BW of spleen, small intestine, liver and Abdominal fat of sampled birds at 12, 22 and 41 d.

Treatment			**Spleen**			**Small intestine**			**Liver**			**Abdominal fat**
Fresh[3]		n[2]	d12	d22	d41	d12	d22	d41	d12	d22	d41	d41
			% BW									
	D[5]	4	0.082	0.075	0.093[a]	5.00[b]	3.60[b]	2.11	3.93	2.99	2.10[b]	2.13
	25OHD[6]	4	0.073	0.082	0.085[a]	5.16[b]	4.42[a]	2.25	3.69	3.27	2.17[b]	2.02
	D+25OHD[7]	4	0.077	0.093	0.080[a]	5.19[b]	3.76[b]	2.24	3.71	3.26	2.39[a]	1.97
	Dx2[8]	4	0.067	0.085	0.095[a]	4.82[b]	4.12[b]	2.25	3.80	3.37	2.17[b]	2.12
Reused[4]												
	D	4	0.079	0.090	0.076[b]	7.00[a]	3.89[b]	2.13	3.91	3.26	2.05[b]	1.97
	25OHD	4	0.082	0.086	0.078[b]	6.68[a]	4.37[a]	2.02	4.02	3.4	2.11[b]	2.12
	D+25OHD	4	0.078	0.083	0.075[b]	6.26[a]	4.00[b]	1.97	3.78	3.25	2.40[a]	2.13
	Dx2	4	0.077	0.080	0.075[b]	6.63[a]	3.59[b]	2.12	3.52	3.15	2.05[b]	2.02
	Pooled SEM		0.005	0.005	0.006	0.247	0.164	0.070	0.145	0.139	0.076	0.069
Source of variance		DF	P-value									
Diet		3	0.494	0.673	0.629	0.111	0.001	0.353	0.586	0.508	0.001	0.490
Litter		1	0.274	0.762	0.006	<0.001	0.902	0.133	0.871	0.661	0.326	0.587
Diet*Litter		3	0.568	0.067	0.512	0.533	0.055	0.630	0.500	0.339	0.851	0.060

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

[1] Pen was the experimental unit.

[2] Fresh pine shavings litter

[3] Reused pine shaving litter; litter was used one previous flock

[4] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[7] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

Table 2.8 Effect of vitamin D source in Fresh and Reused litter on absolute weights relative to BW of carcass parts at processing (42 d)

Treatment			BW	Carcass	Breast[1]	Pectoralis major	Pectoralis minor	Legs	Wings
Fresh[3]		n[2]				g			
	D[5]	4	3133	2049	605	496	109	665	242
	25OHD[6]	4	3251	2088	588	480	108	700	243
	D+25OHD[7]	4	2946	1906	549	451	99	626	227
	Dx2[8]	4	3169	2070	595	489	107	680	240
Reused[4]									
	D	4	3157	2016	606	497	109	640	235
	25OHD	4	3135	2001	574	474	100	653	238
	D+25OHD	4	3259	2103	617	509	109	680	249
	Dx2	4	3210	2037	588	483	105	671	238
Pooled SEM			84.99	53.79	20.33	0.348	3.39	18.46	5.83
Source of variance		DF				P-value			
Diet		3	0.642	0.826	0.650	0.703	0.444	0.394	0.982
Litter		1	0.249	0.780	0.043	0.344	0.959	0.639	0.687
Diet*Litter		3	0.064	0.054	0.186	0.220	0.078	0.058	0.053

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

[1] Breast: Pectoralis major and Pectoralis minor, bone and skin not included. Pen was the experimental unit.

[2] Pen was the experimental unit.

[3] Fresh pine shavings litter.

[3] Reused pine shaving litter; litter was used one previous flock.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[7] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[8] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

Table 2. 9 Effect of vitamin D source in Fresh and Reused litter on live BW, carcass, and weights relative to carcass of breast meat Pectoralis major and minor, leg , and wing yield at processing (42 d)

Treatment			**Breast**[1]	**Pectoralis major**	**Pectoralis minor**	**Legs**	**Wings**
					Carcass as		
Fresh[3]		n[2]			% of BW		
	D[5]	4	29.59[a]	24.24	5.34[a]	32.29[b]	11.84
	25OHD[6]	4	28.12[b]	22.96	5.15[b]	33.5[a]	11.66
	D+25OHD[7]	4	28.73[ab]	23.54	5.18[b]	32.89[ab]	11.90
	Dx2[8]	4	28.60[b]	23.44	5.15[b]	32.88[a]	11.61
Reused[4]							
	D	4	29.96[a]	24.58	5.37[a]	31.73[b]	11.69
	25OHD	4	28.55[b]	23.57	4.97[b]	32.56[a]	11.93
	D+25OHD	4	29.31[ab]	24.12	5.17[b]	32.36[ab]	11.86
	Dx2	4	28.71[b]	23.56	5.14[b]	32.99[a]	11.75
Pooled SEM			0.472	0.42	0.086	0.379	0.144
Source of variance		DF			P-value		
Diet		3	0.025	0.055	0.010	0.035	0.564
Litter		1	0.286	0.180	0.490	0.074	0.596
Diet*Litter		3	0.969	0.932	0.647	0.560	0.451

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

[1] Breast: Pectoralis major and Pectoralis minor, bone and skin not included. Pen was the experimental unit.

[2] Pen was the experimental unit.

[3] Fresh pine shavings litter

[3] Reused pine shaving litter; litter was used one previous flock

[5] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[7] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[8] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

3. EFFECTS OF DIETARY 25-HYDROXYCHOLECALCIFEROL ON GUT MORPHOLOGY, INNATE IMMUNE RESPONSE AND BONE MINERALIZATION OF BROILERS ON REUSED LITTER

ABSTRACT

The vitamin D_3 metabolite 25-hydroxycholecalciferol ($25OHD_3$) is associated with innate immunity and bone characteristics in broiler chickens. The effects of dietary $25OHD_3$ on intestinal morphology, innate immune response, and bone mineralization and development of broiler chickens reared on Fresh and Reused litter was studied. Wheat-soybean meal-based diets were fed to Ross 308 chicks (n=512; 4.18 birds/m^2) for 41 d in a 4 x 2 factorial arrangement with 4 pens/treatment. Dietary treatments were: vitamin D_3 at 2,760 IU/kg feed (D; Control); 25OH D_3 at 2,760 IU/kg feed (25OHD); D_3 at 2,760 IU/kg plus 25OH D_3 at 2,760 IU/kg feed (D+25OHD); or D_3 at 5,520 IU/kg feed (Dx2); each diet was fed to broilers grown on either fresh (Fresh) or reused (Reused) pine shavings to increase bacterial exposure. Rectal temperature was determined, plasma nitric oxide (NO), α-1 acid glycoprotein (AGP) as well as haptoglobin (Hp) levels, and duodenum, jejunum and ileum villus length and width, villus surface area (VSA), crypt depth, and villus length to crypt depth ratio (VCR) were measured. Bone breaking strength (BBS) and total, cortical and trabecular bone mineral density (BMD) and cross-sectional area (CSA) using quantitative computed tomography were measured at 12, 22, and 41 d of age. Also, bone mineral content (BMC) was calculated by multiplying the bone cross sectional area (cm^2) by the density (mg/cm^3). Data were analyzed using ANOVA; differences were considered significant at $P \leq 0.05$. At d 41, Hp and AGP production increased in birds reared on Reused

litter relative to those reared on Fresh litter, also, birds fed 25OHD had lower AGP production as compared to those fed D or Dx2. Reused litter relative to Fresh litter resulted in increased villus length and crypt depth throughout the rearing period. Also, VSA and VCR in newly -hatched broilers decreased and then VCR increased in older birds when birds reared on Reused relative to Fresh litter. Birds fed 25OHD had greater villus width and tended to have increased ($P<0.1$) VSA at d 41, and deeper crypts relative to those fed D and Dx2 at d 22 and 41. Shallower crypt depth and increased VSA and VCR in young birds reared on Fresh litter are associated with more nutrient absorption and lower gut maintenance energy relative to those reared on Reused litter. Reused litter decreased BBS, total and trabecular BMD, total and cortical CSA, and total, cortical and trabecular at d 12; and total BMD, trabecular CSA and BMC in 22-d-old chicks in comparison to Fresh litter. Also, $25OHD_3$ as a complete or partial replacement for vitamin D_3 increased total and cortical BMD at 12 and d 22; total and cortical BMC at d 12; tended to increase BBS at d 22 and 41; and tended to increase trabecular BMC at d 41. The decreased VCR and VSA, and reduced bone mineralization in birds reared on Reused litter could be due to an increase in acute phase proteins in this group. Deeper crypt depth in 25OHD-fed birds relative to D-fed birds may indicate lower villus mucosal cell turnover and therefore lower gut maintenance energy requirement. Overall, $25OHD_3$ reduced inflammation, and increased bone mineralization as compared to vitamin D_3, whereas, vitamin D_3 may have decreased gut maintenance energy in older birds.

Keywords: 25-hydroxycholecalciferol, vitamin D_3, bone mineralization, innate immune response, reused litter, inflammation

3.1 INTRODUCTION

Recycling of litter from previous broiler chicken flocks is used commercially, not only to reduce cost but also to protect gut from pathogens via more rapid colonization of the gut by commensal microorganisms (Torok et al.,2009: Cressman et al., 2010: and Coufal et al., 2006). There is a complex microbiota in the digestive tract of chickens, which contributes to growth and health of the birds. Symbiotic microorganisms in the healthy gut of birds can protect them from the presence of enteric pathogens (Nurmi and Rantala, 1973). Beneficial bacteria may increase intestinal antibody production and epithelial cell turnover (Neg et al., 2009; Savage et al., 1981), and increase villus length in broilers, leading to an increase in nutrient absorption in chickens (Dibaji et al 2014; Lee et al., 2010). In spite of these advantages, the gut microbial population alters when birds are reared on reused litter (Lee et al 2011; Lu et al., 2003). Environmental bacteria (*Lactobacillus*) are the predominant microbes in small intestine of birds raised on fresh litter, while, bacteria of intestinal origin (*Clostridiales*) are predominant microbes for chickens raised on reused litter (Lee et al., 2011; Cressman et al., 2010). This change in the predominant microbial species in the gut of birds may result in changes in intestinal immune responses. For example, birds reared on reused litter have shown increased inflammatory response, whereas, birds reared on fresh litter have more anti-inflammatory response (Shanmugasundaram et al., 2012). In particular, the increment in bacterial exposure on reused litter may reduce performance of broiler during the early post-hatch period due to an increase in inflammation (Torok et al., 2009).

Vitamin D is involved calcium and phosphorous absorption from the gut, and bone mineralization and mobilization (Driver et al 2005; Kasim et al 2006). Vitamin D_3 is a fat-soluble

vitamin and its absorption is facilitated by lipase and bile salts. However, hydroxycholecalciferol ($25OHD_3$) is more rapidly absorbed than vitamin D_3 due to greater polarity and less dependency on bile salts (Bar et al., 1980). Vitamin D_3 requires two hydroxylation steps to become the biologically active form. Vitamin D_3 is hydroxylated to $25OHD_3$ by 25-hydroxylase in the liver and later converted to 1, 25-dihydroxylcholecalciferol [$1,25\text{-}(OH)_2D_3$] by the action of 1 α-hydroxylase in the kidney (Shanmugasundaram and Selvaraj, 2012; Atencio et al., 2005). $25OHD_3$ has a higher absorption rate from the gut relative to vitamin D_3 (Phadnis and Nemere, 2003; Bar et al., 1980), increases Ca uptake from the gut (Phadnis and Nemere, 2003), and enhances small intestine morphology in broiler chickens (Ding et al., 201; Chou et al., 2009). In addition, supplemental $25OHD_3$ results in increased calcium content in tibias and cellular immune responses (Gómez-Verduzco et al., 2013), bone ash (Fritts and Waldroup, 2003), and decreases severity of tibial dyschondroplasia (Parkinson and Cransberg, 2004; Fritts and Waldroup, 2003; Rennie and Whithead, 1996) as compared to dietary vitamin D_3 at the same level of inclusion.

Systemic effects of inflammation increased acute phase protein production (Korver, et al., 1998) and decreased bone strength (Mireles, et al., 2005). Increased expression of inflammatory cytokines is associated with increased production of acute phase proteins in broiler chickens (Lee et al., 2010). Birds reared on reused litter had higher expression of inflammatory cytokine IL-1β relative to those grown on fresh litter (Lee et al., 2011). Supplementation of $25OHD_3$ resulted in decreased inflammatory cytokine IL-1β in lipopolysaccharide (LPS)-injected broilers (Morris et al., 2014). The effects of vitamin D_3 or its metabolites in fresh and reused litter on bone characteristics, intestinal morphology, and immunity of broiler chickens have not previously studied. We hypothesized that dietary supplementation of $25OHD_3$ as a complete or partial

replacement for vitamin D_3 would lead to changes in small intestine morphology, and increases in innate immune response and bone characteristics due to greater nutrient absorption and reduced inflammatory responses. Therefore, the objectives of this trial were to investigate the effects of dietary supplementation of $25OHD_3$ on bone mineralization and development, small intestine morphology, and innate immune response of broiler chickens in unsanitary environments.

3.2 MATERIALS AND METHODS

3.2.1 Experimental Diets and Animal Housing

All experimental conditions and animal protocols were approved by University of Alberta Animal Care and Use Committee in accordance with the Canadian Council of Animal Care (2009) guide. An experiment was conducted with 512 1-d-old male Ross 308 broilers chickens with a stocking density of 4.18 birds/m^2. A completely randomized design with factorial arrangement of treatments (4 diets and 2 litters) was used. For each phase, dietary treatments were: vitamin D_3 at 2,760 IU/kg feed (D); $25OHD_3$ at 2,760 IU/kg feed (25OHD); D_3 at 2,760 IU/kg plus $25OHD_3$ at 2,760 IU/kg feed (D+25OHD); or D_3 at 5,520 IU/kg feed (Dx2). Broilers were allocated to 32 pens (1.9 x 2.2 m^2) and each of the 4 dietary treatments was randomly assigned to 8 pens with 8 replicates per dietary treatment. Used litter from a single previous flock was left on the barn floor for 4 weeks before it was moved to the experimental barn for this study. Broilers were reared in an environmentally-controlled room on either fresh (Fresh) or reused (Reused) pine shavings. Also, feed and water were provided for ad-libitum consumption throughout the 41 d experimentation. Standard broiler rearing management

including lighting program, temperature, and ventilation were maintained as specified by the primary breeder company (Aviagen management handbook for Ross 308, 2009).

3.2.2 Measurement of Inflammatory Status

At 12, 22, and 41 d of age rectal temperature was determined in 12 individual replicate birds per treatment. Plasma levels of nitric oxide (NO), as well as the positive acute phase proteins alpha 1 acid glycoprotien (AGP) and haptoglobin (Hp) were determined to identify the effects of treatments on basal inflammatory response. Blood samples (approximately 1.5 ml) were collected by venipuncture into tubes containing EDTA from 12 individual birds per treatments at each of d 12, d 22 and d 41. The blood samples were centrifuged at 3,000 X g for 10 minutes, and the plasma removed and stored at –20°C until day of analysis.

Plasma nitric oxide concentration was determined according to Bowen et al, (2007). Plasma AGP concentration was determined according to the manufacturer's protocol (AGP ELISA kit, Cat. NO. ab15690). Plasma Hp concentration was determined according to the manufacturer's protocol (Hp ELISA kit, Cat. NO. MBS013153). Optical density (OD) at 550 nm (OD550) for NO and at 450 nm (OD450) for Hp and AGP was measured with a SpectraMax M5 Microplate Reader (Molecular Devices, California, USA).

3.2.3 Intestinal Histology

Intestinal histology was measured according to the method of Nain et al. (2012). Intestinal samples (2 cm in length) were excised and fixed in 10% formalin from the mid region of the duodenum, jejunum, and ileum at d 12, 22 and 41 (12 replicates per treatment). Later, samples were gradually dehydrated and embedded in resin, sectioned at 5 mm, and five 5-µm

sections were placed on a microscopic slide stained with hematoxylin. Finally, the slides were measured using a Stereo Discovery v8 microscope (ZEISS, Minneapolis, USA) at 10X magnification and image analysis software Axio Vision Rel 4.8 was used to measure villus height, villus width and crypt depth from four randomly selected villi from five sections on each slide.

The villus height was determined as distance from the tip to the base of the lamina propria, villi width was measured at one-third and two-thirds of the length of the villus, and crypt depth was measured from the base of the villus to the mucosa. For each trait, 4 measurements (4 different locations on each slide) were taken from each bird and the average value for each bird was used for statistical analysis. The ratio of villus height to crypt depth (VCR) was calculated by dividing the villus length by the crypt depth (Kettunen et al., 2001). The surface area was estimated with following formula for all sections (Nain et al., 2012).

$$\text{Villus surface area (VSA)} = 2\pi \times (\text{average villus width}/2) \times \text{villus length};$$

where average villus width is the average of 8 measurements per bird (2 width measurements from the four sample sections examined on each slide).

3.2.4 Bone Characteristics

3.2.4.1 Femur Cross-sectional Area and Bone Mineral Density

Femurs were collected at d 12, 22 and 41 with 12 replicates per treatment at each age. Samples were stored at -20^{o}C until analysis for bone characteristics. Bone mineral density (BMD) and cross-sectional area (CSA) were measured at epiphyseal (proximal; 30% length of femur) and diaphyseal (mid; 50% length of femur) using a Stratec Norland XCT quantitative

computed tomography (QCT) scanner (XCT Reseach SA, Norland, Fort Atkinson, WI), having a 50 kV X-ray tube (Saunders-Blades et al., 2009). Bone mineral content (BMC) was determined as the amount of bone mineral in each 1 mm scan, extrapolated to a 1 cm length of bone. Total, cortical and trabecular bone BMC were calculated by multiplying the cross sectional area (cm^2) by the bone density (mg/cm^3). Later, samples were stored at -20^oC until breaking strength analysis.

3.2.4.2 Femur Breaking Strength

Bone breaking strength (BBS) was conducted for the same bones using an Instron Materials Tester (Saunders-Blades et al., 2009) using Automated Materials Test System software version 8.09, a standard 200 N load plunger, and a modified sheer plate (8 cm in length and 1 mm in width) to measure the perpendicular mechanical force required to break each bone, reported as bone breaking strength (kgF). Samples were thawed at 4 to 6^oC for 24 hours and marked at the midpoint which was determined using a digital caliper. Supports were located at the bone epiphyses and a pressure was applied at the midpoint of each bone, with a crosshead speed of 100 mm/min. The distance between supports was 14, 22 and 32 mm for bones of broilers at 12, 22 41 d of age respectively.

3.2.5 Statistical Analysis

The experimental unit was pen for bone and immunology data. The individual bird was the experimental unit for the small intestine data. All data were analyzed as a two-way analysis of variance with 4 dietary treatments, and 2 litter treatments using the procedure for linear mixed models (PROC MIXED) of SAS (SAS 9.3© for Windows; SAS Institute Inc., Cary, NC). BW was used as a covariate for bone trait data analysis. Differences were considered significant at P

< 0.05. For small intestine and immunology data, ANOVA was in accordance with the following model:

$$Y_{ij} = \mu + D_i + L_j + (DL)_{ij} + E_{ij},$$

Where μ was the population mean; Di was the effect of each dietary treatments (i = 1 to 4); L_j was the effect of litter (j = 1 to 2); $(DS)_{ij}$ was the interaction of each dietary treatment with litter; and Eij was the residual error.

BW was included for bone characteristics as covariate and the ANCOVA was in accordance with the following model:

$$Y_{ij} = \mu + D_i + L_j + (DL)_{ij} + \beta (x_{ij} - \overline{x}_{ij}) + E_{ij},$$

Where μ was the population mean; D_i was the effect of each dietary treatment (i = 1 to 4); L_j was the effect of litter (j = 1 to 2); $(DL)_{ij}$ was the interaction of each dietary treatment with litter; $\beta (x_{ij} - \overline{x}_{ij})$ was a covariate coefficient multiplied by the difference between any individual weight (x_{ij}) and the average of that variable ($\overline{x}_{ij}$); and E_{ij} was the residual error.

3.3 RESULTS AND DISCUSSION

3.3.1 Innate Immune Response

We hypothesized that exposure of broilers to reused litter would increase chronic systemic inflammation. The Hp production was not affected by treatment at d 12 or d 22. However, at d 12, birds fed 25OHD tended to have decreased ($p<0.089$) Hp relative to those fed diets containing vitamin D_3 alone. Additionally, birds reared on Reused litter tended to have

increased (p=0.058) Hp in comparison to Fresh litter at the same day. The Hp production decreased in birds reared on Fresh relative to Reused litter at d 41 (Table 3.1). Birds reared on Reused litter had lower AGP production relative to Fresh litter at d 22. In contrast, APG was increased in birds reared on Reused in comparison to those reared on Fresh litter at d 41. Additionally, birds fed 25OHD had lower AGP as compared to those fed diets containing vitamin D_3 alone; and D+25OHD decreased AGP production relative to 2xD in broilers at d 41 (Table 3.1). There were no differences in rectal temperature and NO among treatments at any ages (Table 3.1). Rectal temperature increases when broilers and layers injected with LPS, indicating systemic inflammation following and acute inflammatory challenge (Leshchinsky et al., 2001). Therefore, Reused litter did not appear to result in greater systemic inflammation than Fresh litter in post hatched broilers in this experiment.

During an acute phase response, acute phase proteins (APP) are produced in response to inflammatory cytokines (Jain et al., 2011; Selvaraj et al., 2016). Chicken APP are produced in the liver in response to infection or tissue injury and their concentrations change with different inflammatory (pathogens, inflammation) and non-inflammatory (gender, age, nutritional status) conditions (Nielsen et al., 1999; Koutsos and Klasing, 2001; Juul-Madsen et al., 2003; Kokosharov, 2006; Kefal and Toker, 2006). Additionally, APP can be increased (positive APP) or decreased (negative APP) in response to inflammatory stimuli. In avian species, several APP have been identified, including AGP, Hp, amyloid A, transferrin, mannan-binding protein, hemopexin, fibrinogen and fibronectin (Chamanza et al., 1999). Therefore, AGP and Hp were investigated to measure inflammation in chickens in this experiment.

In this study, $25OHD_3$ alone reduced inflammatory mediators relative to vitamin D_3 at the same level of inclusion. An increase in expression of inflammatory cytokines is correlated with increased AGP production in chickens during the inflammation (Asasi et al., 2013). Dietary $25OHD_3$ decreased expression of IL-1β in LPS-injected broilers as compared to birds fed vitamin D_3, thus, reducing the acute phase response in the liver (Morris et al., 2014). At d 12, dietary $25OHD_3$ increased the expressions of genes involved in T-cell development and antigen presentation relative to vitamin D_3 (Chapter 4). These genes are linked to the major histocompatibility complex class I molecules, which reduce innate inflammatory responses in mammals (Xu et al., 2012). Therefore, supplementation of $25OHD_3$ at 2,760 IU/kg reduced inflammatory response in broilers relative vitamin D_3 at the same level of inclusion.

The immune system of the early post-hatch chickens is immature, and they are susceptible to enteric pathogens (Crhanova et al., 2011). Poultry litter may contain pathogenic bacteria, which could increase enteric pathogens when chickens are reared on reused relative to fresh litter (Lu et al., 2003; Cressman et al., 2010). The expression of IL-1β increased in birds reared on reused relative to fresh litter (Shanmugasundaram et al., 2012). Increased expression of pro-inflammatory response genes is associated with increased APP and acute inflammation in the liver (Jain et al., 2011); this may explain increased AGP and Hp production in birds reared on Reused relative to Fresh litter. In addition, Reused litter resulted in increased expression of genes are linked to stress response and inflammation at d 12 (Chapter 4).These results indicate that an increase in bacterial exposure in the Reused litter treatment resulted in an increase acute phase response in broiler chickens.

3.3.2 Small Intestine Morphology

At d 12, there were no differences between vitamin D sources on small intestine morphology of broiler chickens; however, birds fed any diet containing $25OHD_3$ tended to have increased ($p< 0.084$) crypt depth in the ileum relative to D and Dx2 at the same day (Table 3.2). Birds reared on Reused litter had longer villus in all sections, greater villus width in the jejunum and ileum, and deeper crypt in the ileum and jejunum at d 12 as compared to birds on Fresh litter. Birds reared on the Fresh litter had greater VCR in the ileum relative to Reused litter at the same day (Table 3.2). At d 22, birds fed 25OHD tended to have increased ($p=0.057$) villus width in the ileum as compared to birds fed D or D+25OHD (Table 3.3). Deeper crypt depth in all three sections and lower VCR in the duodenum were observed for birds fed 25OHD relative to other dietary treatments (Table 3.3). Birds reared on Reused litter had longer jejunal villi, higher VCR in all three sections, and deeper duodenal crypts relative to birds reared on Fresh litter (Table 3.3). At d 41, birds fed 25OHD had greater villus width in the duodenum and deeper jejunal crypts relative to any diet containing vitamin D_3 alone (Table 3.4). Additionally, birds fed 25OHD tended to have increased ($P<0.09$) VSA in the duodenum in comparison to D and Dx2 (Table 3.4). Birds reared on Reused litter had longer jejunal villi and lower VSA in the duodenum and jejunum relative to birds grown on Fresh litter.

Several studies have demonstrated the role of nutrition on morphologic changes in small intestine of chickens (Samanya and Yamauchi, 2002; Shirpoor et al., 2006). Vitamin D has a regulatory role on intestinal function and morphology (Yu et al., 1998). Morphological and functional development of the intestinal villus mucosa is mediated by putrescine (Shinki et al., 1991). There are three main polyamines (putrescine, spermidine, and spermine) having roles in

cell proliferation and differentiation in the small intestine (Tabor and Tabor, 1984). Putrescine can be produced either by ornithine decarboxylase or spermidine N-acetyltransferase from ornithine and spermidine respectively. 1α,25-dihydroxycholecalciferol increases the activity of both enzymes, which increases putrescine production in the duodenum of broilers (Shinki et al., 1981 and 1985). Therefore, increase the serum level of 1,25-$(OH)_2D_3$ may promote putrescine production, leading to increased functional development of gut. Birds fed $25OHD_3$ relative to those fed vitamin D_3 had greater serum $25OHD_3$ concentration (Vignale., et al 2015; Yarger et al. 1995). However, an increased circulating level of $25OHD_3$ did not increase serum 1,25-$(OH)_2D_3$ level (Yarger et al. (1995). This might have been due to the close regulation of the conversion of $25OHD_3$ to 1,25-$(OH)_2D_3$ by serum Ca^{2+} level, parathyroid hormone, vitamin D receptor and concentration of 1,25-$(OH)_2D_3$ in the serum (De Matos, 2008).

Previous studies have indicated that longer villus length is associated with increased nutrient absorption due to an increase in intestinal surface area (Onderci et al., 2006; Perić et al., 2010). Villus length to crypt depth ratio is a good indicator to estimate digestive capacity of the small intestine in animals (Montagne, 2003). The increased crypt depth can indicate an accelerated enterocyte migration rate from the crypt to the villi, resulting greater nutrient absorption (Berlanga et al., 2001). In this experiment, $25OHD_3$ (2,760 IU/kg feed) increased duodenal and jejunal crypt depth, duodenal villus width, and tended to increase VSA, and decreased VCR relative to vitamin D_3 at same level of activity in birds at d 41. Contrary to this, Chou et al. (2009) reported that birds fed $25OHD_3$ at 2,760 IU/kg feed had longer villi and shallower crypts in the duodenum and jejunum and greater VCR, but, smaller villi in the ileum relative to birds fed vitamin D_3 at the same level. Similar to our study, deeper crypt depth was observed for birds fed $25OHD_3$ (Ding et al., 2011). These results indicate that dietary

supplementation of $25OHD_3$ could trigger nutrient absorption in the small intestine due to increased VCA and crypt depth. However, shallower crypts indicate a slower epithelial cell turnover, resulting in lower energy and protein demand for gut maintenance (Yason et al., 1987; Montagne, 2003). Hence, birds with shallower crypts could be more efficient, with lower gut maintenance requirements and higher rate of body weight gain. Deeper crypts in the $25OHD_3$-fed birds may indicate a higher turnover rate of intestinal epithelium, and consequently higher gut maintenance nutrient requirements, which in turn could have reduced BW at d 22, and breast muscle yield at processing (Chapter 2). It has been demonstrated in mammals and chickens that $25OHD_3$ is absorbed more rapidly from the proximal jejunum into the portal vein than vitamin D_3 (Bar et al., 1980; Sitrin et al., 1982). Ding et al. (2011) suggested the increased small intestine crypt depth might be due to increased absorption of $25OHD_3$. Therefore, supplementation of $25OHD_3$ alone relative to vitamin D_3 may result in increased gut maintenance energy requirements and increased potential nutrient absorption in broiler chickens.

No previous study has been conducted on the effects of reused litter on intestinal morphology; however, there is an increase in jejunal villus length (Chichlowski et al., 2007; Awad et al., 2009; Lee et al., 2010; Aliakbarpour et al 2012; Song et al., 2014), crypt depth (Lee et al., 2010), and integrity of the intestinal mucosal barrier of chicken fed probiotics (Aliakbarpour et al 2012). A greater presence of enteric pathogens in reused litter relative to fresh litter can change intestinal morphology. For example, *C. perfringens*-induced necrotic enteritis resulted in shorter villus heights and lower VCR relative to unchallenged broilers (Golder et al., 2011), and chickens challenged with *Salmonella* Typhimurium had lower villus height, VSA, VCR, and crypt depth. Therefore rearing chickens in reused litter may result in decreased morphological parameters of the small intestine by an increase in bacterial exposure.

In this experiment, Reused litter resulted in longer villi and deeper crypts in both post hatch and older birds; and increased VCR at d 22, but decreased VSA and VCR at d 12 and 41 in comparison to Fresh litter. Birds reared on Reused litter had reduced feed intake FI, BW and breast meat yield and increased small intestine weight at d 12; however, FI and gain to d 41tended to increase (0.077; 0.088 respectively) in birds reared on Reused litter relative to those grown on Fresh litter. However, there were no significant effects of litter treatment on broiler performance and breast meat yield at d 22 and d 41 (Chapter 2). Increased expression of AGP and pro-inflammatory response genes resulted in deeper crypt and shorter villus in broilers (Lee et al., 2010). Additionally, intestinal epithelial cell proliferation increased in response to increased inflammatory cytokines in mice (Inan et al., 2000). In this study, Reused litter increased production of Hp and AGP at d 41 and tended to increase (p=0.058) production of Hp at d 12 relative to Fresh litter in broiler chickens. These results showed that birds reared on Reused litter had more inflammation at d 12 and d 41 relative to birds in Fresh litter. The negative effects on small intestine morphology in post hatch and older broilers could be due to increased chronic systemic inflammation and increased expression of pro-inflammatory genes in post hatch and older broilers.

3.3.3 Bone characteristics

3.3.3.1 Bone Mineral Density

At d 12, birds fed D+25OHD had 10% higher total BMD relative to the other dietary treatments, and 3% higher cortical BMD in comparison to D and Dx2 at 30% of femur length. Additionally, birds fed 25OHD tended to have increased (by 7%) total BMD relative to birds fed any diet containing vitamin D_3 alone (Table 3.5). Total BMD at 30% was decreased by 8% in

birds reared on Reused relative to Fresh litter (Table 3.5). Birds fed D had 6.5% lower cortical BMD relative to other dietary treatments at 50% of femur length (Table 3.6). Trabecular BMD increased by 121% in birds reared on Fresh as compared to Reused litter at 50% of femur length (Table 3.6). Total BMD at 30% of femur length increased by 9.5% in birds fed D+25OHD relative to D and Dx2 in the Reused litter (Table 3.7). At d 22, birds fed D+25OHD had 8.5% higher total BMD relative to the other dietary treatments (Table 3.5). Total BMD at 50% of femur length decreased by 4% in birds reared on Reused as compared to Fresh litter (Table 3.6).

3.3.3.2 Cross Sectional Area

At d 12, birds reared on Reused litter had 4% lower total CSA at 30% of femur length relative to birds reared on Fresh litter (Table 3.8). At 30% of femur length Birds fed Dx2 had 8% higher total CSA relative to D and 25OHD in Fresh litter; and birds fed D+25OHD had 12% higher total CSA relative to any diet containing vitamin D_3 alone in the Reused litter (Table 3.7). Interestingly, birds fed D_3 had lower cortical CSA at 30% relative to those fed any diets containing $25OHD_3$ in the Reused litter (Table 3.7), the same pattern was observed for d 12 broiler performance (Chapter 2). Cortical CSA at 50% of femur length tended to be increased by around 8.5% ($P< 0.094$) in birds fed D+25OHD in comparison birds fed D. Additionally, birds reared on Fresh litter had 8% higher cortical CSA relative to birds reared on Reused litter (Table 3.9). There was a nearly significant ($P< 0.087$) interaction in trabecular CSA at the 50% scan location between diet and litter at d 12. Trabecular CSA tended to be increased (by 18%) in birds fed D+25OHD relative to Dx2 in the Fresh litter at the 50% scan location, but, trabecular CSA did not differ between dietary treatment in the Reused litter (Table 3.7). At d 22, trabecular CSA at 30% of femur length was reduced in birds fed D+25OHD relative to D and Dx2. Additionally,

birds reared on Fresh litter had higher trabecular CSA at each scan location relative to Reused litter (Tables 3.8 and 3.9).

3.3.3.3 Bone Mineral Content

At d 12, there was a significant interaction for total and cortical BMC at 30% of the length of femur between diet and litter (Table 3.7). Total BMC was increased by around 11% in birds fed D+25OHD or Dx2 relative to D and 25OHD in the Fresh litter. However, birds fed Dx2 had 14% lower total BMC as compared to birds fed any diet containing $25OHD_3$ on Reused litter. Cortical BMC increased by around 11.5% in birds fed Dx2 relative to D or 25OHD in the Fresh litter; and it increased by around 14.5% in birds fed D+25OHD relative to other dietary treatments in the Reused litter. Cortical BMC decreased by around 2% in birds fed D in comparison to other dietary treatments at the 50% scan location. Cortical BMC increased by 9% in birds reared on Fresh relative to Reused litter at the 50% scan location. Trabecular BMC increased by 89% in birds grown on Fresh as compared to Reused litter at the 50% femur length (Table 3.7). At d 22, birds reared on Fresh litter had 98% higher trabecular BMC relative to birds reared on Reused litter at 50% of femur length (Table 3.11). There was a nearly significant ($P< 0.082$) interaction in trabecular BMC between diet and litter at d 41. Birds fed 25OHD tended to have increased trabecular BMC relative to birds fed D in the Reused litter, but there were no differences in trabecular BMC between dietary treatments in Fresh litter.

The tibia is an indicator of overall skeletal growth (Jendral et al., 2008), whereas the femur may provide a stronger indicator of changes in bone mineral density than tibia (Melton et al., 2009). Applegate and Lilburn (2002) reported that the epiphyseal or proximal region of a long bone is linked to longitudinal bone growth with lower percentage of bone ash than the

diaphyseal region; however, diaphyseal or middle region is comprised of higher bone ash in broiler chickens. Therefore, comparison of ash content in these different regions of the bone could be an index for skeletal development differences throughout the life of the birds.

3.3.3.4 Bone Breaking Strength

Bone breaking strength (BBS) increased by 5.5% in birds reared on Fresh relative to Reused litter at d 12 (Table 3.12). At d 22, birds fed $25OHD_3$ tended to have increased BBS ($p=0.092$) by 7% in $25OHD_3$ fed birds relative to Dx2. At d 41, birds fed $25OHD_3$ tended to have increased ($p=0.083$) by 10% relative to D.

Quantitative computed tomography is a technique which provides a 3-dimensional calculation of volume of total, trabecular, and cortical BMD and CSA; and is correlated with percentage of ash content in chicken bones (Korver et al., 2004). No previous research has investigated effect of reused litter on bone mineralization and development in poultry; however, in chickens reared on reused litter, pathogenic bacteria such as the *Clostridiales* family are the predominat microbes (Lee et al., 2011; Cressman et al., 2010) and inflammatory response is increased (Shanmugasundaram et al., 2012). Injection of LPS increased production of pro-inflammatory cytokines in chickens (Nii et al., 2011); and cytokines such as IL-1 and IL-6 are associated with acute inflammation and increased bone resorption (Rath et al., 2000; Roux and Orcel, 2000). Mireles et al. (2005) reported decreased tibia weight and total Ca percentage, bone breaking strength, and distance to break in LPS injected birds and hypothesized that systematic inflammation may cause a loss of bone minerals from the extracellular matrix and reduce bone breaking strength and distance to break. Reused litter resulted in decreased bone breaking strength at d 12 and bone mineralization at d 12 an 22 in comparison to Fresh litter. Therefore,

the reason for this reduction in bone mineralization and development in birds reared on Reused litter might have been due to an increase in systemic inflammation.

In this study, $25OHD_3$ as a complete or partial replacement for vitamin D_3 tended to increased BBS at d 22 and 41, also we saw that the dietary combination of $25OHD_3$ and vitamin D_3 had a greater impact on bone mineralization than $25OHD_3$ alone for broilers at early ages, however greater increases in bone mineralization and breaking strength were observed with $25OHD_3$ alone in older broilers. Increased broiler bone quality has been reported when a combination of $25OHD_3$ and vitamin D_3 was fed, particularly during the first week post-hatch (Papešová et al., 2008). Supplementation of $25OHD_3$ as a complete or partial replacement for vitamin D_3 in broiler diets increased bone strength and quality by increased load and stiffness, bone ash, and bone calcification relative to vitamin D_3 at the same level of inclusion (Świątkiewicz and Koreleski, 2005; 2006; Gómez-Verduzco et al., 2013: Fritts and Waldroup, 2003;). Additionally, $25OHD_3$-injected *in ovo* to male broilers had increased bone breaking strength relative to non-injected birds at d 28 (Bello et al., 2014). However, bone mineral density and bone breaking strength did not differ at 41 d in broiler chickens when $25OHD_3$ was provided to the breeder hens at 1,380 IU/L water as compared to vitamin D_3 at 3,000 IU/kg feed (Saunders-Blades and Korver, 2014). In mammals, 1 α-hydroxylase, which converts $25OHD_3$ to 1,25-$(OH)_2D_3$ has been found in bone tissue (Andeson et al., 2005). Increased expression of 1 α-hydroxylase in bone tissue is correlated with bone mineralization, but not circulating 1,25-$(OH)_2D_3$ serum levels. However, the effect of dietary $25OHD_3$ on expression of 1 α-hydroxylase was not measured in the current study. Thus, the increased bone strength and mineralization due to feeding $25OHD_3$ as a complete or partial replacement for vitamin D_3 in current study could be due to greater bio-potency on a per unit basis than vitamin D_3 (Fritts and Waldroup, 2003), or

more rapid absorption rate in small intestine (Bar et al., 1980). Dietary supplementation of $25OHD_3$ increased villus and crypt morphology in this study, which may have increased nutrient absorption. Therefore, from a biological standpoint, the accumulated increased villus and crypt morphology in $25OHD_3$-fed birds may contribute to more nutrients being available for bone minimization as compared to those fed D.

In conclusion, supplementation of $25OHD_3$ as a complete or partial replacement for vitamin D_3 reduced inflammation in birds relative to vitamin D_3 alone. Additionally, $25OHD_3$ at 2,760 IU/kg feed relative to vitamin D_3 at the same level of activity increased potential nutrient absorption by increasing in VSA and villus width in older birds. Therefore, the reduction in inflammation in post-hatch and older birds and increased potential nutrient absorption in birds might have caused increased bone quality by an increase in bone mineralization and bone breaking strength in birds fed $25OHD_3$ at 2,760 IU/kg feed relative to those fed vitamin D_3 alone. However, $25OHD_3$-fed birds may have been less efficient due to increased crypt depth at each age, resulting in increase in gut maintenance energy requirements. This result may explain reduced breast meat yield at processing in 25OHD-fed birds relative to D-fed birds (Chapter 2). The increased crypt depth in this study could be due to increased absorption of $25OHD_3$ in upper of small intestine however we did not measure the rate of intestinal absorption $25OHD_3$ or vitamin D_3 in this study. Reused litter resulted in increased inflammation in broiler chickens relative to Fresh litter. Also, Reused litter relative to Fresh litter decreased villus and crypt morphology in post hatch birds. These results could have explained the decreased in bone mineralization and strength in post-hatch birds reared on Reused litter. Although inflammation still was higher in birds reared on Reused litter relative to Fresh litter, increased in nutrient absorption in older birds could have allowed them to overcome the negative effect on BBS at d

22 and 41. In this study, the effects of dietary supplementation of $25OHD_3$ were more apparent as increased bone mineralization and development, and reduction in inflammation than on changes in small intestine morphology in broiler chickens.

3.4 REFERENCES

Aliakbarpour, H. R., M. Chamani, G. Rahimi, A. A. Sadeghi, and D. Qujeq. 2012. The *bacillus subtilis* and lactic acid bacteria probiotics influences intestinal mucin gene expression, histomorphology and growth performance in broilers. Asian-Aust. J. Anim. Sci. 25:1285-1293.

Applegate, T. J., and M. S. Lilburn. 2002. Growth of the femur and tibia of a commercial broiler line. Poult. Sci. 81:1289-1294.

Asasi, K., A. Mohammadi, Z. Boroomand, S. A. Hosseinian, and S Nazifi. 2013. Changes of several acute phase factors in broiler chickens in response to infectious bronchitis virus infection. Poult. Sci. 92:1989-1996.

Atencio, A., G. M. Pesti, and H. M. Edwards. 2005. Twenty-five hydroxycholecalciferol as a cholecalciferol substitute in broiler breeder hen diets and its effect on the performance and general health of the progeny. Poult. Sci. 84:1277-1285.

Aviagen. 2009. Ross Broiler Management Manual. Aviagen Ltd., Newbridge, UK.

Awad, W. A., K. Chareeb, S. Abdel-Raheem and J. Bohm. 2009. Effects of dietary inclusion of probiotic and symbiotic on growth performance, organ weight and intestinal histomorphology of broiler chickens. Poult Sci. 88:49-56.

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.

Bello, A., P. Y. Hester, P. D. Gerard, W. Zhai, and E. D. Peebles. 2014. Effects of commercial in ovo injection of 25-hydroxycholecalciferol on bone development and mineralization in male and female broilers. Poult. Sci. 93:2734-2739.

Berlanga-Acosta, J., R. J. Playford, N. Mandir, R. A. Goodlad. 2001. Gastrointestinal cell proliferation and crypt fission are separate but complementary means of increasing tissue mass following infusion of epidermal growth factor in rats. Gut. 48:803-807.

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

Canadian Council on Animal Care. 2009. A guide to the care and the use of experimental animals. CCAC, Ottawa, ON, Canada.

Chamanza, R., L. Van Veen, M.T. Tivapasi and M.J. Toussaint. 1999. Acute phase proteins in the domestic fowls. World's Poult Sci J. 55:61-71.

Chichlowski, M., W. J. Croom, F. W. Edens, B. W. McBride, R. Qiu, C. C. Chiang, L. R. Daniel, G. B. Havenstein, and M. D. Koci. 2007. Microarchitecture and spatial relationship between bacteria and ileal, cecal, and colonic epithelium in chicks fed a direct-fed microbial, PrimaLac, and salinomycin. Poult. Sci. 86:1121-1132.

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.

Coufal, C. D., C. Chavez, P. R. Niemeyer, and J.B. Carey. 2006. Effects of top-dressing recycled broiler litter on litter production, litter characteristics, and nitrogen mass balance. Poult. Sci. 85:392-397.

Cressman, M. D., Z. Yu, M. C. Nelson, S. J. Moeller, M. S. Lilburn, and H. N. Zerby. 2010. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. Appl. Environ. Microbiol.76:6572-6582.

Crhanova, M., H. Hradecka, M. Faldynova, M. Matulova, H. Havlickova, F. Sisak, and I. Rychlik. 2011. Immune response of chicken gut to natural colonization by gut microflora and to Salmonella enterica serovar enteritidis infection. Infect. Immun. 79:2755-2763.

De Matos, R. 2008. Calcium metabolism in birds. Vet. Clin. North Am. Exot. Anim. Pract. 11:59-82.

Dibaji, S. M., A. Seidavi, L. Asadpour, and F. M. da Silva. 2014. Effect of a synbiotic on the intestinal microflora of chickens. J. Appl. Poult. Res. 23:1-6.

Ding. B. A., A. Pirone, C. Lenzi, A. Baglini, and I. Romboli. 2011. Effect of hen diet supplemented with 25-OH-D_3 on the development of small intestinal morphology of chick. J. Anim. Feed. Sci. 20: 420-431.

Driver, J. P., G. M. Pesti, R. I. Bakalli, and H. M. Jr. Edwards. 2005. Calcium requirements of the modern broiler chicken as influenced by dietary protein and age. Poult. Sci. 84:1629-1639.

Dusso, A. S., A. J. Brown, and E. Slatopolsky. 2005. Vitamin D. Am. J. Physiol. Renal Physiol. 289:8-28.

Fasina, Y. O., F. J. Hoerr, S. R. McKee, and Conner. D. E. 2010. Influence of *Salmonella enterica* serovar Typhimurium infection on intestinal goblet cells and villous morphology in broiler chicks. Avian. Dis. 54:841-847.

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.

Fritts, C. A., and P. W. Waldroup. 2005. Comparison of cholecalciferol and 25-hydroxycholecalciferol in broiler diets designed to minimize phosphorus excretion. J. Appl. Poult. Res. 14:156-166.

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

Gomaa, M. H., D. Yoo, D. Ojkic, and J. R. Barta. 2009. Infection with a pathogenic turkey coronavirus isolate negatively affects growth performance and intestinal morphology of young turkey poults in Canada. Avian. Pathol. 38:279-286

Gómez-Verduzco. G., R. Morales-López, and E. Avila-Gozàlez. 2013. Use of 25 hydroxycholecalciferol in diets of broiler chickens: effects on growth performance, immunity and bone calcification J. Poult. Sci. 50: 60-64.

Hystad, M. E., J. H. Myklebust, T. H. Bo, E. A. Sivertsen, E. Rian, L. Forfang, E. Munthe, A. Rosenwald, M. Chiorazzi, I. Jonassen, L. M. Staudt, and E. B. Smeland. 2007. Characterization of early stages of human B cell development by gene expression profiling. J. Immunol. 179:3662–3671.

Inan, M. .S., V. Tolmacheva, Q. S. Wang, D. W. Rosenberg, and C. Giardina, 2000. Transcription factor NF-kappaB participates in regulation of epithelial cell turnover in the colon. Am. J. Physiol. Gastrointest. Liver Physiol. 279, G1282–G1291.

Jain, S., V. Gautam, and S. Naseem. 2011. Acute phase proteins: as diagnostic tool. J. Pharm. Bioallied. Sci. 3:118-127.

Jendral, M. J., D. R. Korver, J. S. Church, and J. J. R. Feddes. 2008. Bone mineral density and breaking strength of white leghorns housed in conventional, modified, and commercially available colony battery cages. Poult. Sci. 87:828–837.

Juul-Madsen, H. R., M. Munch, K. J. Handberg, P. Sørensen, A. A. Johnson, L. R. Norup, and P. H. Jørgensen. 2003. Serum levels of mannan-binding lectin (MBL) in chickens prior to and during experimental infection with avian infectious bronchitis virus (IBV). Poult. Sci. 82:235-241.

Kasim, S., B. L. Blake, and X. Fan. 2006. The role of dopamine receptors in the neurobehavioral syndrome provoked by activation of L-type calcium channels in rodents. Dev. Neurosci. 28:5055-17.

Kefal. S, and N. Y. Toker, 2006. Effects of probiotics on some acute phase proteins in broilers exposed to *Salmonella* Typhimurium lipopolysaccharides. Arch. Geflügelk. 70:270-277

Kettunen, H., K. Tiihonena, S. Peuranena, M. T. Saarinena, and J. C. Remusb. 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. Part A Mol. Integr. Physiol. 130:759–769.

Kokosharov, T. 2006. Changes in the protein profile in birds with experimental acute fowl typhoid. Bulg. J. Vet. Med. 9:189–192.

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. Poultry Science 77:1217-1227.

Korver, D. R., J. L. Saunders-Blades, and K. L. Nadeau. 2004. Assessing bone mineral density in vivo: quantitative computed tomography. Pult. Sci. 83:222-229.

Koutsos, E. A., and K. C. Klasing. 2001. The acute phase response in Japanese quail (*coturnix coturnix japonica*). Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 128:255-263.

Lee, K. W., S. H. Lee, H. S. Lillehoj, G. X. Li, S. I. Jang, U. S. Babu, M. S. Park, D. K. Kim, E. P. Lillehoj, A. P. Neumann, T. G. Rehberger, and G. R. Siragusa. 2010. Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. Poult. Sci. 89:203-216.

Lee, K. W., H. S. Lillehoj, S. H. Lee, S. I. Jang, G. D. Ritter, D. A. Bautista, and E. P. Lillehoj. 2011. Impact of fresh or used litter on the posthatch immune system of commercial broilers. Avian. Dis. 55:539-544.

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

Lu, J., S. Sanchez., C. Hofacre, J. J. Maurer, B. G. Harmon, and M. D. Lee. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. Appl. Environ. Microbiol. 69:901-908.

Melton, L. J., B. S. Coan, and S. C. Lee. 1999. Prediction of the strength of the elderly proximal femur by bone mineral density and quantitative ultrasound measurements of the heel and tibia. Bone. 24:49-55.

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. 84:553-60.

Montagne, L., J. R. Pluske, and D. J. Hampson. 2003. A review of interactions between dietary fiber and the intestinal mucosa, and their consequences on digestive health in young nonruminant animals. Anim. Feed. Sci. Technol. 108:95–117.

Morris, A., R. Shanmugasundaram, M. S. Lilburn, and R. K. Selvaraj. 2014. 25-Hydroxycholecalciferol supplementation improves growth performance and decreases inflammation during an experimental lipopolysaccharide injection. Poult. Sci. 93:1951-1956.

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.

Nazifi, S., A. Rezakhani, M. Koohimoghadam, M. Ansari-Lari, and Z. Esmailnezhad. 2008. Valuation of serum haptoglobin in clinically healthy cattle and cattle with inflammatory diseases in Shiraz, a tropical area in southern Iran. Bul. J. Vet. Med. 11:95-101.

Ng, S. C, A. L. Hart, M. A. Kamm, A. J. Stagg, and S. C. Knight. 2009. Mechanisms of action of probiotics: recent advances. Inflamm. Bowel. Dis. 15:300-310.

Nielsen, O. L., J. Jensenius, P. H. Jørgensen, and S. B. Laursen. 1999. Serum levels of chicken mannan-binding lectin (MBL) during virus infections: Indication that chicken MBL is an acute phase reactant. Vet. Immunol. Immunopathol. 70:309-316.

Nii, T.; Sonoda, Y, N. Isobe, and Y. Yoshimura. 2011. Effects of lipopolysaccharide on the expression of proinflammatory cytokines and chemokines and the subsequent recruitment of immunocompetent cells in the oviduct of laying and molting hens. Poult. Sci. 90:2332-2341.

North, C. M., R. B. Crawford, H. Lu, and N. E Kaminski. 2009. Simultaneous in vivo time course and dose response evaluation for TCDD-induced impairment of the LPS-stimulated primary IgM response. Toxicol. Sci. 112:123-132.

Nurmi, E., and M. Rantala. 1973. New aspects of *Salmonella* infection in broiler production. Nature. 241:210-211.

Onderci, M., N. Sahin, K. Sahin, G. Cikim, A. Aydìn, I. Ozercan, and S. Aydìn. 2006. Efficacy of supplementation of α-amylase producing bacterial culture on the performance, nutrient use, and gut morphology of broiler chickens fed a corn-based diet. Poult. Sci. 85:505–510.

Papešová, L., A. Fucikova, M. Pipalova, and P. Tupy. 2008. The synergic effect of vitamin D_3 and 25-hydroxycholecalciferol/calcidiol in broiler diet. Sci. Agric. Bohemica 39:273-277.

Parkinson, G. B., and P. H. Cransberg. 2004. Effect of casein phosphopeptide and 25-hydroxycholecalciferol on tibial dyschondroplasia in growing broiler chickens. Br. Poult. Sci. 45:802:806.

Perić, L., N. Milosevic, D. Zikic, S. Bjecov, D. Cvetkovic, S. Markov, M. Mohnl, and T. Steiner. 2010. Effects of probiotic and phytogenic products on performance, gut morphology and cecal microflora of broiler chickens. Arch. Anim. Breed. 53:350-359.

Phadnis, R., and I. Nemere, I. 2003. Direct, rapid effects of 25-hydroxyvitamin D_3 on isolated intestinal cells. J. Cell. Biochem. 90:287-293.

Raman, K., H. Wang, M. J. Troncone, W. I Khan, G Pare, and J. Terry. 2015. Overlap chronic placental inflammation is associated with a unique gene expression pattern. PLoS One. 10:e0133738-e0133750.

Rath, N. C., G. R. Huff, W. E. Huff, and J. M. Balog. 2000. Factors regulating bone maturity and strength in poultry. Poult. Sci. 79:1029-1032.

Rennie, J. S., and C. C. Whitehead. 1996. Effectiveness of dietary 25 and 1 hydroxycholecalciferol in combating tibial dyschondroplasia in broiler chickens. Br. Poult. Sci. 37:413-421.

Roux, S., and P. Orcel. 2000. Bone loss: factors that regulate osteoclast differentiation: an update. Arthritis. Res. 2:451-456.

Samanya M., Yamauchi K., 2002. Histological alterations of intestinal villi in chickens fed dried *Bacillus subtilis var. natto*. Comp. Biochem. Physiol. 133:95-104.

Saunders-Blades, J. L., J. L. MacIsaac, D. R. Korver, and D. M. Anderson. 2009. The effect of calcium source and particle size on the production performance and bone quality of laying hens. Poult. Sci. 88:338–353.

Saunders-Blades, J., and, D. R. Korver. 2014. The effect of maternal vitamin D source on broiler hatching egg quality, hatchability, and progeny bone mineral density and performance. Poult Sci. 23:773-783.

Savage, D. C., J. E. Siegel, J. E. Snellen, and D. D. Whitt. 1981. Transit time of epithelial cells in the small intestines of germfree mice and ex-germfree mice associated with indigenous microorganisms. Appl. Environ. Microbiol. 42:996-1001.

Selvaraj, R. K., R. Shanmugasundaram, and R. Rengasamy. 2016. The use and abuse of immune indices in nutritional immunology studies. J. Appl. Poult. Res. 00:1-8.

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

Shanmugasundaram, R., M. S. Lilburn, and R. K. Selvaraj. 2012. Effect of recycled litter on immune cells in the cecal tonsils of chickens. Poult. Sci. 91:95-100.

Shinki, T., N. Takahashi, C. Miyaura, K. Samejima, Y. Nishii, and T. Suda. 1981. Ornithine decarboxylase activity in chick duodenum induced by 1α,25-dihydroxycholecalciferol. Biochem. J. 195:685-690.

Shinki, T., N. Takahashi, T. Kadofuku, T. Sato, and T. Suda. 1985. Induction of spermidine N1-acetyltransferase by 1α, 25-dihydroxyvitamin D_3 as an early common event in the target tissues of vitamin D. J. Biol. Chem. 260:2185-2190.

Shinki, T., H. Tanaka, J. Takito, A. Yamaguchi, Y. Nakamura, S. Yoshiki, and T. Suda. 1991. Putrescine is involved in the vitamin D action in chick intestine. Gastroenterology. 100:113-122.

Shirpoor, A., B. Ilkhanizadeh, R. Saadatian, B. S. Darvari, F. Behtaj, M. Karimipour, F. Ghaderi-Pakdel, and E. Saboori. 2006. Effect of vitamin E on diabetes-induced changes in small intestine and plasma antioxidant capacity in rat. J. Physiol. Biochem. 62:171-177.

Shojadoost, B., S. Behboudi, A. I. Villanueva, J. T. Brisbin, A. A. Ashkar, S. Sharif. 2015. Vitamin D_3 modulates the function of chicken macrophages. Res. Vet. Sci.100:45-51.

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.

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

Song, J., K. Xiao, Y. L. Ke , L. F. Jiao, C. H. Hu , Q. Y. Diao, B. Shi, and X. T. Zou. 2014. Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. Poult. Sci. 93:581–588.

Sitrin, M. D., K. L. Pllack, M. J. Bolt, and I. H. Roseberg. 1982. Comparison of vitamin D and 25-hydroxyvitamin D absorption in the rat. Amer. J. Physiol. 242:326-332.

Świątkiewicz, S, and J. Koreleski. 2005. The effect of particulate limestone and 25-hydroxy-cholecalciferol in broiler chicken diets on mechanical properties of bones. J. Anim. Feed Sci. 14:495-498.

Świątkiewicz, S., J. Koreleski, and J. Kopowski. 2006. Effect of phytase and 25-hydroxycholecalciferol on the performance and bone quality in broiler chickens. Med. Weter. 62:81-84.

Tabor, C. W., and H. Tabor. 1984. Polyamines. Annu. Rev. Biochem. 53:749–790.

Taira, I., T. Fujinaga, and M. Okumura. 1992. Equine haptoglobin isolation, characterization and the effects of ageing, delivery, inflammation on the serum concentration. J. Vet. Med. Sci. 54:435-442.

Torok, V. A., R. J. Hughes, K. Ophel-Keller, M. Ali, and R. MacAlpine. 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. Poult. Sci. 88:2474–2481.

Vignale, K., E. S. Greene, J. V. Caldas, J. England, N. Boonsinchai, P. Sodsee, E. D. Pollock, S. Dridi, and C. N. Coon. 2015. 25-Hydroxycholecalciferol enhances male broiler breast meat yield through the mTOR pathway. J. Nutr. 145:855-863.

Xu, S., X. Liu, Y. Bao, X. Zhu, C. Han, P. Zhang, X. Zhang, W. Li, and X. Cao. 2012. Constitutive MHC class I molecules negatively regulate TLR-triggered inflammatory responses via the Fps-SHP-2 pathway. Nat. Immunol. 13:551-559.

Yang, Y., P. A. Iji, A. Kocher, L. L. Mikkelsen, and M. Choct. 2008. Effects of dietary mannanoligosaccharide on growth performance, nutrient digestibility, and gut development of broilers given different cereal-based diets. J. Anim. Physiol. Anim. Nutr. (Berl.) 92:650–659.

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.

Yason, C. V., B. A. Summers, and K. A. Schat. 1987. Pathogenesis of rotavirus infection in various age-groups of chickens and turkeys-Pathology. Am. J. Vet. Res. 48:927–938.

Yu, B., C.C. Tsai, J.C. Hsu, and P.W.S. Chiou. 1998. Effect of different sources of dietary fiber on growth performance, the intestinal morphology and caecal carbohydrases of the domestic geese. Br. Poultry Sci. 39:560-567.

3.5 TABLES

Table 3.1 Effect of vitamin D source and Fresh or Reused litter on plasma nitric oxide, haptoglobin and α-1 acid glycoprotein concentrations of broiler chickens

		AGP [1]			**Hp[2]**			**NO[3]**			**Rectal temperature**		
		d 12	d 22	d 41	d 12	d 22	d 41	d 12	d 22	d 41	d 12	d 22	d 41
Dietary treatment	n[4]		μM			g/L			μg/mL			c°	
D[5]	8	138	159	295[ab]	0.323	0.386	0.369	27.08	18.41	16.5	41.0	41.2	41.4
25OHD[6]	8	155	147	251[c]	0.226	0.419	0.325	26.26	20.95	15.8	41.0	41.2	41.5
D+25OHD[7]	8	135	159	272[bc]	0.286	0.359	0.283	25.78	23.77	12.2	41.0	41.3	41.4
Dx2[8]	8	138	164	305[a]	0.333	0.422	0.265	24.79	21.19	17.2	41.0	41.2	41.5
Litter													
Fresh[9]	16	147	169[a]	255[b]	0.261	0.373	0.260[b]	25.15	22.10	16.3	41.1	41.2	41.7
Reused[10]	16	136	145[b]	306[a]	0.323	0.419	0.360[a]	26.80	20.06	16.1	41.0	41.2	41.5
Pooled SEM		9.8	8.9	14.8	0.0323	0.0430	0.0445	9.68	1.66	1.66	1.66	0.10	0.06
Source of variance	DF						p-value						
Diet	3	0.160	0.289	0.002	0.089	0.690	0.357	0.927	0.419	0.592	0.954	0.625	0.282
Litter	1	0.104	0.001	<0.001	0.058	0.280	0.031	0.824	0.368	0.822	0.171	0.759	0.920
Diet*Litter	3	0.840	0.346	0.698	0.531	0.435	0.692	0.121	0.300	0.282	0.490	0.415	0.363

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

[1] Alpha 1 acid glycoprotein concentration.

[2] Haptoglobin concentration.

[3] Nitric oxide concentration.

[4] Pen was the experimental unit.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed a diet containing 2,760 IU/kg complete feed of 25-OH- D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[7] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of 25-OH- D_3 from 0 to 41 d.

[8] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.
[9] Fresh wood shavings litter.
[10] Reused wood shavings litter; litter was used by one previous flock.

Table 3.2 Effect of vitamin D source and Fresh or Reused litter on small intestine morphology of broiler chickens at d 12

		Duodenum					Jejunum					Ileum				
		Crypt depth	Villus length	Villus width	VSA[2]	VCR[3]	Crypt depth	Villus length	Villus width	VSA	VCR	Crypt depth	Villus length	Villus width	VSA	VCR
Dietary treatment	n[1]		µm		mm^2	Length /Depth		µm		mm^2	Length /Depth		µm		mm^2	Length /Depth
D[4]	8	208	1770	133	0.23	8.84	144	1032	118	0.37	7.55	119	701	110	0.49	6.37
25OHD[5]	8	221	1784	139	0.25	8.25	188	1075	131	0.38	6.11	131	711	117	0.52	5.57
D+25OHD[6]	8	225	1885	132	0.22	8.64	165	999	114	0.37	6.24	122	686	112	0.52	5.70
Dx2[7]	8	254	1818	127	0.22	7.52	154	993	127	0.41	6.73	116	678	116	0.55	6.17
Litter																
Fresh[8]	16	218	1735^b	129	0.24	8.21	138^b	976	113^b	0.37	7.38	102^b	649^b	107^b	0.52	6.59^a
Reused[9]	16	236	1893^a	136	0.23	8.41	188^a	107	132^a	0.40	5.93	141^a	738^a	121^a	0.52	5.31^b
Pooled SEM		16.2	54.3	8.8	0.016	0.672	15.9	75.3	9.2	0.290	0.575	6.4	24.3	5.6	0.033	0.420
Source of variance	DF	P-value														
Diet	3	0.262	0.459	0.816	0.681	0.534	0.273	0.859	0.549	0.724	0.303	0.418	0.770	0.771	0.736	0.506
Litter	1	0.279	0.008	0.499	0.568	0.761	0.004	0.209	0.046	0.421	0.019	<0.001	0.001	0.019	0.978	0.006
Diet*Litter	3	0.633	0.155	0.644	0.466	0.412	0.774	0.496	0.135	0.493	0.534	0.084	0.691	0.752	0.938	0.342

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

[1] Pen was the experimental unit.

[2] Villus surface area (VSA) calculated with average villus length and width = $2\pi \times (\text{width}/2) \times \text{length}$.

[3] Ratio of villus length to crypt depth.

[4] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[7] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.
[8] Fresh pine shavings litter.
[9] Reused pine shavings litter; litter was used by one previous flock.

Table 3.3 Effect of vitamin D source and Fresh or Reused litter on small intestine morphology of broiler chickens at d 22

		Duodenum					Jejunum					Ileum				
		Crypt depth	Villus length	Villus width	VSA[2]	VCR[3]	Crypt depth	Villus length	Villus width	VSA	VCR	Crypt depth	Villus length	Villus width	VSA	VCR
Dietary treatment	n[1]		µm		mm^2	Length /Depth		µm		mm^2	Length /Depth		µm		mm^2	Length /Depth
D[4]	8	148^{c}	1,827	162^{b}	0.21	13.8^{a}	129^{b}	1,335	137	0.34	10.4	115^{b}	821	125	0.49	7.18
25OHD[5]	8	226^{a}	1,760	204^{a}	0.25	9.8^{b}	174^{a}	1,334	161	0.38	7.8	154^{a}	895	150	0.54	6.00
D+25OHD[6]	8	189^{b}	1,870	178^{ab}	0.24	12.2^{ab}	147^{b}	1,327	154	0.37	9.5	124^{b}	805	115	0.46	6.60
Dx2[7]	8	165^{bc}	1,744	155^{b}	0.23	12.9^{a}	136^{b}	1,268	139	0.35	9.4	121^{b}	798	127	0.50	6.81
Litter																
Fresh[8]	16	202^{a}	1,739	183	0.24	10.9^{a}	150	1,235^{b}	144	0.37	8.5^{b}	141^{a}	809	126	0.49	6.14^{b}
Reused[9]	16	163^{b}	1,860	167	0.23	13.5^{b}	143	1,397^{a}	152	0.35	10.1^{a}	85^{b}	850	133	0.51	7.17^{a}
Pooled SEM		12.0	67.8	11.8	0.018	0.87	9.2	73.0	13.4	0.038	0.66	6.7	45.2	8.9	0.042	0.457
Source of variance	DF	P-value														
Diet	3	0.001	0.809	0.025	0.542	0.023	0.010	0.901	0.521	0.787	0.067	0.002	0.416	0.057	0.604	0.335
Litter	1	0.004	0.252	0.162	0.608	0.007	0.427	0.036	0.554	0.601	0.027	0.041	0.367	0.433	0.851	0.034
Diet*Litter	3	0.202	0.700	0.679	0.270	0.586	0.634	0.464	0.631	0.655	0.444	0.080	0.282	0.564	0.694	0.290

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

[1] Pen was the experimental unit.

[2] Villus surface area (VSA) calculated with average villus length and width = $2\pi \times (\text{width}/2) \times \text{length}$.

[3] Ratio of villus length to crypt depth.

[4] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[7] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[8] Fresh pine shavings litter.

[9] Reused pine shavings litter; litter was used by one previous flock.

Table 3.4 Effect of vitamin D source and Fresh or Reused litter on small intestine morphology of broiler chickens at d 41.

		Duodenum					**Jejunum**					**Ileum**				
		Crypt depth	Villus length	Villus width	VSA[2]	VCR[3]	Crypt depth	Villus length	Villus width	VSA	VCR	Crypt depth	Villus length Villus width		VSA	VCR
Dietary treatment	n[1]		µm		mm^2	Length /Depth		µm		mm^2	Length /Depth		µm		mm^2	Length /Depth
D[4]	8	166	2,007	162^{b}	0.29	11.04	110^{b}	1,333	125	0.31	12.83	110	818	123	0.47	7.65
25OHD[5]	8	204	2,154	204^{a}	0.37	8.68	163^{a}	1,497	155	0.34	9.19	134	909	136	0.47	6.95
D+25OHD[6]	8	183	2,155	178^{ab}	0.30	10.71	136^{ab}	1,301	139	0.35	9.71	136	812	115	0.45	6.20
Dx2[7]	8	170	2,075	155^{b}	0.29	9.97	131^{ab}	1,426	135	0.30	11.53	114	767	133	0.56	6.74
Litter																
Fresh[8]	16	174	2,131	183	0.34^{a}	10.27	126	1,256^{b}	137	0.35^{a}	10.23	125	850	131	0.49	7.02
Reused[9]	16	188	1,970	167	0.29^{b}	9.93	144	1,522^{a}	140	0.29^{b}	11.41	122	804	123	0.48	6.75
Pooled SEM		11.4	103.4	11.3	0.025	1.04	11.4	96.3	10.7	0.025	1.15	9.01	51.0	9.5	0.036	0.533
Source of variance	DF	P-value														
Diet	3	0.100	0.367	0.025	0.090	0.192	0.027	0.473	0.271	0.534	0.121	0.118	0.275	0.379	0.160	0.307
Litter	1	0.232	0.330	0.162	0.046	0.680	0.143	0.011	0.762	0.021	0.311	0.717	0.377	0.413	0.726	0.629
Diet*Litter	3	0.503	0.957	0.680	0.836	0.373	0.316	0.222	0.500	0.486	0.551	0.742	0.267	0.236	0.525	0.911

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

[1] Pen was the experimental unit.

[2] Villus surface area (VSA) calculated with average villus length and width = $2\pi \times (\text{width}/2) \times \text{length}$.

[3] Ratio of villus length to crypt depth.

[4] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of 25-OH- D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of 25-OH- D_3 from 0 to 41 d.

[7] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[8] Fresh wood shavings litter.

[9] Reused wood shavings litter; litter was used by one previous flock.

Table 3.5 Effect of vitamin D source and Fresh or Reused litter on femur bone mineral density as measured by quantitative computed tomography at 30% of femur length from the proximal epiphysis at 12, 22, and 41 d. Dependent variable adjusted to a common BW with analysis of covariance.

		Total[1]			Cortical[2]			Trabecular[3]		
	n[4]	12 d	22 d	41 d	12 d	22 d	41 d	12 d	22 d	41 d
Dietary treatment						mg/cm^3				
D[5]	8	338^{b}	356^{b}	346	685^{c}	753	790	38.7	43.2	67.5
25OHD[6]	8	340^{b}	370^{b}	373	701^{ab}	746	796	19.1	58.1	75.4
D+25OHD[7]	8	372^{a}	390^{a}	353	716^{a}	763	796	44.1	47.5	65.7
Dx2[8]	8	337^{b}	351^{b}	348	698^{bc}	748	793	29.4	38.8	67.2
Litter										
Fresh[9]	16	361^{a}	361	352	702	756	798	41.0	45.8	66.0
Reused[10]	16	332^{b}	372	358	698	749	790	23.3	48.0	72.0
Pooled SEM		7.9	10.2	8.5	5.3	6.1	5.1	8.97	6.98	4.18
Source of variance	DF					P-value				
Diet	3	0.005	0.047	0.062	0.002	0.169	0.831	0.130	0.300	0.356
Litter	1	0.001	0.289	0.403	0.409	0.222	0.116	0.050	0.766	0.152
Diet*Litter	3	0.183	0.604	0.610	0.162	0.918	0.739	0.367	0.547	0.408
Covariate BW	1	0.002	0.015	0.032	0.689	0.767	0.001	0.109	0.120	0.056

[a-c] Treatment means within the same column within effect with no common superscripts are significantly different ($P < 0.05$).
[1]Total = Comprised of cortical and trabecular bone tissue in a 1 mm thick cross-sectional scan.
[2]Cortical = outer shell of the femur with a mineral density >500 mg/cm^3.
[3]Trabecular = measurements taken in the inner part of the bone in the trabecular space.
[4] Pen was the experimental unit.
[5]Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.
[6]Birds fed a diet containing 2,760 IU/kg complete feed of 25OHD_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.
[7]Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of 25OHD_3 from 0 to 41 d.
[8]Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.
[9]Fresh wood shavings litter
[10]Reused wood shavings litter; litter was used by one previous flock

Table 3.6 Effect of vitamin D source and Fresh or Reused litter on femur bone mineral density as measured by quantitative computed tomography at 50% of femur length from the proximal epiphysis at 12, 22, and 41 d. Dependent variable adjusted to a common BW with analysis of covariance.

		Total[1]			Cortical[2]			Trabecular[3]		
	n[4]	12 d	22 d	41 d	12 d	22 d	41 d	12 d	22 d	41 d
Dietary treatment		mg/cm^3								
D[5]	8	543[b]	552	453	774[b]	813	886	60.4	32.5	46.8
25OHD[6]	8	548[b]	567	479	793[a]	812	897	32.4	27.1	56.2
D+25OHD[7]	8	566[a]	578	477	801[a]	831	894	35.2	23.0	54.7
Dx2[8]	8	553[ab]	551	464	794[a]	809	895	39.2	20.4	50.4
Litter										
Fresh[9]	16	567[a]	551[b]	471	793	819	891	57.6[a]	31.6	50.5
Reused[10]	16	538[b]	572[a]	466	788	814	895	26.0[b]	48.3	53.6
Pooled SEM		7.5	11.0	7.9	6.3	8.5	6.5	11.04	7.18	5.35
Source of variance	DF	P-value								
Diet	3	0.162	0.183	0.092	0.022	0.143	0.539	0.261	0.662	0.596
Litter	1	0.001	0.045	0.539	0.393	0.525	0.549	0.004	0.130	0.564
Diet*Litter	3	0.034	0.781	0.678	0.109	0.110	0.900	0.854	0.697	0.110
Covariate BW	1	0.049	0.009	0.036	0.918	0.690	<0.001	0.464	0.600	0.113

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

[1] Total = Comprised of cortical and trabecular bone tissue in a 1 mm thick cross-sectional scan.

[2] Cortical = outer shell of the femur with a mineral density >500 mg/cm^3.

[3] Trabecular = measurements taken in the inner part of the bone in the trabecular space.

[4] Pen was the experimental unit.

[5] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed a diet containing 2,760 IU/kg complete feed of 25OHD_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[7] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of 25OHD_3 from 0 to 41 d.

[8] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[9] Fresh wood shavings litter.

[10] Reused wood shavings litter; litter was used by one previous flock

Table 3.7Significant and nearly significant interactions between diets and litter for femur bone mineral density, cross sectional area and bone mineral content as measured by quantitative computed tomography at 30% and 50% of femur length from the proximal epiphysis at 12, 22, and 41d of broilers. Dependent variables adjusted to a common BW with analysis of covariance.

		At 30%			At 50%		
		CSA[2] (mm^2)	BMC[3] (mg/cm)	BMC (mg/cm)	BMD[1] (mg/cm^3)	CSA (mm^2)	BMC (mg/cm)
		Cortical[4]	Total[5]	Trabecular[6]	Total	Trabecular	Trabecular
		d12					d41
Treatment							
Fresh[7]							
	D[9]	6.61[b]	55.27[b]	44.93[cd]	5.61[ab]	2.84	18.67
	25OHD[10]	6.65[b]	54.41[b]	46.95[bc]	5.62[ab]	2.95	15.87
	D+25OHD[11]	7.01[ab]	61.92[a]	49.85[ab]	5.63[ab]	3.14	23.9
	Dx2[12]	7.23[a]	61.03[a]	51.23[a]	5.81[a]	2.78	21.48
Reused[8]							
	D	5.97[c]	51.52[bc]	41.04[e]	5.26[c]	3.03	18.98
	25OHD	6.15[bc]	52.04[b]	42.87[de]	5.35[bc]	3.16	27.52
	D+25OHD	6.56[b]	54.80[b]	47.23[bc]	5.68[a]	2.92	19.83
	Dx2	5.72[c]	46.69[c]	39.56[e]	5.24[c]	3.15	20.56
	Pooled SEM	0.176	2.038	1.273	0.104	0.119	2.249
	Interaction P-value	0.008	0.016	0.002	0.034	0.087	0.082

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

[1] Bone mineral density

[2] Cross sectional area

[3] Bone mineral content

[4]Cortical = Outer shell of the femur with a density >500 mg/cm3.

[5]Total = All bone mineralization in 1 inch cross-sectional scan.

[6]Trabecular = Measurements taken in the inner part of the bone in the trabecular space.

[7] Fresh wood shavings litter.

[8] Reused wood shaving litter; litter was used one previous flock.

[9]Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[10] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.
[11] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.
[12] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

Table 3.8 Effect of treatments on femur cross-sectional area as measured by quantitative computed tomography at 30% of femur length from the proximal epiphysis at 12, 22, and 41d. Dependent variable adjusted to a common BW with analysis of variance

		Total[1]			Cortical[2]			Trabecular[3]		
	n[4]	12d	22d	41d	12d	22d	41d	12d	22d	41d
Dietary treatment		mm^2								
D[5]	8	15.77	39.05	93.60	6.29^{b}	15.48	32.72	7.58	20.28^{a}	54.39
25OHD[6]	8	15.61	37.85	91.26	6.37^{a}	15.25	34.28	7.54	19.21^{ab}	50.37
D+25OHD[7]	8	15.70	37.30	92.19	6.79^{ab}	16.00	33.26	7.28	18.01^{b}	52.78
Dx2[8]	8	15.82	39.60	95.51	6.48^{b}	15.82	33.69	7.66	20.57^{a}	55.34
Litter										
Fresh[9]	16	16.05^{a}	39.13	92.95	6.88^{a}	15.55	33.12	7.41	20.31^{a}	53.62
Reused[10]	16	15.40^{b}	37.77	93.32	6.10^{b}	15.72	33.85	7.62	18.72^{b}	52.82
Pooled SEM		7.938	0.678	1.608	0.126	0.551	0.841	0.195	0.626	1.634
		P-value								
Source of variance	DF									
Diet	3	0.960	0.084	0.272	0.033	0.633	0.6034	0.519	0.024	0.124
Litter	1	0.035	0.055	0.820	***	0.686	0.3875	0.306	0.015	0.605
Diet*Litter	3	0.234	0.455	0.351	*	0.783	0.5483	0.630	0.296	0.190
Covariate BW	1	***	***	***	***	***	***	0.004	0.001	0.023

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

[1]Total =For the entire bone.

[2]Cortical = outer shell of the femur with a mineral density >500 mg/cm3.

[3]Trabecular = measurements taken in the inner part of the bone in the trabecular space.

[4] Pen was the experimental unit.

[5]Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[7]Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[8]Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[9]Fresh wood shavings litter

[10]Reused wood shaving litter; litter was used one previous flock

Probabilities of interaction: *$P < 0.05$; **$P < 0.1$; ***$P < 0.0001$.

Table 3.9 Effect of treatments on femur cross-sectional area as measured by quantitative computed tomography at 50% of femur length from the proximal epiphysis at 12, 22, and 41d. Dependent variable adjusted to a common BW with analysis of variance

		Total[1]			Cortical[2]			Trabecular[3]		
	n[4]	12d	22d	41d	12d	22d	41d	12d	22d	41d
Dietary treatment		mm^2								
D[5]	8	11.81	33.85	84.5298	7.27	21.34	39.36	2.94	9.67	41.08
25OHD[6]	8	12.12	32.71	82.14	7.54	21.24	39.39	3.05	8.75	38.54
D+25OHD[7]	8	12.27	32.68	82.61	7.86	21.16	39.73	3.03	8.81	39.05
Dx2[8]	8	11.97	34.27	84.50	7.54	21.86	39.61	2.97	9.68	41.03
Litter										
Fresh[9]	16	12.28	33.86	83.22	7.86[a]	21.15	39.76	2.93	10.00[a]	39.46
Reused[10]	16	11.81	32.90	83.67	7.24[b]	21.65	39.29	3.06	8.46[b]	40.38
Pooled SEM		0.264	0.711	1.60	0.167	0.488	0.884	0.085	0.381	1.210
Source of variance	DF	P-value								
Diet	3	0.625	0.332	0.611	0.094	0.757	0.989	0.733	0.184	0.226
Litter	1	0.095	0.196	0.775	0.001	0.331	0.598	0.131	0.001	0.403
Diet*Litter	3	0.933	0.292	0.202	0.537	0.742	0.476	**	0.638	0.129
Covariate BW	1	***	***	***	***	***	***	0.003	0.016	***

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

[1]Total =For the entire bone.

[2]Cortical = outer shell of the femur with a mineral density >500 mg/cm3.

[3]Trabecular = measurements taken in the inner part of the bone in the trabecular space.

[4] Pen was the experimental unit.

[5]Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6] Birds fed a diet containing 2,760 IU/kg complete feed of 25OHD$_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[7]Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of 25OHD$_3$ from 0 to 41 d.

[8]Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[9]Fresh wood shavings litter

[10]Reused wood shaving litter; litter was used one previous flock

Probabilities of interaction: *P < 0.05; **P < 0.1; ***P < 0.0001.

Table 3.10 Effect of treatments on femur bone mineral content as measured by quantitative computed tomography at 30% of femur length from the proximal epiphysis at 12, 22, and 41d. Dependent variable adjusted to a common BW with analysis of variance

		Total[1]			Cortical[2]			Trabecular[3]		
	n[4]	12d	22d	41d	12d	22d	41d	12d	22d	41d
Dietary treatment		mg/cm								
D[5]	8	53.39[b]	138.6	322.7	42.98[b]	116.4	258.3	2.81	8.47	36.61
25OHD[6]	8	53.23[b]	140.1	339.5	44.91[b]	114.1	272.9	1.46	11.64	38.51
D+25OHD[7]	8	58.36[a]	145.0	324.7	48.54[a]	122.3	264.0	3.35	9.08	34.44
Dx2[8]	8	53.86[b]	139.2	331.8	45.39[b]	118.2	267.1	1.96	7.71	37.06
Litter										
Fresh[9]	16	58.16[a]	140.9	325.7	48.24[a]	117.7	263.8	2.85	9.49	35.47
Reused[10]	16	51.26[b]	140.5	333.6	42.67[b]	117.8	267.3	1.94	8.96	37.84
Pooled SEM		1.458	4.085	6.953	1.458	4.235	6.926	0.690	1.495	0.247
Source of variance	DF	P-value								
Diet	3	0.035	0.6208	0.3100	0.0004	0.4078	0.5079	0.1831	0.3082	0.7070
Litter	1	***	0.9131	0.2594	***	0.9633	0.6143	0.2020	0.7137	0.3361
Diet*Litter	3	*	0.5459	0.5438	*	0.7799	0.5430	0.178	0.5296	0.4186
Covariate BW	1	***	***	***	***	***	***	0.0908	0.0198	0.0065

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

[1]Total =For the entire bone.

[2]Cortical = outer shell of the femur with a mineral density >500 mg/cm3.

[3]Trabecular = measurements taken in the inner part of the bone in the trabecular space.

[4] Pen was the experimental unit.

[2]Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[3] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[4]Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[5]Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6]Fresh wood shavings litter

[7]Reused wood shaving litter; litter was used one previous flock

Probabilities of interaction: *P < 0.05; **P < 0.1; ***P < 0.0001.

Table 3.11 Effect of treatments on femur bone mineral content as measured by quantitative computed tomography at 50% of femur length from the proximal epiphysis at 12, 22, and 41d. Dependent variable adjusted to a common BW with analysis of variance

		Total[1]			Cortical[2]			Trabecular[3]		
	n[4]	12d	22d	41d	12d	22d	41d	12d	22d	41d
Dietary treatment		mg/cm								
D[5]	8	6429	18669	38353	5620[b]	17301	34816	173.8	299.4	1882
25OHD[6]	8	6668	18557	39177	5976[a]	17267	35281	101.2	244.7	2170
D+25OHD[7]	8	6933	18841	39220	6297[a]	17597	35478	105.4	207.9	2187
Dx2[8]	8	6664	18860	39009	6007[a]	17645	35412	113.3	222.6	2102
Litter										
Fresh[9]	16	6976[a]	18642	39021	6235[a]	17303	35388	161.6[a]	323.3[a]	1998
Reused[10]	16	6371[b]	18822	38858	5715[b]	17602	35105	85.2[b]	163.9[b]	2172
Pooled SEM		145.8	411.5	911	127.3	378.4	800.97	33.50	70.73	224.9
Source of variance	DF	P-value								
Diet	3	0.149	0.954	0.900	0.004	0.863	0.937	0.382	0.810	0.762
Litter	1	0.001	0.672	0.857	0.001	0.443	0.724	0.007	0.036	0.441
Diet*Litter	3	0.480	0.418	0.843	0.135	0.638	0.447	0.844	0.704	**
Covariate BW	1	***	***	***	***	***	***	0.285	0.405	0.972

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

[1]Total =For the entire bone.

[2]Cortical = outer shell of the femur with a mineral density >500 mg/cm3.

[3]Trabecular = measurements taken in the inner part of the bone in the trabecular space.

[4] Pen was the experimental unit.

[2]Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[3] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[4]Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[5]Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6]Fresh wood shavings litter

[7]Reused wood shaving litter; litter was used one previous flock

Probabilities of interaction: *$P < 0.05$; **$P < 0.1$; ***$P < 0.0001$.

Table 3.12 Effect of treatments on femur bone breaking strength at 12, 22, and 41d of age. Bone breaking strength adjusted to a common BW with analysis of covariance.

		bone breaking strength		
		12d	22d	41d
Dietary treatment	n[1]	KgF		
D[2]	8	7.26	16.91	36.54
25OHD[3]	8	7.45	17.31	40.27
D+25OHD[4]	8	7.79	17.28	41.80
Dx2[5]	8	7.46	15.50	38.61
Litter				
Fresh[6]	16	7.70[a]	16.80	39.27
Reused[7]	16	7.28[b]	16.70	39.34
Pooled SEM		0.19	0.57	1.66
		P-value		
Source of variance	DF			
Diet	3	0.268	0.092	0.083
Litter	1	0.047	0.870	0.960
Diet*Litter	3	0.312	0.554	0.871
Covariate BW	1	< 0.001	< 0.001	0.005

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

[1] Pen was the experimental unit.

[2]Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[3] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[4]Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.

[5]Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[6]Fresh wood shavings litter

[7]Reused wood shaving litter; litter was used one previous flock

4. EFFECTS OF DIETARY 25-HYDROXCHOLECALCIFEROL ON BREAST MUSCLE GENE EXPRESSION OF BROILER CHICKENS RAISED ON REUSED LITTER

ABSTRACT

The vitamin D_3 metabolite 25-hydroxycholecalciferol ($25OHD_3$) increases broiler chicken breast meat yield and innate immunity by relatively unknown mechanisms. This study investigated the effect of an unsanitary environment on expression of genes associated with immune function and breast muscle synthesis and development of broilers fed 2 sources of vitamin D activity. Day-old Ross 308 chicks were placed in pens (n=512) in a 4 x 2 factorial arrangement with 4 pens/treatment. Dietary treatments were: D_3 at 2,760 IU/kg feed (D; Control); 25OH D_3 at 2,760 IU/kg feed (25OHD); D_3 at 2,760 IU/kg plus $25OHD_3$ at 2,760 IU/kg feed (D+25OHD); or D_3 at 5,520 IU/kg feed (Dx2). Each diet was fed to broilers on either fresh (Fresh) or reused (Reused) pine shavings to increase bacterial exposure. At d 12, 12 pectoralis major samples per treatment from birds fed either D or 25OHD and reared on either Fresh or Reused litter, and 8 samples per diet and litter treatment combination (n=32) were converted to a uniquely indexed cDNA library; the resulting cDNA libraries were pooled and global transcriptome analysis was performed using Illumina RNA sequencing. On average approximately 15 million sequence reads per sample were obtained. Low quality reads and 5 outliers were removed, and RNA-Seq mapping was analyzed by TopHat and HTSeq. The remaining samples were aligned to the chicken reference genome (Galgal4). A total of 73 differentially-expressed (DE) genes were detected, with 34 DE genes between the D and 25OHD treatments, and nine DE genes between Fresh and Reused litters. Gene ontology results revealed

that 25OHD$_3$ increased the expression of genes participating in muscle synthesis and adaptive immunity, and decreased the expression of genes involved in amino acid metabolism in comparison to vitamin D$_3$ in breast muscle of broiler chickens. Also, gene ontology results suggested that the Reused litter increased expression of genes that were associated with pro-inflammatory response relative to Fresh litter. CaMKIIA (related to cell growth) and BFIV21 (linked to adaptive immunity) were tested by traditional real-time PCR. Dietary 25OHD$_3$ increased the expression of CaMKIIA relative to vitamin D$_3$ in broilers; 25OHD relative to vitamin D$_3$ increased the expression of BFIV21 in birds reared on Reused litter, but there were no differences in birds reared on Fresh litter at d 12. These results indicate that dietary 25OHD$_3$ increased expression of genes linked to growth and adaptive immunity of post-hatched broilers, particularly in Reused litter.

Key Words: broiler chicken, 25-hydroxycholecalciferol, breast muscle, adaptive immunity, Reused litter.

4.1 INTRODUCTION

The poultry industry often reuses litter from previous flocks because of the economic advantages due to reduced cost of removal, disposal, and acquisition of new litter and also due to the presence of commensal microorganisms within it (Volkova et al., 2009; Coufal et al., 2006). These bacteria may enhance small intestine morphology, immune response, and performance of broiler chickens (Dibaji et al 2014; Lee et al., 2010; Waititu et al., 2014). This may involve the concept of competitive exclusion in which commensal bacteria colonize the gastro-intestinal tract and protect gut from enteric pathogens (Nurimi and Rantala, 1973). Feeding of a *Lactobacillus*-based probiotic culture increased expression of genes that function in apoptosis in

order to reduce pathogenic infection in gut (Higgins et al., 2001). Additionally, direct-fed microbials altered gene expression profiles to decrease the percentage of B-cell markers and increase intestinal intraepithelial lymphocyte (IEL) T-cell surface markers (Lee et al., 2010). Taken together, these data are consistent with direct-fed microbials having the potential to enhance immune response development in the small intestine of broiler chickens. However, the composition of the intestinal microbiota profile is altered when broilers are raised on reused in comparison to fresh litter (Lu et al., 2003). Pathogenic bacteria such as the *Clostridiales* family were dominant in reused litter, whereas, *Lactobacillus* were the predominate microbes in fresh litter (Cressman et al., 2010). In addition, expression of pro-inflammatory cytokines such as IL-1β increases in reused vs. fresh litter (Shanmugasundaram et al., 2012). Broiler BW and feed intake were reduced when they were reared on reused litter (Torok et al., 2009). The authors suggested that the negative effects of reused litter on broiler performance may have been due to an increase in bacteria exposure and a change in gut microbiota composition.

Cholecalciferol (vitamin D_3) is widely used as the source of vitamin D in poultry nutrition. Vitamin D_3 requires two hydroxylation steps to become the active form of vitamin D. In the liver, vitamin D_3 is converted to 25-hydroxycholecalciferol ($25OHD_3$) by 25-hydroxylase and then in the kidney is hydroxylated to 1, 25- dihydroxylcholecalciferol [$1,25\text{-}(OH)_2D_3$] by the action of 1 α-hydroxylase (Shanmugasundaram and Selvaraj, 2012). The conversion of $25OHD_3$ to $1,25\text{-}(OH)_2D_3$ is highly regulated by Ca serum level vitamin D receptor (VDR) and $1,25\text{-}(OH)_2D_3$ itself in chicken (De Matos, 2008; Russell et al., 1993). Dietary supplementation of $25OHD_3$ enhances broiler breast meat yield (Yarger et al, 1995; Vignale et al. 2015), protein synthesis rate (Vignale et al. 2015), and density of satellite cells in breast muscle (Hutton et al., 2014), in comparison to vitamin D_3 at the same level of inclusion. Additionally, $25OHD_3$

decreases expression of pro-inflammatory cytokines relative to vitamin D_3 when broiler chickens are challenged with lipopolysaccharide (Morris et al., 2014).

Several studies in mammals and chickens have demonstrated that VDR via 1,25-$(OH)_2D_3$ regulates a number of genes associated with muscle hypertrophy and development, immune response, bone formation, and calcium and phosphorous uptake and metabolism in the intestine (Boland et al., 2011; Shojadoost et al 2014; Kim et al., 2005; Healy et al., 2003). According to the study by Vignale et al. (2015), supplementation of $25OHD_3$ enhances breast muscle yield through the mechanistic target of rapamycin (mTOR)/S6 kinase (S6K) pathway in response to increased expression of VDR, mTOR, S6K, and the quail myoblast cell line QM7. However, previous research on enhanced breast muscle synthesis in broiler chickens did not investigate the gene expression response of the entire chicken genome when birds were fed various vitamin D sources. Additionally, there is little gene expression information about breast muscle development in birds reared on reused litter. Broiler performance and breast meat yield were reduced at d 12 in birds reread on reused as compared to those reared on fresh litter, also, birds fed $25OHD_3$ had lower BW relative to those fed vitamin D_3 alone in our study (Chapter 2). Therefore, the objective of this study was to determine the effects of fresh and reused litters on expression of genes associated with immune function, and breast muscle synthesis and development of 12 d old broiler chickens fed vitamin D_3 or $25OHD_3$.

4.2 MATERIALS AND METHODS

4.2.1. Experimental Diets and Animal Housing

All experimental conditions and animal protocols were approved by University of Alberta Animal Care and Use Committee: Livestock in accordance with the Canadian Council of

Animal Care (2009) guide. An experiment was undertaken with 512 1-day-old (1d) male Ross 308 broilers chickens. A two level factorial arrangement of treatments (4 diet and 2 litter treatments) was conducted. It was assumed that vitamin D_3 and $25OHD_3$ have the same physiological activity in chicken diets (NRC, 1994). For each dietary phase the dietary treatments were: vitamin D_3 at 2,760 IU/kg feed (D); $25OHD_3$ at 2,760 IU/kg feed (25OHD); D_3 at 2,760 IU/kg plus $25OHD_3$ at 2,760 IU/kg feed (D+25OHD); or D_3 at 5,520 IU/kg feed (Dx2). Each of the 4 dietary treatments was randomly assigned to 32 pens (1.9 x 2.2 m^2) in an environmentally controlled room with 8 replicates per treatment. Chickens were allowed free access to feed and clean water throughout the 41 d experiment. Broilers were reared on either fresh (Fresh) or pine shavings that had been used by one previous flock (Reused). The reused litter was stored for approximately 4 weeks before it was moved to the experimental barn.

4.2.2. Tissue Collection, RNA Isolation, and Library Preparation

The pectoralis (P.) major was collected from chicks at d 12 (12 replicates per treatment) for RNA isolation. Approximately 10 to 20 g at P. major was collected a cut from the medial side of the muscle. The tissue was immediately frozen in liquid nitrogen and stored at – 80°C. Total RNA was isolated using the TRIzol® procedure (Invitrogen, Carlsbad, USA) using 1 mL TRIzol® to every 30 mg of tissue according to the manufacturer`s recommendations. A NanoDrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, Wilmington USA) was used to quantify the RNA, and it was also tested for integrity on the Agilent 2200 TapeStation (Agilent Technologies, Waldbronn, Germany). The minimum RNA integrity number (RIN) score was ≥ 7 for Real-Time PCR samples and ≥ 8 for RNA-seq samples. If any sample was below the acceptable limit, that sample was re-extracted. A total of 32 RNA isolates (n=8 replicates per treatment) from breast muscle samples obtained from D and 25OHD birds only on

either in Fresh or Reused litter at d 12 were selected for RNA-seq. Two-hundred nanograms of each total RNA sample was used in the construction of cDNA libraries using the Truseq Stranded Total RNA Prep kit (Illumina, San Diego, CA) according to the manufacturer's instruction as described by Zhou et al. (2015). Briefly, oligo (dT) magnetic beads were used to purify the poly-A containing mRNA molecules and then mRNA was subsequently fragmented. Then Super Script II reverse transcriptase (Invitrogen™; Austin, TX) was used to reverse transcribe the purified RNA into first-strand cDNA. dUTP was used instead of dTTP to synthesize the second-strand cDNA, and then cDNA samples were adenylated at the 3' end. Later, the cDNA samples were ligated with the Illumina indexed adaptors. Finally, cDNA was amplified by PCR. After PCR enrichment, the cDNA quantity was estimated using a Quant-iT™ PicoGreen® (Invitrogen – Molecular Probes, Eugene, OR, USA) as described by Sedlackova et al. (2013). Libraries were quantified on an Agilent 2200 TapeStation (Agilent Technologies, Waldbronn, Germany). Individual cDNA libraries were grouped into 4 pools with each pool including duplicates of all treatments and then cDNA libraries were normalized to 4 nM.

4.2.3. Whole-Transcriptome Sequencing

Each of the four pools were sequenced in two separate lanes of an Illumina Hiseq 2000 sequencer (Illumina, San Diego, US), using 101 bp paired-end libraries. Before read alignment, the quality control for the raw sequence reads was performed using the FastQC program (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). RNA-Seq reads flagged as low quality by the chastity filter in Illumina's CASAVA software v1.8 were removed as well as reads with an average read quality score below 1.5, and reads in which over 30 of the last 40 bases had a Phred quality score below 2. After filtering, 97% of the total reads were retained and the remaining reads had high FastQC quality scores (median>30). Next, the reads were aligned to

the chicken reference genome sequence assembly (Galgal4; Zhou et al., 2015) using TopHat 2.0.12 (Kim et al., 2013) with default parameters except for maximum mismatches in a read and maximum edit distance which were both set to 3. For annotation of genes, we used the GTF file for Galgal4 from Ensembl version 77 (Piórkowska et al., 2016). The HTSeq- count program (version 0.5.3p4) was used to count the number of reads mapped to each gene for each sample as described by Anders et al. (2015).

4.2.4. Analysis of Differential Gene expression

Differentially expressed (DE) genes were determined using EdgeR v3.8.5 (Bioconductor v3.0; R v3.1.1; Robinson et al., 2010; Tam et al., 2015). Data exploration on read counts (normalized to counts per million (CPM)) performed using hierarchical clustering and multi-dimensional scaling (MDS) plots (using the *plotMDS* function in the limma (version 3.14.4; Kommadath et al., 2014) revealed some evidence of clustering by pool and batch cDNA libraries. Samples that were identified as outliers in the MDS plot were removed from further analysis. Genes expressed at very low levels were identified and filtered out, keeping only those genes that achieved CPM above five in at least seven of the samples. Trimmed mean of M-values (TMM) normalisation was then applied to account for compositional differences between the libraries. Using specific functions in edgeR, negative binomial generalized log-linear models based on a 2x2 factorial design (diet and litter) were fitted to the read count data for each gene after first estimating the dispersions (variance parameters). The models also accounted for effects of pool and batch of samples. Gene-wise likelihood ratio tests were then conducted to extract the DE contrasts of interest. DE genes were determined based on criteria: false discovery rate (FDR) below 0.1 and logCPM above 1. The functional annotation of differentially expressed genes was performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID;

http://david.abcc.ncifcrf.gov; Huang da et al., 2009); and Ingenuity Pathway Analysis software (IPA; Ingenuity Systems; Redwood City, CA; www.ingenuity.com).

4.2.4.1 DAVID

Before importing the dataset genes, if no information was available for *Gallus Gallus* genes, annotations from other gene orthologues such as those from *Homo sapiens* were used in DAVID. The list of all DE genes from the different contrasts was imported into DAVID's Functional Annotation tool, and all *Gallus Gallus* or *Homo sapiens* genes were selected as the background list. Then, Functional Annotation Clustering was performed to measure relationships among the annotation terms based on the degrees of their co-association genes to group the similar, redundant, and heterogeneous annotation contents from the same or different resources into annotation groups. The p-values associated with each annotation term inside each cluster were exactly the same meaning/values as p-values (Fisher Exact/EASE Score Threshold, (p-value< 0.1); Huang da et al., 2009) obtained from the Functional Annotation tool, except that each annotation cluster was also given a Group Enrichment Score which is the geometric mean (in -log scale) of member's p-values in a corresponding annotation cluster, and this new score was used to rank the cluster's biological significance. Thus, the top ranked annotation clusters most likely have consistently lower p-values for their annotation members. This allowed us to obtain an overarching view of the most significant functional pathways, from different database sources that were represented by our DE genes. In addition, DAVID Pathway Viewer was performed using KEGG pathways in order to facilitate biological interpretation of DE genes list.

4.2.4.2 IPA

IPA is designed for the functional annotation and mapping to canonical metabolic and regulatory pathways based on human and rat gene bank sources (Krämer al., 2014; Zhou et al., 2015). Through IPA's Core Analysis function, DE genes were mapped to the Ingenuity Knowledge Base (IKB), and all genes that were associated with a biological term in the IKB were analyzed. From the Core Analysis report, a summary of molecular networks and identified biological functions and pathways in the DE gene list were displayed. In addition, signaling and metabolic canonical pathways were determined using a Fisher's exact test with the cut-off p-value < 0.05 (Zhou et al., 2015). Later, reports from the Core Analysis subcategories; Associated Network Function, Molecular and Cellular Functions (top five), Diseases and Disorders, and Physiological System Development and Function, that were overrepresented in DE gene lists were investigated.

4.2.4.3 Gene Network Analysis

The list of DE genes from the various contrasts studied from chicken breast muscle was submitted to DAVID and IPA software to determine metabolic and regulatory pathways overrepresented in the differential expression analysis. To conduct a meaningful canonical pathway analysis using gene network tools, the DE gene list should contain a minimum of 7 genes. Using this criteria, four contrasts were selected for individual pathway analysis in both IPA and DAVID: 1) birds reared on Fresh relative to Reused litter when fed vitamin D_3 (Fresh vs Reused* D) with seven DE genes; 2) birds fed vitamin D_3 in comparison to $25OHD_3$ when they were reared on Reused litter (D vs 25OHD* Reused) with 17 DE genes; 3) birds fed vitamin D_3 in comparison to $25OHD_3$ (D vs 25OHD) with 34 DE genes; and 4) birds reared on Fresh vs Reused litter (Fresh vs Reused) with nine DE genes. Additionally, all 73 DE genes were used in

a separate pathway analysis to increase the potential overrepresented pathways that could be detected in our data. The list of DE genes was imported into IPA software for gene network generation. The results revealed that "Amino Acid Metabolism" was among the top molecular and cellular function identified in five contrasts (Tables 4.1, 4.2, 4.3, 4.4 and 4.5). However, the Associated Network Function did not reveal specific individual biological networks linked to either muscle synthesis or immunity in the RNA-seq data, although the lack of genes classified as significantly DE between comparison groups was a contributing factor. Therefore, four DE gene lists based on interesting biological functions were prepared and imported separately to IPA to have better understanding of gene relationships. Since our objective was to investigate effects of supplementation of vitamin D_3 and $25OHD_3$ to birds reared in either Fresh or Reused litter on breast muscle development and immunity pathways of broiler chickens, DE genes that were linked to these two areas across the contrasts were separately imported into IPA and run through the Core Analysis to identify canonical pathways or relative gene networks related to our hypothesis. Additionally, genes associated with amino acid metabolism were obtained from the 73 DE genes and imported to IPA as another data set since this category was the first subcategory identified by IPA in almost all contrasts. Lastly, 36 DE genes which have been shown to link directly or indirectly to growth, muscle development and immunity were imported.

4.2.5. Reverse Transcription and Designing and Testing of Real-Time PCR Primers for Verification of DE Genes

All individual P. major RNAs (96) from the four dietary treatments (D, 25OHD, D+25OHD, and Dx2) and two environments (Fresh and Reused) at d 12 were included in the real-time PCR experiment. First, these RNAs were diluted to 200 ng/µl with RNase-free water and stored at -80 °C until cDNA synthesis. cDNA was synthesized according to High Capacity

cDNA Reverse Transcription Kit protocol (Applied Biosystems, Foster City, USA). Before the cDNA reaction, a reference RNA was prepared by pooling 4 µl of each individual RNA (200 ng/µl). Later, 10 µl of reverse transcription (RT) mix was prepared for either individual or reference RNAs by mixing 2 µl of 10X RT buffer, 0.8 µl of 25X dNTP Mix (100 mM), 2 µl of 10X RT Random Primer, 1 µl of MultiScribe™ Reverse Transcriptase (50 U/µl), 1 µl of RNase inhibitor (20 U/µl), and 3.2 µl of RNase free water. Then 10 µl of RT was added and gently mixed with10 µl of each RNA (200 ng/µl). Each 20 µl reaction was amplified by thermo cycler (Applied Biosystems® Veriti®, Foster City, US) at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. The resulting cDNA was stored at –20°C until real time PCR.

In total, three endogenous or "housekeeping" genes, plus two target genes associated with immune response and growth were identified for real-time PCR (Table 4.6). The forward and reverse primers of each gene were designed according to the following protocol: the reference sequence of all of the isomers of each selected *Gallus Gallus* gene were aligned with the *Homo sapiens*, using Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were designed using Primer3 software (Version 0.4.0; http://bioinfo.ut.ee/primer3-0.4.0) with the following quality parameters: product size within the range of 90-150 bp, primer size between 18-22 bp, primer Tm (melting temperature) between 55-60 ^{o}C, primer GC% between 45-60. Afterwards, the forward and reverse primers were tested for the minimum probability of hairpin, delta G self-dimer (lower than -6 kcal/mole), and delta G hetro-dimer (lower than -6 kcal/mole) through the IDT Oligoanalyzer 3.1 (http://www.idtdna.com/calc/analyzer).

A serial dilution of reference cDNA was prepared at 20, 2, 0.2, 0.02, and 0.002 ng/µl to test the primer amplification. The primer testing reactions were carried out in 96-well optical reaction plates (Applied Biosystems, Foster City, USA) using the Applied Biosystems Step-One-Plus real-time PCR system (Applied Biosystems, Foster City, USA). Each reaction was prepared in a total volume of 20 µl containing 2 µl of each reference cDNA template, 0.6 µl of each forward and reverse primer (200 nM), 0.4 µl of 50X ROX High (Applied Biosystems® 5700HT), 10 µl of the 2× KAPA SYBR® FAST qPCR Master Mix ABI Prism™ (Kapa Biosystems, Boston, USA), and 6.4 µl of RNase-free water. To check for contamination, two control samples were included in each run; a no RT control which was the reference RNA without conversion to cDNA by reverse transcriptase, and a no template control in which water replaced template cDNA. Amplification conditions consisted of: [1] hold stage for 30 s at 95°C for enzyme activation; [2] cDNA amplification stage of 3 s at 95°C and then 30 s at 58°C for 40 cycles; and [3] a final melt curve stage which consisted of 15 s at 95°C, 1 min at 58°C, and then heating (melt) of the reaction with a ramp rate of + 0.3°C per 15 s from 58°C to 95°C to obtain fluorescence measurements that reflect the Tm of the main product while minimizing interference from primer-dimer or other non-specific amplification products. Primers were evaluated using the following criteria: agreement between duplicates in the standard curve with standard deviation (SD) ≤0.4 between CT values of technical replicate, quality of the amplification plot, having a single, distinct, and product length appropriate peak in melt curve, a reaction efficiency of between 90% and 120%. The target gene data was normalized using NormFinder software version 20 (Andersen et al., 2004). Actin, beta [ACTB], Glyceraldehyde-3-phosphate dehydrogenase [GAPDH], and 18S ribosomal RNA [18S] were tested for gene

expression normalization suitability. The 18S was found to be the most suitable endogenous gene to normalize the data for target genes.

4.2.6. Statistical Analysis

Housekeeping gene normalized real-time PCR data were tested for normality by PROC UNIVARIATE and were all normally distributed. Then data were analyzed as a two-way ANOVA with 4 dietary treatments and 2 environments using the procedure for linear mixed models (PROC MIXED) of SAS (SAS 9.3© for Windows; SAS Institute Inc., Cary, NC). Differences were considered significant at $P < 0.05$. The model used was:

$$Y_{ij} = \mu + D_i + L_j + (DL_{ij}) + e_{ij},$$

Where μ was the population mean; D_i was the effect of each dietary treatments ($i = 1$ to 4); L_j was the effect of litters ($j = 1$ to 2); $(DL)_{ij}$ was the interaction of each dietary treatment with litter treatment; and e_{ij} was the residual error.

4.3 RESULTS

4.3.1. Differential Gene Expression

In pools 1 and 2, approximately 15 million short sequence reads (101 bp nucleotides) were produced; in pool 3 approximately 5 million; and in pool 4 approximately 35 million short sequence reads were produced for each sample by the RNA-Seq experiment. In total, 17,108 unique genes were counted in the samples. After removing the low expressed genes with cpm <5 in 27 samples, and 5 identified outlier samples, from the remaining 27 samples 7,611 unique genes were identified. Within this set of genes we found 73 DE genes (p-value < 0.1) across all treatment comparisons in P. major of broiler chickens (Table 4.7). Going forward, this list of 73

DE genes obtained from all contrasts was used for pathway analysis in order to increase the chance of finding related DE canonical pathways from DE gene list (Appendix 1).

4.3.2. Results of DAVID Analysis

Functional Annotation Clustering analysis of D vs 25OHD and all DE genes by DAVID revealed three and six significant clusters respectively. Prominent among the top clusters for both datasets were terms associated with amino acid transfer and biosynthesis. Cluster 1 of D vs 25OHD contained the GO (Gene ontology, http://www.geneontology.org) terms amino acid transport (GO:0006865), and regulation of developmental growth (GO:0048638). Cluster 2 contained inhibition of host tapasin by virus (GO:0039591). Cluster 3 was related to ATP binding (GO:0005524). Similar to the results from the D vs 25OHD Functional Annotation Clustering, cluster 1 resulting from the analysis of 73 DE genes contained the functions of amino acid transport (GO:0006865) and regulation of developmental growth (GO:0048638). Cluster 2 contained the GO terms related to positive regulation of nitrogen compound metabolic process (GO:0051173), muscle organ development (GO:0007517), myogenic basic muscle-specific protein (GO:0007517), skeletal muscle tissue development (GO:0007519), and skeletal muscle organ development (GO:0060538). Cluster 4 was linked to calcium channel inhibitor activity (GO:0019855), calcium channel activity (GO:0005262), and calcium ion transmembrane transport (GO:0070588). Cluster 5 contained signal peptide processing (GO:0006465), and Cluster 6 was linked to ATP binding (GO:0005524).

4.3.3. Results of IPA Analysis

The IPA Canonical Pathway analysis of the 73 DE genes revealed the calcium/calmodulin-dependent protein kinase II alpha (CaMKIIA) was involved in calcium

signaling pathway (Figure 4.1). The gene interaction network of the 11 DE genes associated with muscle development is depicted in Figure 4.2. The major hub molecule in this network was extracellular signal-regulated kinases (ERK1/2) linked to 10 molecules; however, only fibroblast growth factor receptor 2 (FGFR2) from our DE gene list was linked to that molecule. The gene interaction network of the nine DE genes linked to immune response and inflammation is displayed in Figure 4.3. The hub molecule in this network was tumour necrosis factor (TNF) which was linked to 11 molecules. The gene interaction network of 8 of the DE genes associated with amino acid metabolism is illustrated in Figure 4.4. The key modules in this network were transcription factor activating transcription factor 4 (ATF4) and TNF which were linked with 15 and 10 molecules respectively. In the last analysis of 36 DE genes that were directly or indirectly related to growth, muscle development and immunity, 11 DE genes were related to organ and skeletal muscle development or immunity (Figure 4.5). The major hub molecule was insulin, which was linked to 11 modules. The other two main molecules in this network were VEGF and extracellular signal-regulated kinases (ERK1/2) which both were linked to 7 molecules including insulin.

4.3.4. Validation of RNA-seq Expression Data by Real-Time PCR

Two significantly DE genes, CaMKIIA and BFIV21, that were identified by RNA-seq analysis as being related to muscle development, and adaptive immunity, respectively, were selected for SYBRgreen real-time PCR verification. Real-time PCR was performed on all 96 individual breast muscle RNA samples from the four dietary and two litter treatments. Birds fed $25OHD_3$ alone displayed higher expression of (CaMKIIA) relative to those birds fed vitamin D_3 at the same level; also D+25OHD fed birds tended to have higher CaMKIIA expression as compared to D (P=0.07). There was an interaction between diet and litter treatments for the

expression of BFIV21 ($P< 0.05$). In the Reused litter, birds fed vitamin D_3 at 2,760 IU/kg feed had lower expression of BFIV21 relative to any other dietary treatments, but, there were no difference between dietary treatments in the Fresh litter (Table 4.8). The results from Real-time PCR confirmed those from RNA-seq for both CaMKIIA and BFIV21.

4.4 DISCUSSION

4.4.1 Growth Properties in Breast muscle

Investigating traits at the level of gene function in different physiological stages or genetic backgrounds, can lead to a better understanding of the molecular mechanisms that are linked to economically-important traits and variants affecting the phenotype. In this study, broiler chickens fed 25OHD had higher expression of MyoD1 relative to birds fed D; and birds fed $25OHD_3$ had higher expression of MyoG as compared to those fed vitamin D_3 alone in the Reused litter (Appendix 4.1). In both chickens and mammals, there are four myogenic regulatory factor genes, including myogenic determination protein (MyoD) and myogenin (MyoG), and these are involved in postnatal and prenatal muscle development (Arnold and Winter, 1998; Yablonka-Reuveni and Paterson, 2001; Liu et al., 2012). In mammals, myogenic differentiation 1 (MyoD1) is involved in the differentiation of myoblasts into myotubes during myogenesis (Edmondson and Olsen, 1989); MyoG is associated with an increase in breast muscle and leg muscle yields in chickens (Genxi et al., 2014). Additionally, both MyoD1 and MyoG are associated with satellite cell proliferation in avian muscle (Clark et al., 2016). Satellite cells are present in adult muscle and play important roles in hypertrophic skeletal muscle growth (Armand, 1983). Dietary $25OHD_3$ did not increase breast muscle yield but it increased leg muscle yield at d 41 (Chapter 2). Therefore, increased expression of two of the main myogenic

regulatory factors may be linked to the increased thigh muscle synthesis in those fed 25OHD relative to those fed D at d 41. However, since $25OHD_3$ did not affect breast meat yield in this study, these genes may not have been translated into protein in breast muscle to increase muscle synthesis. It would be interesting to look at the expression of myogenic regulatory genes in both breast and leg muscle when birds fed vitamin D sources to investigate actual differences on gene expression between thigh and breast muscle.

Other muscle growth-related genes, CaMKIIA and corticotropin releasing hormone receptor 2 (CRHR2) had higher expression, and fibroblast growth factor receptor 2 (FGFR2) had lower expression, in birds fed 25OHD relative to those fed vitamin D_3 at the same level of activity. These genes are involved in proliferation of smooth muscle cells (Agrotis et al., 2004; Cipolletta et al., 2010; and Bale et al., 2002). Cholinergic receptor, nicotinic, delta (CHRND) was also expressed higher in birds fed 25OHD relative to those fed vitamin D_3 at same level of activity, and is linked to skeletal muscle tissue growth (GO:0048630). The glucosidase, alpha; acid (GAA) was more highly expressed in birds reared on Reused litter as compared to birds on Fresh litter. This gene is linked to muscle weakness, and skeletal muscle abnormality in quail (Kunita et al., 1998), and abnormality of myofibrillogenesis in striated muscle in mouse (Rucker et al., 2004). Dietary supplementation of $25OHD_3$ at 5,520 IU/kg feed relative to vitamin D_3 at the same level of inclusion increased protein synthesis and breast muscle yield, mediated through mTOR/S6K pathway (Vignale et al., 2015). However, the DE genes identified in this study are individually linked to muscle development and are not involved in the mTOR pathway (Figures 4.3 and 4.6). However, the DE genes identified in this study were most likely linked to insulin secretion and the cAMP pathway rather than mTOR pathway. For example, homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein (HERPUD1)

was highly expressed in birds fed D relative to those fed 25OHD; and birds fed vitamin D had higher expression of HERPUD1 as compared to 25OHD-fed birds in the Reused litter. HERPUD1 regulates insulin secretion through interaction of nicotinamide nucleotide transhydrogenase in mice (Wong et al., 2013). The growth-stimulating effect of insulin is mediated by cyclic adenosine monophosphate (cAMP) signaling in the chicken. Also, insulin is involved in muscle cell differentiation and muscle contraction by regulating glycogenesis (Kuznetsova et al., 2004; Sibut et al, 2011). Broiler performance was increased in vitamin D_3 fed birds relative to those fed $25OHD_3$ at d 12 and 22 (Chapter 2). Thus, an increase in insulin secretion in response to higher expression of HERPUD1 in vitamin D_3 fed birds may have contributed to greater growth performance production in those birds.

CaMKIIA was highly expressed in birds fed $25OHD_3$ relative to those fed vitamin D_3 alone. Through IPA analyses and follow-up literature searches it appears that CaMKIIA could be associated with growth or muscle development via molecules of the cAMP pathway. These effects may be explained by two possibilities. One possibility is via an association with insulin secretion. CaMKIIA was linked to insulin through phosphoinositide 3-kinase though (Akt) and ERK1/2 molecules (Figure 4.5). An increase in intracellular Ca^{2+} serum triggers Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which is involved in insulin secretion in mammals (Easom, 1999). In mammals, activation of CaMKII is regulated by secretion of insulin (Easom et al., 1997) and inhibition of CaMKII resulted in decrease in insulin secretion (Vest et al., 2007). The activity of ERK1/2 also is regulated by CaMKII (Nguyen et al., 2004), and ERK1/2 is involved in protein synthesis in response to insulin in chicken (Tesseraud et al., 2006). ERK1/2 has been identified in chicken myoblasts (Leshem et al., 2002) and activated ERK1/2 is essential to induce insulin-like growth factor-1 in order to promote skeletal muscle

hypertrophy in the rat (Haddad and, Adams, 2004). Insulin stimulates protein synthesis in embryo fibroblasts, and myoblasts or myotubes of skeletal muscle in chickens (Tesseraud et al., 2006; Simon, 1989). The second possibility is through regulation of the phosphorylation of cAMP responsive element binding protein (CREB). CaMKIIA was involved in the calcium signaling pathway and growth cellular signals that can regulate transcription factors such as CREB by phosphorylation (Figure 4.1). CREB as a cAMP-responsive activator needs to be phosphorylated in response to growth factor signals such as calcium/calmodulin kinases II (CaMKII) to promote cellular gene expression (Sun et al., 1994). CaMKIIA belongs to the CaMKs family which participates in inositol 1,4,5-triphosphate (IP3) and cAMP pathways in response to insulin (Dixit et al., 2013). The cAMP pathway is associated with growth and muscle development in mammals (Berdeaux and Stewart, 2012). These results indicate that CaMKIIA may have a regulatory effect on cAMP/CREB signaling pathway or could be linked to insulin secretion leading to regulation of growth and muscle development. Therefore, increased leg meat yield in 25OHD-fed birds relative to D-fed birds at processing (Chapter 2) could likely be regulated through the cAMP pathway by activation of CaMKII family genes such as, CaMKIIA in this study.

4.4.2 Immune Function Genes in the Breast Muscle

Relative to vitamin D, feeding a similar physiological activity level of $25OHD_3$ was accompanied by an increase in the expression of predicted genes for class I histocompatibility antigen, F10 alpha chain-like mRNA (LOC769716), and class I histocompatibility antigen, F10 alpha chain-like, transcript variant X6 (LOC417083). These genes are chicken homologues of HLA-A which has been shown to activate CD4 + T lymphocyte and CD8 + T lymphocyte (Billerbeck et al., 2013), cytotoxic T lymphocyte cell lines in mammals (Denkberg et al., 2000),

and the development of T-cells and adaptive immunity in the chicken (Ewald and Livant, 2004). Another DE gene related to immune response was MHC class I alpha chain 2 (BFIV21), which was more highly expressed in birds fed 25OHD as compared to D in the Reused litter. This gene was not mapped by either IPA or DAVID, but according to the literature it is involved in the MHC family which contributes to adaptive immunity by inducing cytotoxic T lymphocytes in the chicken (Fulton et al., 1995). Additionally, the MHC I molecule is associated with antigen presentation and enteric pathogen protection (Afanassieff et al., 2001). Increased expression of HLA-A is also linked to reduced chronic phase hepatitis (Mosaad et al., 2010). Acute phase hepatitis is correlated to acute phase protein serum concentrations such as alpha 1-antitrypsin, alpha 1-acid glycoprotein (AGP), and C-reactive protein (Gödl et al., 1990). Several acute inflammatory biomarkers such as haptoglobin (Hp) and AGP have been identified in chicken (Chamanza et al., 1999), which are produced in response to inflammatory stimuli (Kefal and Toker, 2006). Supplementation of $25OHD_3$ at 2,760 IU/kg feed tended to decrease ($p<0.1$) Hp production relative to vitamin D_3 at the same level of activity in broiler chickens at d 12 (Chapter 3). These results suggest that dietary supplementation of $25OHD_3$ as compared to vitamin D_3 triggered T-cell development and the antigen presentation leading to an increase in adaptive immune response, and reduced inflammation in broiler chickens.

Poultry litter may contain pathogenic bacteria, such as *Salmonella* spp., *Campylobacter* spp., *Escherichia coli*, *Clostridium perfringens*, and *Staphylococcus aureus* (Lu et al., 2003; Cressman et al., 2010). Hence, chickens raised on reused litter likely have increased early bacteria exposure relative to birds reared on fresh litter. In the IPA canonical pathway analysis, immunoglobulin J (IgJ) is part of the joining chain of the multimeric IgA and IgM (JCHAIN; Figure 4.3). Broiler chickens reared on Reused litter had an increase in expression of IgJ as

compared to birds reared on Fresh litter. IgJ is involved in B cell development (Hystad et al., 2007) and is highly expressed in inflamed tissues and in chronic inflammation in humans (Raman et al., 2015). In addition, the expression of IgJ was increased in avian influenza virus-infected birds relative to non-infected birds (Wang et al., 2014). Plasma Hp concentration tended to increase (p=0.054) at d 12 in birds reared on Reused compared to Fresh litter (Chapter 3). This result showed an increase in inflammation may result in increased expression of IgJ when birds were reared on Reused litter. The performance and meat yield of broiler chickens raised on Reused litter was reduced at d 12 relative to birds on Fresh litter. Therefore this reduction in performance might have been due to inflammation in birds reared on Reused litter, as indicated by the increased serum concentration haptoglobin and increased the expression of IgJ.

4.4.3 Amino Acid Metabolism and Inflammation in Breast Muscle

Several DE genes in this study were associated with amino acid metabolism (Figure 4.4). Birds fed D relative to those fed 25OHD had higher expression of aldehyde dehydrogenase 18 family, member A1 (ALDH18A1), ANSA, phosphoglycerate dehydrogenase (PHGDH), solute carrier family 1 (glutamate/neutral amino acid transporter) member 4 (SLC1A4), solute carrier family 7 (cationic amino acid transporter, y+ system) member 3 (SLC7A3), and solute carrier family 6 (neurotransmitter transporter, glycine) member 9 (SLC6A9), in their breast muscle. Additionally, ALDH18A1, ANSA, PHGDH, CTH, and SLC6A9 were more expressed in birds fed D relative to those fed 25OHD in the Reused litter. ALDH18A1 is associated with biosynthesis of L-proline (Baumgartner et al., 2000), ANSA is involved in biosynthesis of aspargine (Balasubramanian et al., 2013), PHGDH is involved in biosynthesis of serine family amino acids (Achouri et al., 1997), and CTH is associated with increased plasma concentrations of cysteine by playing a role in the transsulfuration pathway (Lysne et al., 2015). Additionally,

the SLC gene superfamily participates in amino acid metabolism (Lim et al., 2012; He et al., 2009). SLC7A3 is in charge of transportation of basic amino acids, like arginine and L-lysine in mammals (Ito and Groudine, 1997; Hosokawa et al., 1997; Lim et al., 2012). SLC38A3 is involved in transportation of alpha amino acids such as asparagine, L-alanine, L-histidine, and L-glutamine (Fei et al., 2000; Gu et al., 2000; Chaudhry et al., 1999). SLC1A4 is involved in transportation of L-serine, L-cysteine, L-alanine, and L-threonine in chickens (Gao et al., 2009; Lim et al., 2012). Many studies have indicated that increasing the essential or nonessential amino acid plasma levels in the chicken may increase growth and performance in amino acid-deficient chickens (Pesti, 2009). Breast meat yield and broiler performance increased in male Ross 508 broilers when fed a high-amino acid density diet as compared to low- and moderate–amino acid density diets (Dozier et al., 2004). This could be due to more amino acids being available for protein synthesis and muscle deposition. Additionally, increased expression of genes related to amino acid transportation resulted in increased performance and higher serum amino acid concentrations in pigs housed in a thermoneutral environment relative to heat stressed pigs (Morales et al., 2014). Therefore, an increased expression of genes related to amino acids metabolism in birds fed D relative to 25OHD might reflect increased amino acid transport and biosynthesis in the D-fed chickens. This could explain part of the increased growth performance in birds fed vitamin D relative to $25OHD_3$ alone at d 12.

The DE genes that were involved in amino acid metabolism were also linked to inflammatory response modules. For example, ASNS, CTH, PHGDH, SLC1A4, SlC38A2, and SLC6A9 were directly or indirectly linked to TNF (Figure 4.4), which is involved in activation of inflammatory immune response (Bradley, 2008). Evstafieva et al. (2014), reported that ASNS, SLC6A9, SlC38A2, and CTH are transcriptional target genes of ATF4 and are involved in amino

acid metabolism (Evstafieva et al., 2014; Han et al., 2013). However, ATF4 is also associated with TNF-α signaling which triggers pro-inflammatory responses (Chen et al., 2013; Juknat et al., 2012). As well, PHGDH was highly expressed when birds were fed D relative to those fed 25OHD, or those reared on Reused *vs* Fresh litter. Liu et al. (2013), reported that inhibition of PHGDH in tumor cells down-regulates expression of VEGF. VEGF is associated with activation of inflammatory response genes such as IL-8 and IL-6 (Angelo and Kurzrock, 2007). Birds fed vitamin D_3 tended to have increased ($P<0.1$) chronic inflammation as compared to those fed $25OHD_3$ at d 12 (Chapter 3). Therefore, the tendency towards increased inflammation in birds fed vitamin D_3 relative to $25OHD_3$ might have been associated with genes involved in amino acid metabolism and inflammatory response.

In conclusion, we investigated the effect of dietary inclusion vitamin D_3 and $25OHD_3$ on birds raised in either Fresh or Reused litter, on breast muscle gene expression of broilers at d 12. Vitamin D_3 increased expression of genes linked to amino acid transport and biosynthesis leading to protein synthesis. However, this increase in protein synthesis appeared to be linked to expression of inflammatory response genes. The supplementation of $25OHD_3$ at 2,760 IU/kg feed relative to vitamin D_3 at the same level of inclusion resulted in higher expression of genes associated with proliferation and development of T-cells and antigen presentation (LOC417083 and LOC769716). The DE genes identified were not part of clearly defined pathways in muscle development; however, MoyD1 and MoyG, which are associated with satellite cell proliferation, were more highly expressed in breast muscle of birds fed $25OHD_3$ alone as compared to those fed D. CaMKIIA could be linked to increased growth via the cAMP/CREB signaling pathway, which had higher expression in breast muscle when birds were fed $25OHD_3$ in comparison to vitamin D_3 alone. Increased expression of genes involved in amino acid biosynthesis and

transport in birds fed vitamin D_3 may have resulted in the increased performance in this group of birds as compared to $25OHD_3$-fed birds. However, genes involved in muscle synthesis and development that were more highly expressed in $25OHD_3$-fed birds relative to D_3-fed birds, possibly indicating a lack of subsequent translation of the genes into protein, thus resulting in no change in breast muscle synthesis at d 12. Birds reared on Reused litter had increased expression of genes associated with inflammatory response and antibody synthesis. Birds fed $25OHD_3$ at 2,760 IU/kg feed maintained production performance in the Reused litter, however, performance of broilers fed vitamin D_3 at the same level of inclusion was reduced in the Reused as compared to Fresh litter (Chapter 2). This difference might have been due to increased expression of the myogenic regulatory factor genes MyoG and BFIV21, which are linked to muscle development and adaptive immunity respectively, in birds fed $25OHD_3$ alone as compared to birds fed vitamin D_3 alone in the Reused litter. In addition, the reduced growth performance and breast meat yield in birds grown on Reused litter in comparison with Fresh litter could be due to more energy being partitioned to immune/inflammatory response, as indicated by higher expression of inflammatory response genes IgJ and PHGDH.

4.5 REFERENCES

Achouri, Y., M. H. Rider, E. V. Schaftingen, and M .Robbi. 1997. Cloning, sequencing and expression of rat liver 3-phosphoglycerate dehydrogenase. Biochem. J. 323:365-370.

Agrotis, A., P. Kanellakis, G. Kostolias, G. Di Vitto, C. Wei, R. Hannan, G. Jennings, and A. Bobik. 2004. Proliferation of neointimal smooth muscle cells after arterial injury. Dependence on interactions between fibroblast growth factor receptor-2 and fibroblast growth factor-9. J. Biol. Chem. 279:42221-42229.

Afanassieff, M., M. A. Sherman, L. Zhong, C. Auffray, F. Coudert, R. Zoorob, and M. M. Miller. 2001. At least one class I gene in restriction fragment pattern-Y (Rfp-Y), the second MHC gene cluster in the chicken, is transcribed, polymorphic, and shows divergent specialization in antigen binding region. J. Immunol. 166:3324-3333.

Anders, S., P. T . Pyl, and W. Huber. 2015. HTSeq—a python framework to work with high-throughput sequencing data. Bioinformatics. 31:166-169.

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.

Angelo, L. S., and R. Kurzrock. 2007. Vascular endothelial growth factor and its relationship to inflammatory mediators. Clin. Cancer. Res. 13:2825-2830.

Arnold, H. H., and B. Winter. 1998. Muscle differentiation: more complexity to the network of myogenic regulators. Curr. Opin. Genet. Dev. 8:539-544.

Balasubramanian, M. N., E. A. Butterworth, and M. S. Kilberg. 2013. Asparagine synthetase: regulation by cell stress and involvement in tumor biology. Am. J. Physiol. Endocrinol. Metab. 304:E789-E799.

Bale, T. L., F. J. Giordano, R. P. Hickey, Y. Huang, A. K. Nath, K. L. Peterson, W. W. Vale, and K. F. Lee. 2012. Corticotropin-releasing factor receptor 2 is a tonic suppressor of vascularization. Proc. Natl. Acad. Sci. USA. 99:7734-7739.

Baumgartner, M. R., C. A. Hu, S. Almashanu, G. Steel, C. Obie, B. Aral, D. Rabier, P. Kamoun, J. M. Saudubray, and D. Valle. 2000. Hyperammonemia with reduced ornithine, citrulline,

arginine and proline: a new inborn error caused by a mutation in the gene encoding delta (1)-pyrroline-5-carboxylate synthase. Hum. Mol. Genet. 9:2853-2858.

Berdeaux, R., and R. Stewart. 2012. cAMP signaling in skeletal muscle adaptation: hypertrophy, metabolism, and regeneration. Am. J. Physiol. Endocrinol. Metab. 303:E1-E17.

Billerbeck, E., J. A. Horwitz, R. N. Labitt, B. M. Donovan, K. Vega, W. C. Budell, G. C. Koo, C. M. Rice, and A. Ploss. 2013. Characterization of human antiviral adaptive immune responses during hepatotropic virus infection in HLA-transgenic human immune system mice. J. Immunol. 191:1753-1764.

Biressi, S., C. R. Bjornson, P. M. Carlig, K. Nishijo, C. Keller, and T. A. Rando. 2013. Myf5 expression during fetal MyoGenesis defines the developmental progenitors of adult satellite cells. Dev. Biol. 379:195-207.

Boland, R. L. 2011. VDR activation of intracellular signaling pathways in skeletal muscle. Mol. Cell. Endocrinol. 347:11-16.

Bradley, J. R. 2008. TNF-mediated inflammatory disease. J. Pathol. 214:149-160.

Canadian Council on Animal Care. 2009. A guide to the care and the use of experimental animals. CCAC, Ottawa, ON, Canada.

Chamanza, R., L. Van Veen, M.T. Tivapasi and M.J. Toussaint. 1999. Acute phase proteins in the domestic fowls. Worlds. Poult. Sci. J. 55:61-71.

Chaudhry, F. A., R. J. Reimer, D. Krizaj, D. Barber, J. Storm-Mathisen, D. R. Copenhagen, R. H. Edwards. 1999. Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. Cell. 99:769-780.

Chen, Y., H. Gao, Q. Yin, L. Chen, P. Dong, X. Zhang, and J. Kang. 2013. ER stress activating ATF4/CHOP-TNF-α signaling pathway contributes to alcohol-induced disruption of

osteogenic lineage of multipotential mesenchymal stem cell. Cell. Physiol. Biochem. 32:743-754.

Cipolletta, E., S. Monaco, A. S. Maione, L. Vitiello, P. Campiglia, L. Pastore, C. Franchini, E. Novellino, V. Limongelli, K. U. Bayer, A. R. Means, G. Rossi, B. Trimarco, G. Iaccarino, and M. Illario. 2010. Calmodulin-dependent kinase II mediates vascular smooth muscle cell proliferation and is potentiated by extracellular signal regulated kinase. Endocrinology. 151:2747-2759.

Clark, D. L., C. S. Coy, G. M. Strasburg, K. M. Reed, and S. G. Velleman. 2016. Temperature effect on proliferation and differentiation of satellite cells from turkeys with different growth rates. Poult. Sci. 95:934–947.

Coufal, C. D., C. Chavez, P. R. Niemeyer, and J. B. Carey. 2006. Effects of top-dressing recycled broiler litter on litter production, litter characteristics, and nitrogen mass balance. Poult. Sci. 85:392-397.

Cressman, M. D., Z. Yu, M. C. Nelson, S. J. Moeller, M. S. Lilburn, and H. N. Zerby. 2010. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. Appl. Environ. Microbiol.76:6572-6582.

Deng, B., M. Wehling-Henricks, S. A. Villalta, Y. Wang, and J. G . Tidball. 2012. IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. J. Immunol. 189:3669-3680.

Denkberg, G., C. J. Cohen, D. Segal, A. F. Kirkin, and Y. Reiter. 2000. Recombinant human single-chain MHC-peptide complexes made from *E. coli* by in vitro refolding: functional single-chain MHC-peptide complexes and tetramers with tumor associated antigens. Eur. J. Immunol. 30:3522-3532.

De Matos, R. 2008. Calcium metabolism in birds. Vet. Clin. North. Am. Exot. Anim. Pract. 11:59-82.

Dibaji, S. M., A. Seidavi , L. Asadpour ,and F. M. da Silva. 2014. Effect of a synbiotic on the intestinal microflora of chickens. J. Appl. Poult. Res. 23:1-6.

Dixit, S. S., T. Wang, E. J. Manzano, S. Yoo, J. Lee, D. Y. Chiang, N. Ryan, J. L. Respress, V. K. Yechoor, and X. H. Wehrens. 2013. Effects of CaMKII-mediated phosphorylation of ryanodine receptor type 2 on islet calcium handling, insulin secretion, and glucose tolerance. Plos. One. 8: e58655- e58667.

Dozier, W. A., M. T. Kidd, and A. Corzo. 2004. Dietary amino acid responses of broiler chickens. J. Appl. Poult. Res. 17:157-167.

Easom R. A., N. R. Filler, E. M. Ings, J. Tarpley, and M. Landt. 1997. Correlation of the activation of Ca2+/calmodulin-dependent protein kinase II with the initiation of insulin secretion from perifused pancreatic islets. Endocrinology. 138:2359-2364.

Easom, R., 1999. CaM kinase II: a protein kinase with extraordinary talents germane to insulin exocytosis. Diabetes. 48:675-684.

Edmondson, D. G., and E. N. A. Olson. 1989. Gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes. Dev. 3:628-640.

Evstafieva, A. G., A. A. Garaeva, A. A. Khutornenko, A. V. Klepikova, M. D. Logacheva, A. A. Penin, G. E. Novakovsky, I. E. Kovaleva, and P. M. Chumakov. 2014. A sustained deficiency of mitochondrial respiratory complex III induces an apoptotic cell death through the p53-mediated inhibition of pro-survival activities of the activating transcription factor 4. Cell. Death Dis. 5:e1511-e1521.

Ewald, S. J., and E. J. 2004. Livant+ distinctive polymorphism of chicken B-FI (major histocompatibility complex class I) molecules. Poult. Sci. 83:600-605.

Fei, Y. J., M. Sugawara, T. Nakanishi, W. Huang, H. Wang, P. D. Prasad, F. H. Leibach, and V. Ganapathy. 2000. Primary structure, genomic organization, and functional and electrogenic characteristics of human system N 1, a Na+- and H+-coupled glutamine transporter. J. Biol. Chem. 275:23707-23717.

Fulton, J. E., E. L. Thacker, L. D. Bacon, and H. D. Hunt. 1995. Functional analysis of avian class I (BFIV) glycoproteins by epitope tagging and mutagenesis in vitro. Eur. J. Immunol. 25:2069-2076.

Gao, H., G. Wu, T. E. Spencer, G. A. Johnson, F. W. Bazer. 2009. Select nutrients in the ovine uterine lumen. IV. Expression of neutral and acidic amino acid transporters in ovine uteri and peri-implantation conceptuses. Biol. Reprod. 80:1196-1208.

Genxi Z., T. Ying, Z. Tao, W. Jinyu, and W. Yongjuan. 1990. Expression profiles and association analysis with growth traits of the MyoG and Myf5 genes in the Jinghai yellow chicken. Mol. Biol. Rep. 11:7331-7338.

Gödl, I., C. Müller, H. Wolf, and M. M. Eibl. 1990. Acute phase reaction in acute viral hepatitis. Padiatr. Padol. 25:147-155.

Gu, S., H. L. Roderick, P. Camacho, and J. X. Jiang. 2000. Identification and characterization of an amino acid transporter expressed differentially in liver. Proc. Natl. Acad. Sci. USA. 97:3230-3235.

Guernec A, C. Berri, B. Chevalier, N. Wacrenier-Ceré, E. Le Bihan-Duval, and M. J. Duclos. 2003. Muscle development, insulin-like growth factor-I and myostatin mRNA levels in chickens selected for increased breast muscle yield. Growth. Horm. IGF. Res. 13:8-18.

Haddad, F., and G. R. Adams. 2004. Inhibition of MAP/ERK kinase prevents IGF-I-induced hypertrophy in rat muscles. J. Appl. Physiol. 96:203-210.

Han, J., S. H. Back, J. Hur, Y. H. Lin, R. Gildersleeve, J. Shan, C. L. Yuan, D. Krokowski, S. Wang, M. Hatzoglou, M. S. Kilberg, M. A. Sartor, R. J. Kaufman. 2013. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. Nat. Cell. Biol. 15:481-490.

He, L., K. Vasiliou, and D. W . Nebert. 2009. Analysis and update of the human solute carrier (SLC) gene superfamily. Hum. Genomics. 2:195-206.

Healy, K. D., J. B. Zella, M. J. Prahl, and H. F. De Luca. 2003. Regulation of the murine renal vitamin D receptor by 1,25-dihydroxyvitamin D_3 and calcium. Proc. Natl. Acad. Sci. 100:9733-9737.

Higgins, S. E., A. D. Wolfenden, G. Tellez, B. M. Hargis, and T. E. Porter. 2011. Transcriptional profiling of cecal gene expression in probiotic and *Salmonella*-challenged neonatal chicks. Poult. Sci. 90:901-913.

Hosokawa, H., T. Sawamura, S. Kobayashi, H. Ninomiya, S. Miwa, and T. Masaki. 1997. Cloning and characterization of a brain-specific cationic amino acid transporter. J. Biol. Chem. 272:8717-8722.

Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4:44-57.

Hutton, K. C., M. A. Vaughn, G. Litta, B. J. Turner, and J. D. Starkey. 2014. Effect of vitamin D status improvement with 25-hydroxycholecalciferol on skeletal muscle growth characteristics and satellite cell activity in broiler chickens. J. Anim. Sci. 92:3291-3299.

Hystad, M. E., J. H. Myklebust, T. H. Bo, E. A. Sivertsen, E. Rian, L. Forfang, E. Munthe, A. Rosenwald, M. Chiorazzi, I. Jonassen, L. M. Staudt, and E. B. Smeland. 2007. Characterization of early stages of human B cell development by gene expression profiling. J. Immunol. 179:3662–71.

Ito, K., and M. Groudine. 1997. A new member of the cationic amino acid transporter family is preferentially expressed in adult mouse brain. J. Biol. Chem. 272:26780-26786.

Juknat, A., M. Pietr, E. Kozela, N. Rimmerman, R. Levy, G. Coppola, D. Geschwind, and Z.Vogel. 2012. Differential transcriptional profiles mediated by exposure to the cannabinoids cannabidiol and Δ9-tetrahydrocannabinol in BV-2 microglial cells. Br. J. Pharmacol. 165:2512-2528.

Kefal. S, and N. Y. Toker. 2006. Effects of probiotics on some acute phase proteins in broilers exposed to *Salmonella* typhimurium lipopolysaccharides. Arch. Geflügelk. 70:270-277.

Kim, S., N. K. Shevde, and J. W. Pike. 2005. 1,25-Dihydroxyvitamin D_3 stimulates cyclic vitamin D receptor/retinoid X receptor DNA binding, co-activator recruitment, and histone acetylation in intact osteoblasts. J. Bone. Miner. Res. 20:305-317.

Kim, D., G. Pertea, C. Trapnell, H. Pimente, R. Kelley, and S. L. Salzberg. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome. Biol. 14:R36-R49.

Kommadath, A., H. Bao, A. S. Arantes, G. S. Plastow, C. K. Tuggle, S. M. Bearson, L. Guan le, and P. Stothard. 2014. Gene co-expression network analysis identifies porcine genes associated with variation in Salmonella shedding. BMC Genomics. 15:452-467.

Krämer. A., J. Green , J. Pollard, and S. Tugendreich. 2014. Causal analysis approaches in ingenuity pathway analysis (IPA). Bioinformatics. 30:523-530.

Kunita R., O. Nakabayashi, J. Y. Wu, Y. Hagiwara, M. Mizutani, M. Pennybacker, Y. T. Chen, and T. Kikuchi. 1997. Molecular cloning of acid a-glucosidase cDNA of Japanese quail (*Coturnix coturnix japonica*) and the lack of its mRNA in acid maltase deficient quails. Biochim. Biophys. Acta. 1362:269-278.

Kuznetsova, L. A., S. A. Plesneva, A. O. Shpakov, and T. S. Sharova. 2004. Dehydrogenase, and glycogen synthase in skeletal muscles of chick embryos and chickens. J. Evol. Biochem. Phys. 40:409-419.

Lee, K. W., S. H. Lee, H. S. Lillehoj, G. X. Li, S. I. Jang, U. S. Babu, M. S. Park, D. K. Kim, E. P. Lillehoj, A. P. Neumann, T. G. Rehberger, and G. R. Siragusa. 2010. Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. Poult. Sci. 89:203-216.

Leshem, Y., I. Gitelman, C. Ponzetto, and O. Halevy. 2002. Preferential binding of Grb2 or phosphatidylinositol 3-kinase to the met receptor has opposite effects on HGF-induced myoblast proliferation. Exp. Cell. Res. 274:288–298.

Lim, C. H., W. Jeong, W. Lim, J. Kim, G. Song, F. W. Bazer. 2012. Differential expression of select members of the SLC family of genes and regulation of expression by microRNAs in the chicken oviduct. Biol. Reprod. 87:145-166.

Liu, Q. C., X. H. Zha, H. Faralli, H. Yin, C. Louis-Jeune, E. Perdiguero, P. Muñoz-Cànoves, M. A. Rudnicki, M. Brand, C. Perez-Iratxeta, and F. J. Dilworth. 2012. Comparative expression profiling identifies differential roles for MyoGenin and p38a MAPK signaling in myogenesis. J. Mol. Cell. Biol. 4:386-397.

Liu, J., S. Guo, Q. Li, L. Yang, Z. Xia, L. Zhang, Z. Huang, and N. Zhang. 2013. Phosphoglycerate dehydrogenase induces glioma cells proliferation and invasion by stabilizing forkhead box M1. J. Neurooncol. 11:245-255.

Lu, J., S. Sanchez, C. Hofacre, J. J. Maurer, B. G. Harmon, and M. D. Lee. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. Appl. Environ. Microbiol. 69:901-908.

Lysne, V., B. Bjørndal, R. Vik, J. E. Nordrehaug, J. Skorve, O. Nygård, and R. K. Berge. 2015. A protein extract from chicken reduces plasma homocysteine in rats. Nutrients. 7:4498-4511.

Malila Y., K. M. Carr, C. W. Ernst, S. G. Velleman, K. M. Reed, and G. M. Strasburg. 2014. Deep transcriptome sequencing reveals differences in global gene expression between normal and pale, soft, and exudative turkey meat. J. Anim. Sci. 92:1250-1260.

Miraoui, H., K. Oudina, H. Petite, Y. Tanimoto, K. Moriyama, and P. J. Marie. 2009. Fibroblast growth factor receptor 2 promotes osteogenic differentiation in mesenchymal cells via ERK1/2 and protein kinase C signaling. J. Biol. Chem. 284:4897-4904.

Morales, A., F. Grageola, H. García, N. Arce, B. Araiza, J. Yáñez, and M. Cervantes. 2014. Performance, serum amino acid concentrations and expression of selected genes in pair-fed growing pigs exposed to high ambient temperatures. J. Anim. Physiol. Anim. Nutr. 98:928-35.

Morris, A., R. Shanmugasundaram. M. S. Lilburn, and R. K. Selvaraj. 2014. 25-Hydroxycholecalciferol supplementation improves growth performance and decreases inflammation during an experimental lipopolysaccharide injection. Poult. Sci. 93:1951-1956.

Mosaad, Y. M., R. E. Farag, M. M. Arafa, S. Eletreby, H. A. El-Alfy, B. S. Eldeek, and Z. M. Tawhid. 2010. Association of human leucocyte antigen class I (HLA-A and HLA-B) with chronic hepatitis C virus infection in egyptian patients. Scand. J. Immunol. 6:548-553.

Mutryn, M. F., E. M. Brannick, W. Fu, W. R. Lee, and B. Abasht. 2015. Characterization of a novel chicken muscle disorder through differential gene expression and pathway analysis using RNA-sequencing. BMC Genomics. 16:399-418.

Nguyen, A., P. Chen, and H. Cai. 2004. Role of CaMKII in hydrogen peroxide activation of ERK1/2, p38 MAPK, HSP27 and actin reorganization in endothelial cells. FEBS. Lett. 572:307-313.

Nurmi, E., and M. Rantala. 1973. New aspects of *Salmonella* infection in broiler production. Nature. 241:210-211.

Pesti, G. M. 2009. Impact of dietary amino acid and crude protein levels in broiler feeds on biological performance. J. Appl. Poult. Res. 18:477-486.

Piórkowska, K., K. Żukowski, J. Nowak, K. Połtowicz, K. Ropka-Molik, and A .Gurgul. 2016. Genome-wide RNA-Seq analysis of breast muscles of two broiler chicken groups differing in shear force. Anim. Genet. 47:68-80.

Robinson, M. D., D. J. McCarthy, and G. K. Smyth. 2010. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26:139-140.

Raman, K., H. Wang, M. J. Troncone, W. I Khan, G Pare, and J. Terry. 2015. Overlap chronic placental inflammation is associated with a unique gene expression pattern. PLoS One. 10:e0133738-e0133750.

Rucker, M., T. J. Fraites, S. L. Porvasnik, M. A. Lewis, I. Zolotukhin, D. A. Cloutier, B. J. Byrne. 2004. Rescue of enzyme deficiency in embryonic diaphragm in a mouse model of metabolic myopathy: Pompe disease. Development.13:3007-3019.

Russell, J., A. Bar, L. M. Sherwood, and S. Hurwitz. 1993. Interaction between calcium and 1,25-dihydroxyvitamin D_3 in the regulation of preproparathyroid hormone and vitamin D receptor messenger ribonucleic acid in avian parathyroids. Endocrinology. 132:2639-2644.

Sabourin, L. A., A. Girgis-Gabardo, P. Seale, A. Asakura, and M. A. Rudnicki. 1999. Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. J. Cell. Biol. 144:631-643.

Sedlackova, T., G. Repiska, P. Celec, T. Szemes, and G. Minarik. 2013. Fragmentation of DNA affects the accuracy of the DNA quantitation by the commonly used methods. Biological procedures online, fragmentation of DNA affects the accuracy of the DNA quantitation by the commonly used methods. Biol. Proced. Online. 15:5-13.

Sibut, V., C. Hennequet-Antier, E. Le Bihan-Duval, S. Marthey, M. J. Duclos, and C. Berri. 2011. Identification of differentially expressed genes in chickens differing in muscle glycogen content and meat quality. BMC Genomics. 12:112-125.

Simon, J. 1989. Chicken as a useful species for the comprehension of insulin action. Crit. Rev. Poult. Biol. 2:121-148.

Singh, K., and F. J. Dilworth. 2013. Differential modulation of cell cycle progression distinguishes members of the myogenic regulatory factors. FEBS. J. 280:3991-4003.

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

Shanmugasundaram, R., M. S. Lilburn, and R. K. Selvaraj. 2012. Effect of recycled litter on immune cells in the cecal tonsils of chickens. Poult. Sci. 91:95-100.

Shojadoost, B., S. Behboudi, A. I. Villanueva, J. T. Brisbin, A. A. Ashkar, and S. Sharif. 2015. Vitamin D_3 modulates the function of chicken macrophages. Res. Vet. Sci.100:45-51.

Sun, P., H. Enslen, P. Myung, and R. Maurer. 1994. Differential activation of CREB by Ca2+/calmodulin-dependent protein kinase type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev. 8:2527-2539.

Tam, S., M. S. Tsao, and J. D. McPherson. 2015. Optimization of miRNA-seq data preprocessing. Brief. Bioinform. 6:950-963.

Targowski, S. P., W. Klucinski, S. Babiker, and B. J. Nonnecke. 1986. Effect of ammonia on in vivo and in vitro immune responses. Infect. Immun. 43:289-293.

Torok, V. A., R. J. Hughes, K. Ophel-Keller, M. Ali, and R. MacAlpine. 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. Poult. Sci. 88:2474–2481.

Tesseraud, S., M. Abbas, S. Duchene, K. Bigot, P. Vaudin, J. Dupont. 2006. Mechanisms involved in the nutritional regulation of mRNA translation: features of the avian model. Nutr. Res. Rev. 19:104-116.

Vest, R. S., K. D. Davies, H. O'Leary, J. D. Port, and K. U. Bayer. 2007. Dual mechanism of a natural CaMKII inhibitor. Mol. Biol. Cell. 18: 5024–5033.

Vignale, K., E. S. Greene, J. V. Caldas, J. England, N. Boonsinchai, P. Sodsee, E. D. Pollock, S. Dridi, and C. N. Coon. 2015. 25-Hydroxycholecalciferol enhances male broiler breast meat yield through the mTOR Pathway. J. Nutr. 145:855-863.

Volkova, V. V., R. H. Bailey, and R. W. Wills. 2009. Salmonella in broiler litter and properties of soil at farm location. Plos. One. 4:e6403-e6409.

Waititu, S. M., A. Yitbarek, E. Matini, H. Echeverry, E. Kiarie, J. C. Rodriguez-Lecompte, and C. M. Nyachoti. 2014. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poult. Sci. 93:625-635.

Wang, Y., B. Lupiani, S. M. Reddy, S. J. Lamont, and H. Zhou. 2014. RNA-seq analysis revealed novel genes and signaling pathway associated with disease resistance to avian influenza virus infection in chickens. Poult. Sci. 93:485-493.

Wong, N., G. Morahan, M. Stathopoulos, J. Proietto, and S. Andrikopoulos. 2013. A novel mechanism regulating insulin secretion involving HERPUD1 in mice. Diabetologia. 56:1569-1576.

Wouters, B. G., and M. Koritzinsky. 2008. Hypoxia signaling through mTOR and the unfolded protein response in cancer. Nature. 8:851-864.

Yablonka-Reuveni Z., and B. M. Paterson. 2001. MyoD and myogenin expression patterns in cultures of fetal and adult chicken myoblasts. J. Mol. Histol. 49:455-462.

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.

Zhang, J., D Upadhya, L. Lu, and L. W Reneker. 2015. Fibroblast growth factor receptor 2 (FGFR2) is required for corneal epithelial cell proliferation and differentiation during embryonic development. Plos. One. 10:e1371-e1389.

Zhou, N., W. R. Lee, and B. Abasht. 2015. Messenger RNA sequencing and pathway analysis provide novel insights into the biological basis of chickens` feed efficiency. BMC. Genomics. 16:195-215.

4.6 TABLES

Table 4.1 Top biological functions of differentially-expressed genes at d 12 in pectoralis major of broiler chickens fed either vitamin D_3[1] or 25-hydroxycholecalciferol[2]; obtained by Ingenuity Pathway Analysis (IPA).

Diseases and Disorders	p-value Range [3]	Molecules [4]	Genes
Cancer	3.55E-02 - 5.79E-04	19	ART1, CHRND, ENPP2, FGFR2, HDHD3, HLA-A, IDH2, MBD2, MYOD1, PHGDH, PLOB, PPL, PYCRL, RARRES1, SLC1A4, SLC6A9, TNXB, TPPP3, TRIOBP
Hematological Disease	3.51E-02 - 5.79E-04	5	FGFR2, HLA-A, IDH2, MYOD1, PLOB
Organismal Injury and Abnormalities	3.55E-02 - 5.79E-04	21	ALDH18A1, ART1, CaMKIIA, CHRND, ENPP2, FGFR2, HDHD3, HLA-A, IDH2, MBD2, MYOD1, PHGDH, PLOB, PPL, PYCRL, RARRES1, SLC1A4, SLC6A9, TNXB, TPPP3, TRIOBP
Neurological Disease	3.36E-02 - 7.19E-04	17	ALDH18A1, ASNS, CaMKIIA, CHRND, ENPP2, FGFR2, HDHD3, HLA-A, IDH2, MBD2, MYOD1, PHGDH, PITPNC1, PLOB, SLC1A4, SLC6A9, TNXB, TPPP3, TRIOBP
Immunological Disease	3.36E-02 - 1.01E-03	5	FGFR2, HLA-A, IDH2, MYOD1, PLOB
Molecular and Cellular Functions			
Amino Acid Metabolism	2.57E-02 - 3.70E-05	6	ALDH18A1, ASNS, PHGDH, SLC1A4, SLC6A9, SLC7A3
Small Molecule Biochemistry	3.36E-02 - 3.70E-05	14	ALDH18A1, ART1, ASNS, CaMKIIA, CRHR2, ENPP2, FGFR2, PHGDH, PLOB, SLC1A4, SLC6A9, SLC7A3
Molecular Transport	2.97E-02 - 1.74E-04	11	CaMKIIA, CRHR2, ENPP2, FGFR2, HERPUD1, HLA-A, PHGDH, PITPNC1, SLC1A4, SLC6A9, SLC7A3
Cellular Assembly and Organization	2.73E-02 - 9.42E-04	5	CaMKIIA, ENPP2, FGFR2, PLOB, TNXB
Carbohydrate Metabolism	3.36E-02 - 1.62E-03	6	CaMKIIA, ENPP2, FGFR2, IDH2, PHGDH, PITPNCI
Physiological System Development and Function			
Organ Development	3.04E-02 - 1.53E-05	6	CaMKIIA, CHRND, CRHR2, FGFR2, HLA-A, MYOD1

Skeletal and Muscular System Development and Function	3.36E-02 - 1.53E-05	7	CaMKIIA, CHRND, CRHR2, FGFR2, HLA-A, MYOD1 , SLC6A9
Tissue Development	3.36E-02 - 1.53E-05	10	CaMKIIA, CHRND, CRHR2, EDF1, ENPP2, FGFR2, HLA-A, MYOD1, PHGDH, TNXB
Nervous System Development and Function	3.20E-02 - 9.42E-04	8	CaMKIIA, CRHR2, FGFR2, HLA-A, MYOD1 , PHDGH, PLOB, SLC6A9
Tissue Morphology	3.04E-02 - 9.42E-04	5	CaMKIIA, CRHR2, EMPP2, FGFR2, MYOD1

[1]Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[2]Birds fed a diet containing 2,760 IU/kg complete feed of 25OHD_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[3]Range of FDR-corrected p-values for all significant ($P < 0.05$) individual biological function terms grouped under each category, calculated by Fisher's exact test.

Table 4.2 Top biological functions of differentially-expressed genes at d 12 in pectoralis major of broiler chickens raised on Fresh[1] or Reused [2] litter; obtained by Ingenuity Pathway Analysis (IPA).

Diseases and Disorders	**p-value Range [3]**	**Molecules [4]**	**Genes**
Auditory Disease	1.06E-02 - 3.44E-04	1	SLC26A5
Connective Tissue Disorders	4.46E-02 - 3.44E-04	3	GAA, PHGDH, UBI
Developmental Disorder	4.46E-02 - 3.44E-04	3	GAA, PHGDH, UBI
Gastrointestinal Disease	3.03E-02 - 3.44E-04	5	GAA, IGJ, PHGDH, SLC26A5,UBI
Hereditary Disorder	4.98E-02 - 3.44E-04	4	GAA, PHGDH, SLC26A5, UBI
Molecular and Cellular Functions			
Amino Acid Metabolism	5.50E-03 - 3.44E-04	2	PHGDH, SLC38A3
Cellular Assembly and Organization	3.76E-02 - 3.44E-04	4	GAA, PHGDH, SLC26A5, UBI
Cellular Development	2.79E-02 - 3.44E-04	3	GAA, PHGDH, UBI
Cellular Growth and Proliferation	3.44E-04 - 3.44E-04	1	GAA
Molecular Transport	4.43E-02 - 3.44E-04	6	EXOX3L1, GAA, IGJ, PHGDH, SLC26A5, SLC38A3
Physiological System Development and Function			
Embryonic Development	1.64E-02 - 3.44E-04	2	GAA, PHGDH
Organ Development	1.95E-02 - 3.44E-04	3	GAA, PHGDH, SLC26A5
Organismal Development	2.96E-02 - 3.44E-04	2	GAA, PHGDH
Skeletal and Muscular System Development and Function	4.69E-02 - 3.44E-04	2	GAA, UBI
Tissue Development	1.64E-02 - 3.44E-04	3	GAA, PHGDH, UBI

[1] Fresh pine shavings litter.
[2] Reused pine shavings litter; litter was used for one previous flock.
[3]Range of FDR-corrected p-values for all significant ($P < 0.05$) individual biological function terms grouped under each category, calculated by Fisher's exact test.
[4] The number of differentially-expressed genes obtained by IPA.

Table 4.3 Top biological functions of differentially-expressed genes at d 12 in pectoralis major of broiler chickens fed vitamin D_3[3] and raised on either Fresh[1] or Reused litter[2]; obtained by Ingenuity Pathway Analysis (IPA).

Diseases and Disorders	**p-value Range [4]**	**Molecules [5]**	**Genes**
Developmental Disorder	7.38E-04 - 7.38E-04	1	PHGDH
Hereditary Disorder	7.38E-04 - 7.38E-04	1	PHGDH
Metabolic Disease	3.27E-02 - 7.38E-04	1	PHGDH
Neurological Disease	4.24E-02 - 7.38E-04	1	PHGDH
Organismal Injury and Abnormalities	7.38E-04 - 7.38E-04	3	ATP2C2, JCHAIN, PHGDH
Molecular and Cellular Functions			
Cell-To-Cell Signaling and Interaction	1.18E-02 - 1.73E-08	1	MPP7
Cellular Assembly and Organization	2.48E-02 - 1.73E-08	1	MPP7
Cellular Function and Maintenance	4.69E-02 - 7.38E-04	2	JCHAIN, MPP7
Cellular Growth and Proliferation	1.54E-02 - 7.38E-04	1	MPP7
Amino Acid Metabolism	4.34E-02 - 7.38E-04	1	CDK2AP1
Physiological System Development and Function			
Embryonic Development	4.69E-02 - 7.38E-04	2	CDK2AP1, PHGDH
Tissue Morphology	3.20E-02 - 9.38E-04	2	ATP2C2, JCHAIN
Hair and Skin Development and Function	3.91E-02 - 2.21E-03	1	CDK2AP1
Nervous System Development and Function	4.62E-02 - 2.21E-03	1	PHGDH
Organ Morphology	1.47E-02 - 3.69E-03	2	CDK2AP1, PHGDH

[1] Fresh pine shavings litter.
[2] Reused pine shavings litter; litter was used for one previous flock.
[3] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.
[4] Range of FDR-corrected p-values for all significant ($P < 0.05$) individual biological function terms grouped under each category, calculated by Fisher's exact test.
[5] The number of differentially-expressed genes obtained by IPA.

Table 4.4 Top biological functions of differentially-expressed genes at d 12 in pectoralis major of broiler chickens fed either vitamin D_3[1] or 25-hydroxyvitamin D_3[2] and raised on Reused[3] litter; obtained by Ingenuity Pathway Analysis (IPA).

Diseases and Disorders	**p-value Range [4]**	**Molecules [5]**	**Genes**
Connective Tissue Disorders	7.38E-04 - 7.38E-04	1	ALDH18A1
Dermatological Diseases and Conditions	7.38E-04 - 7.38E-04	1	ALDH18A1
Developmental Disorder	3.27E-02 - 7.38E-04	5	ALDH18A1, ASNS, CTH, MYOG, PHGDH
Hereditary Disorder	4.24E-02 - 7.38E-04	5	ALDH18A1, ASNS, CTH, MYOG, PHGDH
Metabolic Disease	7.38E-04 - 7.38E-04	3	ALDH18A1, CTH,PHGDH
Molecular and Cellular Functions			
Amino Acid Metabolism	1.18E-02 - 1.73E-08	5	ALDH18A1, ASNS, CTH, PHGDH, SLC6A9
Small Molecule Biochemistry	2.48E-02 - 1.73E-08	5	ALDH18A1, ASNS, CTH, PHGDH, SLC6A9
Cell-To-Cell Signaling and Interaction	4.69E-02 - 7.38E-04	4	CTH, MPP7, MYOG, SLC6A9
Molecular Transport	1.54E-02 - 7.38E-04	6	CTH, HERPUD1, MYOG, PHGDH, SLC16A6, SLC6A9
Post-Translational Modification	4.34E-02 - 7.38E-04	2	ASNS, CTH
Physiological System Development and Function			
Nervous System Development and Function	4.69E-02 - 7.38E-04	3	CTH, PHGDH, SLC6A9
Respiratory System Development and Function	3.20E-02 - 9.38E-04	3	CTH, MYOG, SLC6A9
Skeletal and Muscular System Development and Function	3.91E-02 - 2.21E-03	1	MYOG
Tissue Morphology	4.62E-02 - 2.21E-03	3	CTH, MYOG, SLC6A9
Cardiovascular System Development and Function	1.47E-02 - 3.69E-03	1	CTH

[1] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[2] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

[3] Reused pine shavings litter; litter was used for one previous flock.

[4] Range of FDR-corrected p-values for all significant ($P < 0.05$) individual biological function terms grouped under each category, calculated by Fisher's exact test.

[5] The number of differentially-expressed genes obtained by IPA.

Table 4.5 Top biological functions of all differentially-expressed genes at d 12 in pectoralis major of broiler chickens obtained by Ingenuity Pathway Analysis (IPA).

Diseases and Disorders	**p-value Range [1]**	**Molecules [2]**	**Genes**
Developmental Disorder	2.26E-02 - 9.95E-05	14	ALDH18A1, ASNS, CHRND, CRHR2 , ENPP2, FGFR2, HLA-A, IDH2, MODY1, MYOG, SLC1A4, TMP2, TNXB, UBI
Skeletal and Muscular Disorders	2.96E-02 - 4.48E-04	14	ART1, CAMK2A, CHRND, FGFR2 , HERPUD1, HLA-A, IDH2, MYOG, SLC1A4, SLC7A3, TMP2, TNXB, UBI
Cancer	2.98E-02 - 1.18E-03	29	ALDH18A1, ART1, ASNS, ATP2X2, CAMK2A, CHRND, CRHR2 , ENPP2, FGFR2, HERPUD1, HLA-A, IDH2, JCHIAN, MPP7, MOYD1, MYOG, NOS1AP, PPL, PPP1R36, SLC16A6, SLC1A4, ALC38A3, SLC7A3, SSH2, TNXB, TMP2, TPPP3, TRIOBP, UBI
Organismal Injury and Abnormalities	2.98E-02 - 1.18E-03	29	ALDH18A1, ART1, ASNS, ATP2X2, CAMK2A, CHRND, CRHR2 , ENPP2, FGFR2, HERPUD1, HLA-A, IDH2, JCHIAN, MPP7, MOYD1, MYOG, NOS1AP, PPL, PPP1R36, SLC16A6, SLC1A4, ALC38A3, SLC7A3, SSH2, TNXB, TMP2, TPPP3, TRIOBP, UBI
Renal and Urological Disease	1.50E-02 - 1.18E-03	10	CAMK2A, CHRND, FGFR2, IDH2, HLA-A, PPL, SLC1A4, TNXB, TRIOBP, UBI
Molecular and Cellular Functions			
Amino Acid Metabolism	2.10E-02 - 2.20E-05	5	ALDH18A1, ASNS, SLC1A4, SLC38A3, SLC7A3,

Molecular Transport	2.77E-02 - 2.20E-05	12	ATP2C2, CAMK2A, CHRND, CRHR2, ENPP2, FGFR2, HERPUD1, HLA-A, SLC16A6, SLC1A4, SLC38A3, SLC7A3
Small Molecule Biochemistry	2.77E-02 - 2.20E-05	10	ALDH18A1, ART1, ASNS, CAMK2A,CRHR2, ENPP2, FGFR2, SLC1A4, SLC38A3, SLC7A3
Cell Cycle	2.39E-02 - 2.62E-04	5	CAMK2A, FGFR2, MYOD1, MYOG, SSH2
Cellular Development	2.83E-02 - 4.44E-04	9	ASNS, CAMK2A, CDK2AP1, CRHR2, FGFR2, IDH2, MYOD1, MYOG, TPM2
Physiological System Development and Function			
Organ Development	2.83E-02 - 1.32E-05	7	CAMK2A, CHRND, CRHR2, FGFR2, HLA-A ,MYOD1, MYOG
Skeletal and Muscular System Development and Function	2.83E-02 - 1.32E-05	8	CAMK2A, CHRND, CRHR2, FGFR2, HLA-A ,MYOD1, MYOG, UBI
Tissue Development	2.83E-02 - 1.32E-05	10	CAMK2A, CHRND, CRHR2, ENPP2, FGFR2, HLA-A ,MYOD1, MYOG, TNXB, TPM2
Tissue Morphology	2.69E-02 - 3.72E-04	9	CAMK2A, CDK2AP1, CRHR2, ENPP2, FGFR2, HLA-A, JCHAIN, MYOD1, MYOG
Embryonic Development	2.83E-02 - 4.44E-04	7	CAMK2A, CDK2AP1, ENPP2, FGFR2, HLA-A , MYOD1, MYOG

[1] Range of FDR-corrected p-values for all significant ($P < 0.05$) individual biological function terms grouped under each category, calculated by Fisher's exact test.
[2] The number of differentially-expressed genes obtained by IPA

Table 4.6 List of the selected endogenous and target genes used for real-time PCR for pectoralis major of broiler chickens at d 12.

Gene symbol	Type		Primer sequence
RNA18S	Endogenous	Forward	TCCCCTCCCGTTACTTGGAT
		Reverse	GCGCTCGTCGGCATGTA
ACTB	Endogenous	Forward	CTGGCACCTAGCACAATGAA
		Reverse	CATCGTACTCCTGCTTGCTG
GAPDH	Endogenous	Forward	GAGGGTAGTGAAGGCTGCTG
		Reverse	CATCAAAGGTGGAGGAATGG
CaMKIIA	Target	Forward	CCCGAAGCGAAAGATCTCATC
		Reverse	TTCAGGCAATCCACCGTC
BFIV21	Target	Forward	CAGTTCCAGAGGCAGTTCC
		Reverse	CCTTCCCGTATTCCACATATCTC

Table 4.7 Number of differentially-expressed genes at d 12 in pectoralis major of broiler chickens obtained from the comparison between birds fed vitamin D_3 or 25-hydroxycholecalciferol and raised on either Fresh or Reused litter from 27 samples.

Contrast	Total DE	Down-regulated	Up-regulated
D vs 25OHD* Reused[1]	17	6	11
D vs 25OHD* Fresh[2]	1	1	-
D vs 25OHD[3]	34	18	16
Fresh vs Reused* D[4]	7	2	5
Fresh vs Reused* 25OHD[5]	3	2	1
Fresh vs Reused[6]	9	6	3

[1] Interaction between vitamin D_3 (2,760 IU/Kg feed) and 25-hydroxycholecalciferol (2,760 IU/Kg feed) in the Reused litter (litter was used by one previous flock).
[2] Interaction between vitamin D_3 (2,760 IU/Kg feed) and 25-hydroxycholecalciferol (2,760 IU/Kg feed) in the Fresh litter.
[3] Interaction between vitamin D_3 (2,760 IU/Kg feed) and 25-hydroxycholecalciferol (2,760 IU/Kg) in the Fresh litter.
[4] Interaction between vitamin D_3 (2,760 IU/Kg feed) and 25-hydroxycholecalciferol (2,760 IU/Kg feed).
[5] Interaction between Fresh and Reused litter (Reused litter was used by one previous flock) in vitamin D_3 (2,760 IU/Kg feed).
[6] Interaction between Fresh and Reused litter (Reused litter was used by one previous flock) in 25-hydroxycholecalciferol (2,760 IU/Kg feed).
[7] Interaction between Fresh and Reused litter (Reused litter was used by one previous flock).

Table 4.8 Differentially-expressed genes at 12 d (p-value < 0.05) in pectoralis major of broiler chickens fed various vitamin D treatments and raised on either Fresh or Reused litter.

			Gene Name	
Treatment			**CaMKIIA**[1]	**BFIV21**[2]
Fresh[4]		n[3]	Fold change	
	D[6]	4	1.75[b]	1.60[a]
	25OHD[7]	4	2.09[a]	1.67[a]
	D+25OHD[8]	4	2.00[ab]	1.62[a]
	Dx2[9]	4	2.00[ab]	1.64[a]
Reused[5]				
	D	4	1.61[b]	1.50[b]
	25OHD	4	2.13[a]	1.74[a]
	D+25OHD	4	1.91[ab]	1.83[a]
	Dx2	4	1.91[ab]	1.55[ab]
	Pooled SEM		0.149	0.120
Source of variance		DF		
Diet		3	0.0408	0.0159
Litter		1	0.3927	0.4542
Diet*Litter		3	0.8711	0.0450

[a,b] Treatment means within the same column within effect with no common superscripts are significantly different (P < 0.05).
[1] Calcium/calmodulin-dependent protein kinase II alpha.
[2] MHC class I alpha chain 2.
[3] Pen was the experimental unit.
[4] Fresh pine shavings litter.
[5] Reused pine shavings litter; litter was used by one previous flock.
[6] Birds fed a diet containing 2,760 IU/kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.
[7] Birds fed a diet containing 2,760 IU/kg complete feed of $25OHD_3$ as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.
[8] Birds fed the vitamin D_3 diet, plus 2,760 IU/kg complete feed of $25OHD_3$ from 0 to 41 d.
[9] Birds fed a diet containing 5,520 IU /kg complete feed of vitamin D_3 as the sole supplemental source of vitamin D_3 activity from 0 to 41 d.

4.7 FIGURES

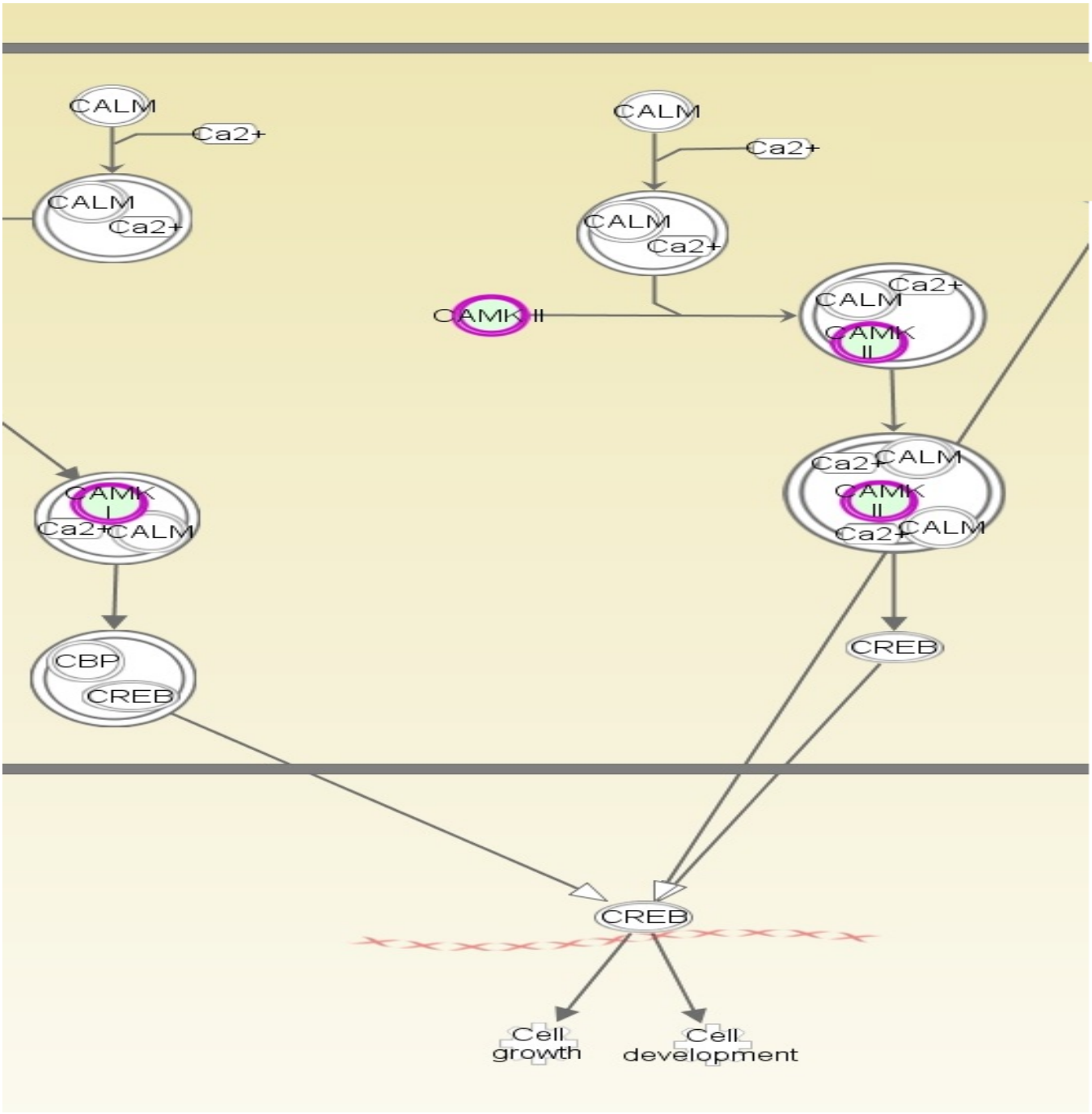

Figure 4.1. Calcium signaling pathway identified by IPA. cAMP responsive element binding protein (CREB), CREB binding protein (CBP) , and cAMP response element modulator (CREM) CAMK (Calmodulin Kinases).

Path Designer Muscle development 2

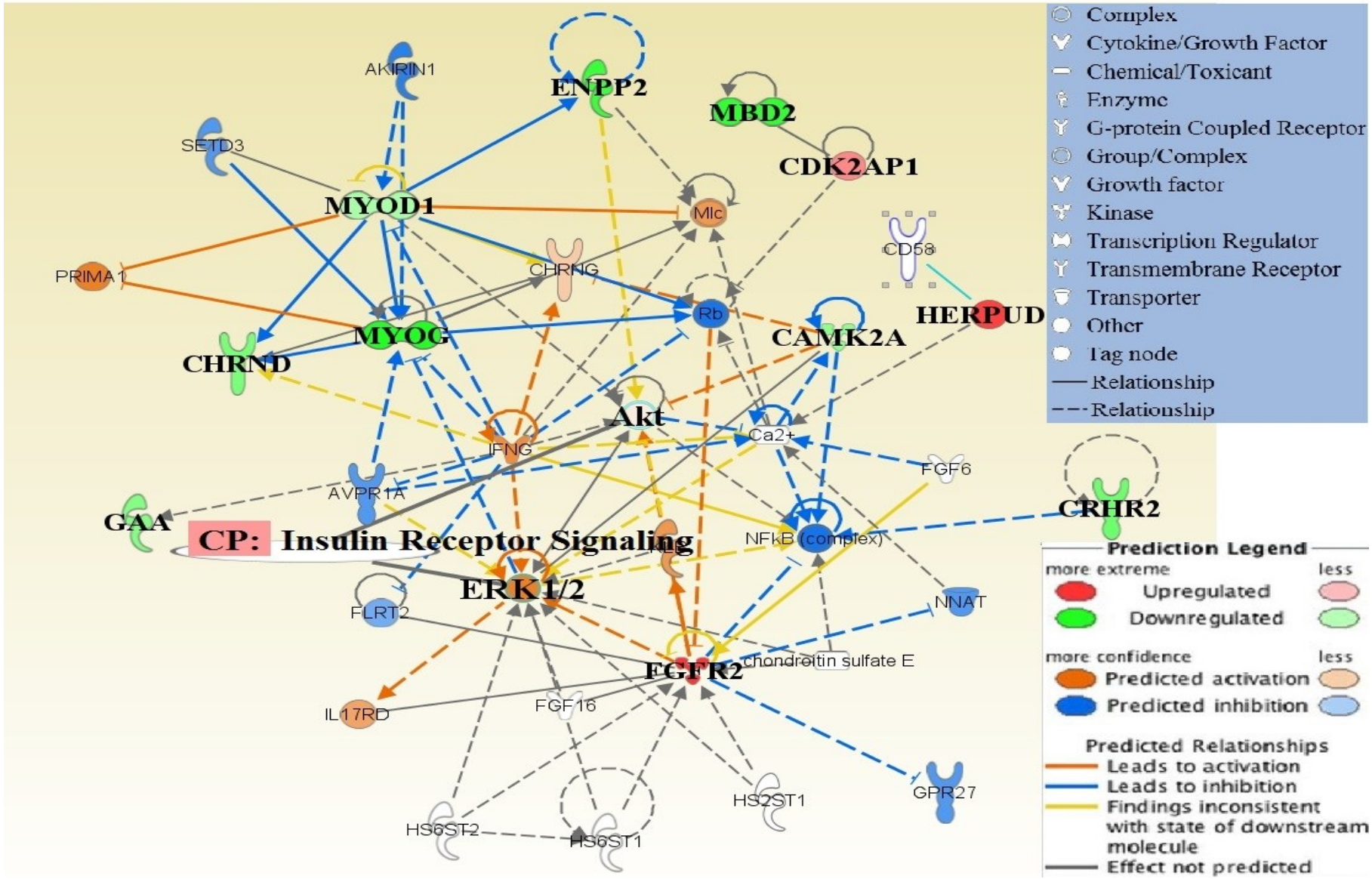

Figure 4. 2 Gene interaction network between the 11 differentially expressed genes (bolded in this network) across treatment effects related to muscle development in pectoralis major of broiler chicken at d 12. Canonical pathway (CP); Calcium/calmodulin-dependent protein kinase II alpha (CaMKIIA); Cyclin-dependent kinase 2 associated protein 1 (CDK2AP1); Corticotropin releasing hormone receptor 2 (CRHR2); Cholinergic receptor, nicotinic, delta (CHRND); Methyl-CpG binding domain protein 2, (MBD2); Ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2); Fibroblast growth factor receptor 2 (FGFR2); Glucosidase, alpha; acid (GAA); Homocysteine-inducible, endoplasmic reticulum stress-inducible (HERPUD1); Myogenic differentiation 1 (MyoD1); and Myogenin (myogenic factor 4) (MyoG). CaMKIIA, CRHR2, CHRND, ENPP2, MBD2, and MyoD1 had higher expression in $25OHD_3$-fed birds relative to those fed vitamin D_3. HERPUD1 and FGFR2 had higher expression in vitamin D_3-fed birds relative to those fed-$25OHD_3$. MyoG had higher expression in birds fed $25OHD_3$ relative to those fed vitamin D_3 in the Reused litter. GAA had higher expression in bird reared on Reused litter as compared to those reared on Fresh litter. CDK2AP1had higher expression in birds reared on Fresh litter relative to those on Reused litter when fed vitamin D_3.

Other genes and molecules are: Akirin 1 (AKIRIN1); phosphoinositide 3-kinase though (Akt); Arginine Vasopressin Receptor 1A (AVPR1A); CD58 Molecule (CD58); Cholinergic receptor, nicotinic, gamma (Muscle) (CHRNG); Extracellular signal-regulated kinases (ERK1/2); Fibroblast growth factor 16 (FGF16); Fibroblast growth factor 6 (FGF6); Fibronectin leucine rich transmembrane protein 2 (FLRT2); Interleukin 17 receptor D (IL17CD); G protein-coupled receptor 27 (CPR27); Heparan sulfate 2-o-sulfotransferase (HS2ST2) ; Heparan sulfate 6-o-sulfotransferase 1 (HS6ST1) ; Heparan sulfate 6-o-sulfotransferase 2 (HS6ST2); Interferon, gamma (INFG); Myc proto-oncogene protein (MIc); Proline rich membrane anchor 1 (PRIMA1); Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFKB); SET domain containing 3 (SETD3).

Path Designer immunity main

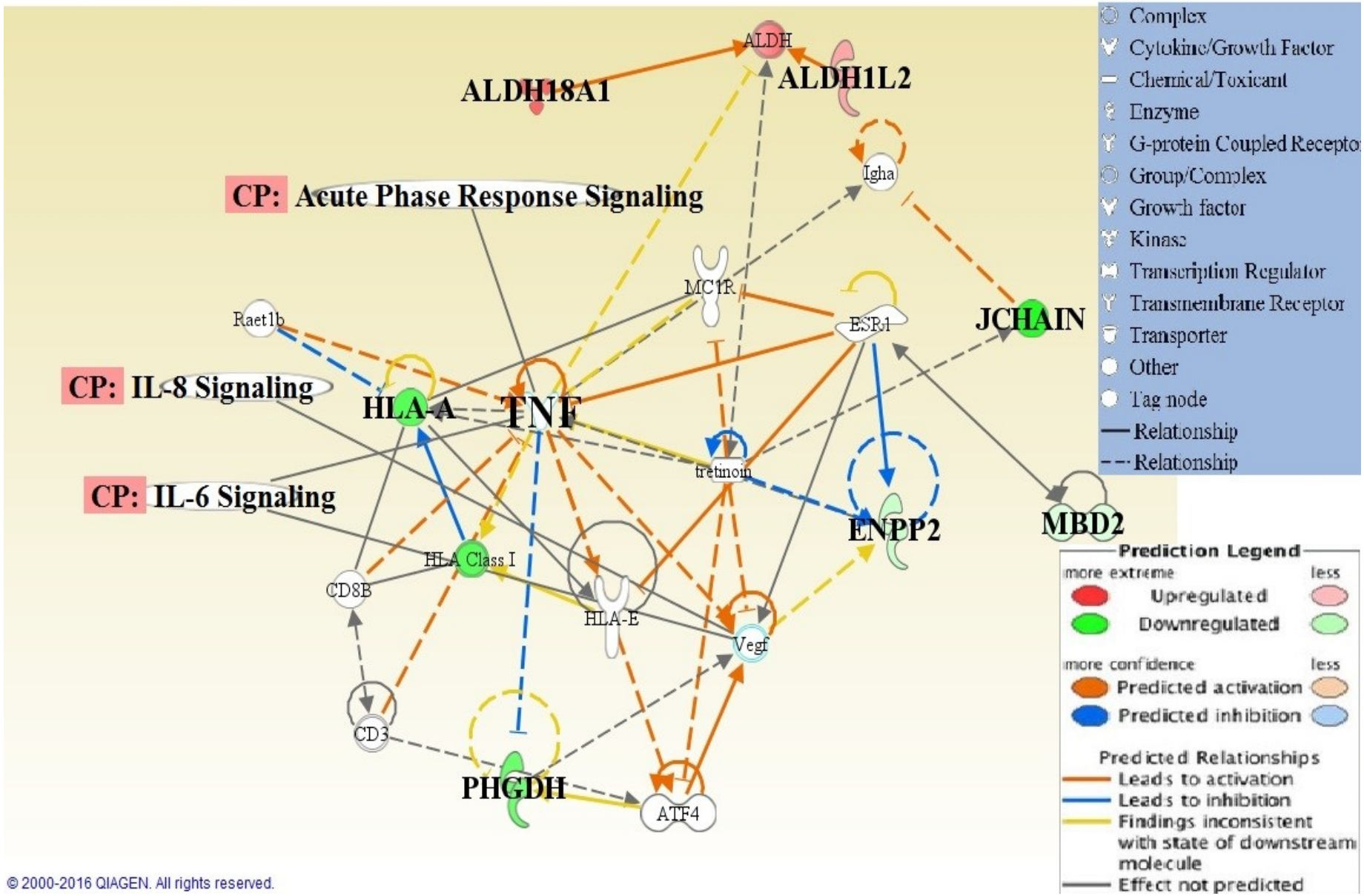

Figure 4. 3. The gene interaction network between the 7 differentially expressed genes (bolded in this network) across treatment effects related to immune response and inflammation in pectoralis major of broiler chicken at d 12. Canonical pathway (CP); Aldehyde dehydrogenase 18 family, member A1 (ALDH18A1); Aldehyde dehydrogenase 1 family, member L2 (ALDH1L2); Ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2); major histocompatibility complex, class I, A, (HLA-A); joining chain of multimeric IgA and IgM (JCHAIN); Methyl-CpG binding domain protein 2, MBD2; Phosphoglycerate dehydrogenase, PHGDH. ENPP2, HLA-A, and MBD2 had higher expression in $25OHD_3$-fed birds relative to those fed vitamin D_3. ALDH18A1 and PHGDH had higher expression in vitamin D_3-fed birds relative to those fed-$25OHD_3$. ALDH18A1 and ALDH1L2 had higher expression in birds fed vitamin D_3 relative to $25OHD_3$ those fed in the Reused litter. JCHAIN and PHGDH had higher expression in bird reared on Reused litter as compared to those reared on Fresh litter.

Other genes and molecules are: Activating transcription factor 4(ATF4); CD3 Molecule (CD3); CD8b Molecule (CD8B); Major histocompatibility complex, class I (HLA- class I); major histocompatibility complex, class E (HLA-E); Estrogen Receptor 1 (ES1R); Immunoglobulin Heavy Constant Alpha (Igha); Melanocortin 1 receptor (MC1R); Retinoic acid early transcript 1B (Raet1b);Tumor necrosis factor (TNF); Vascular endothelial growth factor (Vegf).

Amino Acid Metabolism 2

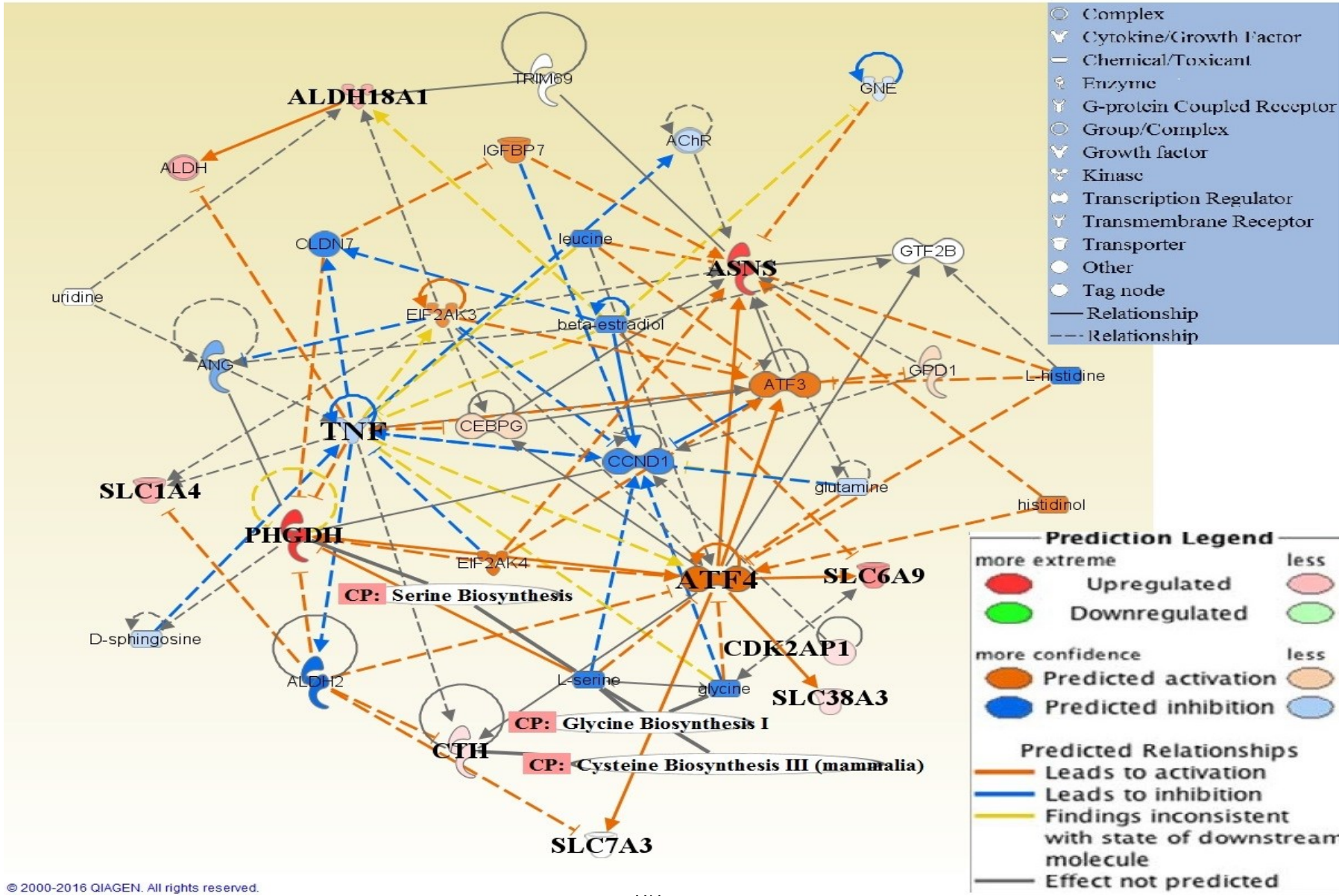

Figure 4. 4 The gene interaction network between the 9 differentially expressed genes (bolded in this network) across treatment effects related to amino acid metabolism in pectoralis major of broiler chicken at d 12. Canonical pathway (CP); Aldehyde dehydrogenase 18 family, member A1, ALDH18A1; Asparagine synthetase (ASNS); Cystathionine gamma-lyase, CTH; Phosphoglycerate dehydrogenase, PHGDH; Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4 (SLC1A4); Solute carrier family 7 (cationic amino acid transporter, y+ system), member 3 (SLC7A3); Solute carrier family 6 (neurotransmitter transporter, glycine), member 9 (SLC6A9); and Solute carrier family 7 (cationic amino acid transporter, y+ system), member 3 (SLC38A3). ALDH18A1, CTH, SLC1A4 and SLC7A3 had higher expression in vitamin D_3-fed birds relative to those fed-25OHD_3. ALDH18A1, ASNS, SLC6A9, and SLC38A3 had higher expression in birds fed vitamin D_3 relative to 25OHD_3 those fed in the Reused litter. PHGDH and SLC38A3 had higher expression in bird reared on Reused litter as compared to those reared on Fresh litter.

Other genes and molecules are: acetylcholine receptor (AChR); Aldehyde dehydrogenase 1 family (ALDH); Angiogenin, pibonuclease, RNase A family, 5 (ANG); Asparagine synthetase (ASNS); Activating transcription factor 4 (ATF4); Activating transcription factor 3 (ATF3); cyclin D1 (CCND1); CCAAT/enhancer binding protein (C/EBP), Gamma (CEBPG); Eukaryotic Translation initiation factor 2-alpha kinase 3; Translation initiation factor 2-alpha kinase 4 (EIF2AK4); Glucosamine (GNE); Glycerol-3-phosphate dehydrogenase 1 (GPD1); General transcription factor IIB (GTF2B); Insulin-like growth factor binding protein 7(IGFBP7);Tumor necrosis factor (TNF); Tripartite Motif Containing 69 (TRIM69).

Path Designer imprtant genes 2

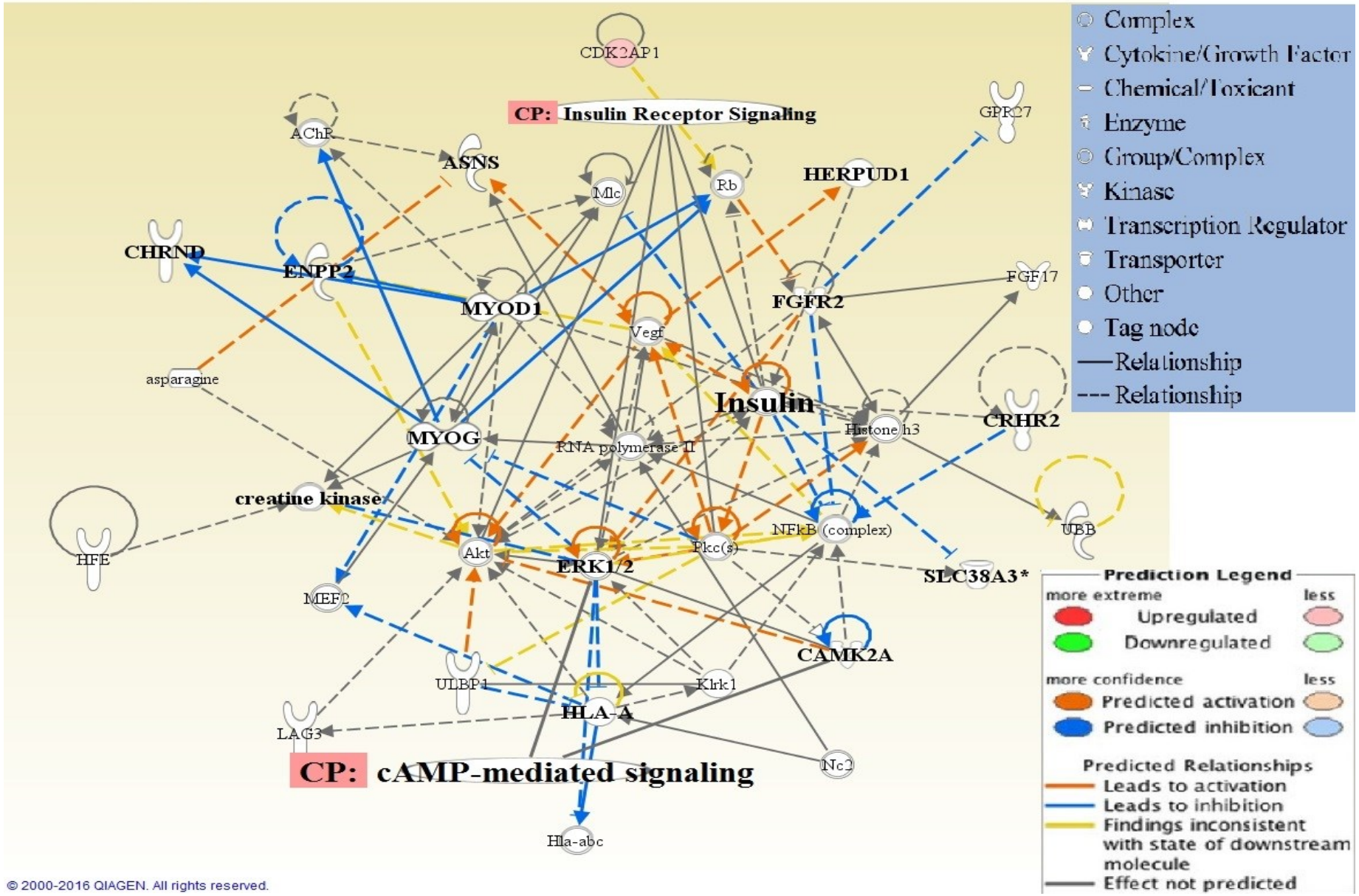

Figure 4.5 The gene interaction network between the 11 differentially expressed genes (bolded in this network) across treatment effects related to growth and immune response in pectoralis major of broiler chicken at d 12. Canonical pathway (CP); Asparagine synthetase (ASNS); Calcium/calmodulin-dependent protein kinase II alpha (CaMKIIA); Corticotropin releasing hormone receptor 2 (CRHR2); Cholinergic receptor, nicotinic, delta (CHRND); Ectonucleotide pyrophosphatase/ phosphodiesterase 2 (ENPP2); Fibroblast growth factor receptor 2 (FGFR2); Homocysteine-inducible, endoplasmic reticulum stress-inducible (HERPUD1); major histocompatibility complex, class I, A, (HLA-A); Myogenic differentiation 1 (MyoD1); and Myogenin (myogenic factor 4) (MyoG); Solute carrier family 7 (cationic amino acid transporter, y+ system), member 3 (SLC38A3). CaMKIIA, CRHR2, CHRND, ENPP2, and MyoD1 had higher expression in $25OHD_3$-fed birds relative to those fed vitamin D_3. ASNS, HERPUD1 and FGFR2 had higher expression in vitamin D_3-fed birds relative to those fed-$25OHD_3$. MyoG had higher and ASNS lower expression in birds fed $25OHD_3$ relative to those fed vitamin D_3 in the Reused litter. SLC38A3 was highly expressed in bird reared on Reused litter as compared to those reared on Fresh litter.

Other genes and molecules are: acetylcholine receptor (AChR); phosphoinositide 3-kinase though (Akt); Arginine Vasopressin Receptor 1A (AVPR1A); Cyclin-dependent kinase 2 associated protein 1 (CDK2AP1); Extracellular signal-regulated kinases (ERK1/2); Fibroblast growth factor 17 (FGF17); G protein-coupled receptor 27 (GPR27); MHC class I antigen (Hla-abc); Lymphocyte-activation gene 3 (LAG3); Myocyte enhancer factor 2 (MEF2); Myc proto-oncogene protein (MIc); Negative cofactor 2 (NC2); Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFKB); Protein kinase C (Pkc) Retinoblastoma (Rb); Tumor necrosis factor-receptor like protein (K1rk1) Ubiquitin B (UBB); UL16 Binding Protein 1 (ULBP1) ; Vascular endothelial growth factor (Vegf).

Appendix 4. 1 The list of differentially-expressed genes obtained from the comparison between dietary inclusion of vitamin D_3 or 25-hydroxycholecalciferol in broiler chickens raised on either Fresh or Reused litter in pectoralis major of at d 12

	Spot Annotation	Log Fold Change	P-value
Fresh vs Reused* D[1]			
	Immunoglobulin J polypeptide (IGJ)	-3.06212	3.54E-08
	Metallothionein 4 (MT4)	1.384241	2.01E-06
	ATPase, Ca++ transporting, type 2C, member 2 (ATP2C2)	1.451476	2.13E-05
	Phosphoglycerate dehydrogenase (PHGDH)	-2.77385	5.12E-05
	Membrane protein, palmitoylated 7 (MPP7)	0.550475	5.24E-05
	Protein phosphatase 1, regulatory subunit 36 (PPP1R36)	0.893942	5.50E-05
	Cyclin-dependent kinase 2 associated protein 1 (CDK2AP1)	0.609579	7.53E-05
Fresh vs Reused* 25OHD[2]			
	Phospholipase A2, group VII (PLA2G7)	0.699898	4.07E-07
	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 3 (SLC38A3)	-0.69645	5.78E-06
	Tubulin, alpha-like 3 (TUBAL3)	-1.31928	1.28E-05
D vs 25OHD* Reused[3]			
	Myogenin (myogenic factor 4) (MYOG)	-0.77638	6.25E-08
	Metallothionein 4 (MT4)	-1.41738	7.95E-07
	Asparagine synthetase (ASNS)	2.540312	1.27E-06
	Phosphoglycerate dehydrogenase (PHGDH)	3.27761	5.72E-06
	WD repeat domain 24 (WDR24)	-0.59113	6.48E-06
	Aldehyde dehydrogenase 18 family, member A1 (ALDH18A1)	1.285109	1.32E-05
	ChaC glutathione-specific gamma-glutamylcyclotransferase 1 (CHAC1)	3.754428	2.42E-05
	MHC class I alpha chain 2 (BFIV21)	-0.96733	4.08E-05
	Pyrroline-5-carboxylate reductase 2-like (PYCR2)	1.099596	6.04E-05
	Solute carrier family 16, member 6 (SLC16A6)	0.917808	1.10E-04
	Cystathionine gamma-lyase (CTH)	0.861556	1.80E-04
	Aldehyde dehydrogenase 1 family, member L2 (ALDH1L2)	0.544284	1.96E-04
	Homocysteine-inducible, endoplasmic reticulum stress-inducible (HERPUD1)	1.312727	1.99E-04
	Membrane protein, palmitoylated 7 (MPP7)	-0.51003	2.07E-04
	Cytoplasmic phosphatidylinositol transfer protein 1-like (PYCRL)	2.146083	2.18E-04
	Solute carrier family 6 (neurotransmitter transporter, glycine), member 9 (SLC6A9)	1.727712	2.19E-04
	Haloacid dehalogenase-like hydrolase domain containing (HDHD3)	-0.50178	2.19E-04
D vs 25OHD* Fresh[4]			
	Methyl-CpG binding domain protein 2 (MBD2)	-0.71624	2.14E-07

D vs 25OHD[5]

Tenascin XB (TNXB)	-0.63789	1.19E-08
Homocysteine-inducible, endoplasmic reticulum stress-inducible (HERPUD1)	0.983578	2.03E-06
Tubulin polymerization-promoting protein family member 3 (TPPP3)	-0.44411	4.86E-06
Periplakin (PPL)	-0.86207	6.19E-06
Phosphatidylinositol transfer protein, cytoplasmic 1 (PITPNC1)	-0.58452	7.11E-06
Nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP)	-0.42509	1.17E-05
Cytoplasmic phosphatidylinositol transfer protein 1-like (PYCRL)	1.744631	1.29E-05
Phosphoglycerate dehydrogenase (PHGDH)	2.394465	1.96E-05
Polymerase (DNA Directed), Beta (POLB)	0.334523	2.80E-05
Calcium/calmodulin-dependent protein kinase II alpha (CaMKIIA)	-0.39706	4.56E-05
Corticotropin releasing hormone receptor 2 (CRHR2)	-0.50827	5.27E-05
Solute carrier family 7 (cationic amino acid transporter, y+ system), member 3 (SLC7A3)	0.618809	6.86E-05
Myogenic differentiation 1 (MYOD1)	-0.30266	8.71E-05
Pyrroline-5-carboxylate reductase 2-like (PYCR2)	0.787053	9.39E-05
Cholinergic receptor, nicotinic, delta (CHRND)	-0.45972	1.30E-04
Tubulin, gamma complex associated protein 4 (TUBGCP4)	0.334023	1.42E-04
Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4 (SLC1A4)	0.931226	1.63E-04
Haloacid dehalogenase-like hydrolase domain containing (HDHD3)	-0.39717	1.65E-04
Endothelial differentiation-related factor 1 (EDF1)	0.328593	1.68E-04
Fibroblast growth factor receptor 2 (FGFR2)	1.05931	1.80E-04
Retinoic acid receptor responder (tazarotene induced) 1 (RARRES1)	0.334567	1.91E-04
Asparagine synthetase (ASNS)	2.138674	2.38E-04
Solute carrier family 6 (neurotransmitter transporter, glycine), member 9 (SLC6A9)	1.28906	3.16E-04
Aldehyde dehydrogenase 18 family, member A1 (ALDH18A1)	0.960164	3.19E-04
Ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2)	-0.68546	3.27E-04
Cationic amino acid transporter-1 (CAT-1)	1.031051	3.72E-04
Slingshot protein phosphatase 2 (SSH2)	-0.41005	3.74E-04
Socitrate dehydrogenase 2 (NADP+), mitochondrial (IDH2)	-0.32268	3.75E-04
ADP-ribosyltransferase 1 (ART1)	-0.50736	3.95E-04
TRIO and F-actin-binding protein-like (TRIOBP)	-0.45284	3.97E-04
Class I histocompatibility antigen, F10 alpha chain-like , mRNA (LOC769716)	-2.19869	4.00E-04
Cha C, cation transport regulator homolog 1 (CHAC1)	2.531763	4.10E-04

	Class I histocompatibility antigen, F10 alpha chain-like, transcript variant X6, misc_RNA (LOC417083)	-2.27074	4.21E-04
	Methyl-CpG binding domain protein 2 (MBD2)	-0.45667	4.35E-04
Fresh vs Reused[6]			
	Immunoglobulin J polypeptide (IGJ)	-2.95125	2.13E-12
	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 3 (SLC38A3)	-0.50377	1.12E-06
	Pancreatic progenitor cell differentiation and proliferation factor (PPDPF)	-0.51596	1.43E-05
	Glucosidase, alpha; acid (GAA)	-0.42813	3.74E-05
	Exocyst complex component 3-like 1 (EXOC3L1)	-0.41029	4.31E-05
	Solute carrier family 26 (anion exchanger), member 5 (SLC26A5)	0.440593	5.10E-05
	Ubiquitin I (UBI)	0.412169	6.19E-05
	Tropomyosin 2 (Beta) (TPM2)	0.726447	7.57E-05
	Phosphoglycerate dehydrogenase (PHGDH)	-1.92721	1.15E-04

[1] Interaction between Fresh and Reused litter (Reused litter was used by one previous flock) in vitamin D_3 (2,760 IU/Kg feed).
[2] Interaction between Fresh and Reused litter (Reused litter was used by one previous flock) in 25-hydroxycholecalciferol (2,760 IU/Kg feed).
[3] Interaction between vitamin D_3 (2,760 IU/Kg feed) and 25-hydroxycholecalciferol (2,760 IU/Kg feed) in the Reused litter (litter was used by one previous flock).
[4] Interaction between vitamin D_3 (2,760 IU/Kg feed) and 25-hydroxycholecalciferol (2,760 IU/Kg) in the Fresh litter.
[5] Interaction between vitamin D_3 (2,760 IU/Kg feed) and 25-hydroxycholecalciferol (2,760 IU/Kg) in the Fresh litter.
[6] Interaction between Fresh and Reused litter (Reused litter was used by one previous flock).

5. RESEARCH SYNTHESIS

5.1 SUMMARY

Used litter may contain pathogenic bacteria which could cause systemic inflammation when birds are grown on this litter (Torok et al., 2009; Shanmugasundaram et al., 2012). Vitamin D is involved in calcium and phosphorous absorption and bone mineralization, as well as having a regulatory effect on the immune system and muscle development in broiler chickens (Rama-Rao et al., 2006; 2009; Morris et al., 2014; Boland et al., 1995). Wheat-soybean meal-based diets were fed to Ross 308 chicks (n=512; 4.18 birds/m^2) for 41 d in a 4 x 2 factorial arrangement with 4 pens/treatment. Dietary treatments were: vitamin D_3 at 2,760 IU/kg feed (D; Control); 25OH D_3 at 2,760 IU/kg feed (25OHD); D_3 at 2,760 IU/kg plus 25OH D_3 at 2,760 IU/kg feed (D+25OHD); or D_3 at 5,520 IU/kg feed (Dx2); each diet was fed to broilers grown on either Fresh or Reused litter to increase bacterial exposure. The objectives of this study were to investigate the effects of vitamin D sources on bone mineralization and development, small intestine morphology, innate immune response, and gene expression of broiler chicken grown on fresh and reused litter. The objectives were addressed with following hypotheses:

1. Dietary 25-hydroxycholecalciferol ($25OHD_3$) would increase growth performance and meat yield throughout the growing period relative to vitamin D_3, and the same parameters would decrease for birds grown on Reused *vs* Fresh litter. This hypothesis was addressed in Chapter 2. Contrary to the hypothesis, broiler chickens fed $25OHD_3$ had lower BW and BW gain from d 0 to d 12 relative to those fed any diet containing vitamin D_3 alone in the Fresh litter, but birds fed D or D+$25OHD_3$ had lower BW and BWG from d 0 to d 12 relative to Dx2 in the Reused litter.

Reused litter resulted in reduced growth performance and meat yield relative to Fresh litter in broiler chickens at d 12. At processing, 25OHD feed increased leg meat yield relative to D, however, breast meat yield increased in birds fed D in comparison to 25OHD and Dx2.

2. Dietary $25OHD_3$ would decrease inflammation; increase bone mineralization and bone breaking strength, and enhance small intestine morphology, also Reused litter would result in increased inflammation, decreased bone mineralization and strength, and reduced absorptive surface area in the small intestine in comparison to Fresh litter. This hypothesis was addressed in Chapter 3. This hypothesis was partly supported, because, $25OHD_3$ reduced inflammation at as indicated by α1 acid glycoprotein d 41 and tended to decrease haptoglobin ($p<0.1$) at d12 relative to birds fed D. Reused litter resulted in higher inflammation at d 41 in broiler chickens and tended to increase ($p<0.1$) inflammation at d 12 relative to Fresh litter. Rectal temperature did not differ between diet and litter treatments during the 41 d experiment. Dietary supplementation of 25OHD increased crypt depth, villus width in the jejunum and tended to increase ($p<0.1$) villus surface area in the duodenum relative to D. Dietary supplementation of $25OHD_3$ increased bone mineralization and bone breaking strength relative to vitamin D_3 throughout the 41 d experiment. Reused litter resulted in deceased in bone quality relative to Fresh litter at d 12 and d 22.

3. Supplementation of $25OHD_3$ would increase the expression of genes involved muscle development and decrease the expression of genes linked to inflammation relative to vitamin D_3; and Reused litter would result in increased inflammatory response genes as compared to Fresh litter in broiler chickens. This hypothesis was addressed in Chapter 4. This hypothesis was supported because supplementation of 25OHD increased expression of myogenic regulatory factor genes, and genes involved in muscle development relative to vitamin D_3. Also, the

expression of genes associated with antigen presentation and adaptive immune response increased in 25OHD-fed birds relative to those fed D. However, expression of genes involved in amino acid biosynthesis and transfer decreased in 25OHD-fed birds relative to those fed D. Additionally, the expression of pro-inflammatory genes increased in birds reared on Reused relative to Fresh litter.

5.2 ANALYSIS AND IMPLICATIONS

The supplementation of $25OHD_3$ did not increase broiler performance or meat yield in comparison to vitamin D_3. This could be due to increased gut maintenance energy requirement or lower expression of genes involved in amino acid metabolism in $25OHD_3$ fed birds relative to vitamin D_3 fed birds. Supplementation of $25OHD_3$ increased the crypt depth, which is linked to increased cell turn over, and therefore greater gut maintenance energy requirement (Yason et al., 1987; Montagne, 2003). Additionally, vitamin D_3 induced expression of genes involved in amino acid biosynthesis and transport (Chapter 4). An increase in amino acid metabolism could result in increased growth performance in both chickens and mammals (Pesti, 2009; Morales et al., 2014). The reason for reduced broiler performance at d12 and bone mineralization and strength during the growing period in birds grown on Reused relative to Fresh litter could be due to increased systemic inflammation, and higher expression of pro-inflammatory genes, which could cause subsequent conditions that increased intestinal epithelial cell turnover and gut maintenance energy requirements. At d12, broiler performance was reduced in D-fed birds in Reused vs Fresh litter, however, 25OHD-fed birds maintained growth performance in both Reused and Fresh litter. This result could be due to the almost significantly increased inflammation and lower expression of myogenic factor gene (MyoG) and anti-inflammatory gene (BFIV21) in D-fed birds as compared to 25OHD-fed birds. MyoG is associated with satellite cells activity and

increased growth performance in chickens (Genxi et al., 2014; Clark et al., 2016). BFIV21 is linked to antigen presentation and enteric pathogen protection (Fulton et al., 1995; Afanassieff et al., 2001).

25OHD$_3$ is converted to the active hormone 1,25-dihydroxylcholecalciferol by the action of 1 α-hydroxylase, also, 1 α-hydroxylase is expressed at similar levels in both thigh and breast muscle (Shanmugasundaram and Selvaraj, 2012). Birds fed 25OHD had higher leg meat yield relative to those fed D, which could be due to an increased expression of 1 α-hydroxylase in thigh muscle in 25OHD-fed birds relative to D-fed birds in this study. It was reported that 25OHD$_3$ increased breast meat yield through the mechanistic target of rapamycin (mTOR) pathway, however, we found another gene, calcium/calmodulin-dependent protein kinase II alpha (CaMKIIA), which may be involved in the cyclic adenosine monophosphate (cAMP) pathway. The cAMP pathway is a Ca signaling pathway associated with growth (Sibut et al, 2011). Additionally, two myogenic regulatory factor genes associated with increased satellite cell proliferation and growth performance in chickens (Genxi et al., 2014; Clark et al., 2016), were highly expressed in 25OHD-fed as compared to D-fed birds (Chapter 4). These results indicate that 25OHD$_3$ stimulated genes or pathways involved in muscle development and cell growth, but those genes may not be translated into protein to increase muscle synthesis. On the other hand, genes involved in immunity were likely translated due to the almost significantly lower inflammation at d12.

The results of the current study indicate that supplementation of 25OHD$_3$ as a complete or partial replacement for vitamin D$_3$ in broiler diet increased innate immune response and bone quality but did not affect growth performance. Dietary 25OHD$_3$ alone increased potential nutrient absorption and gut maintenance energy requirement by increasing the villus parameters and crypt

depth. Also, $25OHD_3$ was able to maintain post-hatch broiler performance in an unsanitary environment due to reduced inflammation in those birds. This may indicate that dietary $25OHD_3$ relative to vitamin D_3 stimulated the partitioning of more nutrients toward bone development and immunity rather than production. The combination of $25OHD_3$ and vitamin D_3 at commercially relevant levels resulted in similar growth performance and meat yield relative to vitamin D_3, but breast meat yield was reduced in broilers fed $25OHD_3$ alone. Also, bone mineralization increased in 25OHD+D-fed birds relative to birds fed vitamin D_3 alone at d 12. This may indicate a synergetic effect of $25OHD_3$ and vitamin D_3 in broiler performance when fed the combination of $25OHD_3$ with vitamin D_3. However, vitamin D_3 at 5,520 IU/kg feed did not increase breast meat yield or growth performance in comparison to vitamin D_3 fed birds at 2,760 IU/kg feed or $25OHD_3$-fed birds. Vitamin D_3 did not affect bone ash and growth performance of broilers from 2,000 to 4,000 IU/kg feed (Fritts and Waldroup, 2003). Therefore, these results may indicate that vitamin D_3 above 2,000 IU/kg feed in broiler diets has the similar effects relative to commercial levels.

The decreased post-hatch broiler performance, breast meat yield and bone mineralization and bone breaking strength in birds reared on Reused litter relative to those grown on Fresh litter could be due to increased inflammation and expression of genes linked to inflammatory response (Chapter 3 and 4). Additionally, variability of the gut microbiota in post-hatch chickens is higher than in birds at d 28 (Torok et al 2009; Lu et al., 2003). Poultry litter may contain pathogenic bacteria which can cause increased bacteria exposure in chicken gut. These may be the reasons for reduced performance and bone characteristic of post-hatched broilers reared Reused relative to Fresh litter. However, the composition of the gut microbiota is more stable in birds at d 28 relative to birds at d 14, and the immune system is more developed (Torok et al 2009; Lu et al.,

2003). These commensal populations may colonize the gut and protect it from enteric pathogens (Nakamura et al., 2002; Zhang et al., 2007). Therefore, a more stable gut microflora predominated by commensal bacteria could be the reason for relatively increased growth performance as compared to post-hatch broilers reared on Reused litter.

In summary, supplementation of $25OHD_3$ relative to vitamin D_3 at commercial levels of inclusion causes increased bone mineralization and bone breaking strength, and reduced systemic inflammation. Dietary $25OHD_3$ can maintain broiler production under stressful conditions, such as in an unsanitary environment through reducing inflammation. Previous studies showed that high stocking density resulted in reduced broiler performance (Feddes et al., 2002). This reduction could be due to an increase in enteric pathogens in the digestive tract of broilers chickens (Guardia et al., 2011). These pathogens can cause systemic inflammation and decrease growth performance of broiler chickens (Lee et al., 2011). Growing broilers at a low stocking density (4.18 birds/m^2) did not provide a severe systemic inflammatory challenge. Therefore, if the effects of $25OHD_3$ in broiler production are mediated at least in part through decreases in inflammation, the current study may not have provided a suitable model to test this hypothesis. The combination of $25OHD_3$ with vitamin D_3 was more effective in promoting bone mineralization of newly hatched broilers relative to vitamin D_3 alone. Vitamin D_3 at twice the level of the standard dose of vitamin D activity did not increase broiler growth performance and innate immune response. Supplementation of $25OHD_3$ can increase the expression of genes involved in growth and immune response, although the increases in gene expression may not have resulted in increases in subsequent translation of the genes into protein to increase muscle synthesis.

5.3 RECOMMENDATION FOR FUTURE RESEARCH

Dietary $25OHD_3$ as a complete or partial replacement for vitamin D_3 did not increase breast meat in Ross 308 and 708 broilers (Hutton et al., 2014; Papešová et al., 2008), however, $25OHD_3$ increased breast meat yield when Cobb 500 broiler chickens were used (Vignale et al., 2015). Additionally, breast meat yield and thigh meat yield increased in one commercial broiler strain relative to another strain under field conditions in Colombia (Lozano-Poveda, 2014). Therefore, it would be useful to investigate the effects of dietary $25OHD_3$ on breast muscle metabolism in different strains of broiler chickens. As observed in Chapter 3, there were no differences observed in rectal temperature during the rearing period due to dietary or litter treatments. Rectal temperature increases during systemic inflammation and is an indicator of systemic inflammation (Leshchinsky et al., 2001). We hypothesized that Reused litter would cause chronic systemic inflammation due to increased bacterial exposure. Additionally, $25OHD_3$ could reduce inflammatory response and activate adaptive immunity leading to increase growth performance and meat yield relative to vitamin D_3. Therefore, for future work, a strong inflammatory response such as that caused by lipopolysaccharide could be used to cause systemic inflammation in birds. As observed in Chapter 4, expression of genes linked to muscle development increased when birds were fed $25OHD_3$ relative to vitamin D_3, although meat yield did not differ between dietary treatments at d 12. This discrepancy could be due to a lack of subsequent translation of the genes into protein, thus resulting in no change in breast muscle synthesis. Also, other genes which were not identified by RNA-seq could have been highly expressed, leading to a lack of subsequent translation of genes associated with muscle synthesis into protein. It would be useful to investigate the expression of proteins involved in muscle development. As reported in Chapter 2, $25OHD_3$ increased leg meat yield relative to breast meat

yield was observed in birds fed $25OHD_3$ at 2,760 IU/kg feed relative to those fed vitamin D_3 at the same level of activity. The 1 α-hydroxylase is expressed in breast and thigh muscles at similar levels (Shanmugasundaram and Selvaraj, 2012). In this study, an increase in expression of 1 α-hydroxylase in thigh muscle in comparison to breast muscle when birds were fed dietary 25OHD relative to D could have resulted in increased leg muscle yield. Therefore it would be interesting to assess the expression of 1 α-hydroxylase in both thigh and breast muscle when birds are fed different vitamin D sources.

5.4 STUDY LIMITATIONS

According to the Ross 308 broiler management guide (Aviagen, 2014), the commercial field stocking density is 40 birds/m^2, however, the stocking density in this study was 4.18 birds/m^2. In the current study, low stocking density and systemic inflammation were some of the limitations that we faced. We hypothesized that increased bacteria exposure would cause systemic inflammation throughout the growing period in broiler chickens, however, we did not see this effect continuously in this study. There could be other two possibilities to cause systemic inflammation during the rearing period in broilers. One possibility would be to increase stocking density, which resulted in decreased broiler performance and bone health (Feddes et al., 2002; Sun et al., 2013). Another possibility would be to challenge the birds with particular pathogenic bacteria like *Escherichia coli* or *Clostridium perfringens*. The microbial population in reused litter is variable (Lee et al., 2011), which can change the development of gut microflora in the newly hatched chicken, and change small intestine and immune system development (Lu et al., 2003; Lee et al., 2011; Pan and Yu, 2014). It would be useful to assess the microbial populations in poultry litters used in this study. We collected liver samples to investigate gene expression related to immunity in broilers fed vitamin sources in fresh *vs* reused litter. Liver is an

appropriate tissue to investigate treatment effects on inflammation due production of inflammatory cytokines and acute phase proteins. However, we did not look at the results for those samples due to time limitations.

5.6 REFERENCES

Afanassieff, M., M. A. Sherman, L. Zhong, C. Auffray, F. Coudert, R. Zoorob, and M. M. Miller. 2001. At least one class I gene in restriction fragment pattern-Y (Rfp-Y), the second MHC gene cluster in the chicken, is transcribed, polymorphic, and shows divergent specialization in antigen binding region. J. Immunol. 166:3324-3333.

Aviagen. 2014. Ross Broiler Management Handbook. Aviagen Ltd., Newbridge, UK.

Boland, R., A. R. de Boland, M. J. Marinissen, G. Santillan, G. Vazquez, and S. Zanello. 1995. Avian muscle cells as targets for the secosteroid hormone 1,25-dihydroxy-vitamin D_3. Mol. Cell. Endocrinol. 114:1-8.

Clark, D. L., C. S. Coy, G. M. Strasburg, K. M. Reed, and S. G. Velleman. 2016. Temperature effect on proliferation and differentiation of satellite cells from turkeys with different growth rates. Poult. Sci. 95:934–947.

Feddes, J. J. R., E. J. Emmanuel, and M. J. Zuidhof. 2002. Broiler performance, body weight variance, feed and water intake, and carcass quality at different stocking densities. Poult. Sci. 81:774–779.

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.

Fulton J. E., E. L. Thacker, L. D. Bacon, and H. D. Hunt. 1995. Functional analysis of avian class I (BFIV) glycoproteins by epitope tagging and mutagenesis in vitro. Eur J Immunol. 25:2069-2076.

Genxi, Z., T. Ying, Z. Tao, W. Jinyu, and W. Yongjuan. 1990. Expression profiles and association analysis with growth traits of the MyoG and Myf5 genes in the Jinghai yellow chicken. Mol. Biol. Rep. 11:7331-7338.

Guardia, S., B. Konsak, S. Combes, F. Levenez, L. Cauquil, J. F. Guillot, C. Moreau-Vauzelle, M. Lessire, H. Juin, and I. Gabriel. 2011. Effects of stocking density on the growth performance and digestive microbiota of broiler chickens. Poult. Sci. 90:1878-1889.

Lee, K. W., H. S. Lillehoj, S. H. Lee, S. I. Jang, G. D. Ritter, D. A. Bautista, and E. P. Lillehoj. 2011. Impact of fresh or used litter on the posthatch immune system of commercial broilers. Avian. Dis. 55:539-544.

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

Lozano-Poveda, C. A. 2014. Effects of dietary combination of 25-OH-D_3 and canthaxanthin on performance, meat yield, bone characteristics and antioxidant status of broilers housed under commercial and experimental conditions. M.Sc. Diss. University of Alberta, Edmonton.

Lu, J., S. Sanchez, C. Hofacre, J. J. Maurer, B. G. Harmon, and M. D. Lee. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. Appl. Environ. Microbiol. 69:901–908.

Montagne, L., J. R. Pluske, and D. J. Hampson. 2003. A review of interactions between dietary fiber and the intestinal mucosa, and their consequences on digestive health in young nonruminant animals. Anim. Feed Sci. Technol. 108:95–117.

Morris, A., R. Shanmugasundaram, M. S. Lilburn, and R. K. Selvaraj. 2014. 25-Hydroxycholecalciferol supplementation improves growth performance and decreases inflammation during an experimental lipopolysaccharide injection. Poult Sci. 93:1951-1956.

Nakamura, A., Y. Ota, A. Mizukami, T. Ito, Y. B. Ngwai, and Y. Adachi. 2002. Evaluation of aviguard, a commercial competitive exclusion product for efficacy and after-effect on the antibody response of chicks to *Salmonella*. Poult Sci. 8:1653-1660.

Pan, D., and Z. Yu. 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut. Microbes. 5:108-119.

Papešová, L., A. Fučíková, M. Pípalová, P. Tupý. 2008. The synergic effect of vitamin D_3 and 25-hydroxycholecalciferol/calcidiol in broiler diet. SAB. 39: 273-277.

Rama-Rao, S. V., M.V. L. N. Raju, A. K. Panda, A. K., G. Shyam Sunder, and R. P. Sharma. 2006. Effect of high concentrations of cholecalciferol on growth, bone mineralization, and mineral retention in broiler chicks fed suboptimal concentrations of calcium and nonphytate phosphorus. J. Appl. Poult. Res. 15:493-501.

Rama-Rao, S. V., M.V. L. N. Raju, A. K. Panda, A. K., G. Shyam Sunder, and R. P. Sharma. 2009. Performance and bone mineralization in broiler chicks fed on diets with different concentrations of cholecalciferol at a constant ratio of calcium to non-phytate phosphorus. Br. Poult. Sci. 50:528-535.

Shanmugasundaram, R., M. S. Lilburn, and R. K. Selvaraj. 2012. Effect of recycled litter on immune cells in the cecal tonsils of chickens. Poult. Sci. 91:95-100.

Sibut, V., C. Hennequet-Antier, E. Le Bihan-Duval, S. Marthey, M. J. Duclos, and C. Berri. 2011. Identification of differentially expressed genes in chickens differing in muscle glycogen content and meat quality. BMC Genomics. 12:112-125.

Sun, Z. W., L. Yan, Y. Y. G, J. P. Zhao, H. Lin, and Y. M. Guo. 2013. Increasing dietary vitamin D_3 improves the walking ability and welfare status of broiler chickens reared at high stocking densities. Poult. Sci. 92:3071-3079.

Torok, V. A., R. J. Hughes, K. Ophel-Keller, M. Ali, and R. MacAlpine. 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. Poult. Sci. 88:2474–2481.

Vignale, K., E. S. Greene, J. V. Caldas, J. England, N. Boonsinchai, P. Sodsee, E. D. Pollock, S. Dridi, and C. N. Coon. 2015. 25-Hydroxycholecalciferol enhances male broiler breast meat yield through the mTOR pathway. J. Nutr. 145:855-863.

Yason, C. V., B. A. Summers, and K. A. Schat. 1987. Pathogenesis of rotavirus infection in various age-groups of chickens and turkeys-Pathology. Am. J. Vet. Res. 48:927–938.

Zhang, G., L. Ma and, M.P. Doyle. 2007. Potential competitive exclusion bacteria from poultry inhibitory to *Campylobacter jejuni* and *Salmonella*. J. Food. Prot.70:867-873.

Bibliography

Achouri, Y., M. H. Rider, E. V. Schaftingen, and M .Robbi. 1997. Cloning, sequencing and expression of rat liver 3-phosphoglycerate dehydrogenase. Biochem. J. 323:365-370.

Agrotis, A., P. Kanellakis, G. Kostolias, G. Di Vitto, C. Wei, R. Hannan, G. Jennings, and A. Bobik. 2004. Proliferation of neointimal smooth muscle cells after arterial injury. Dependence on interactions between fibroblast growth factor receptor-2 and fibroblast growth factor-9. J. Biol. Chem. 279:42221-42229.

Afanassieff, M., M. A. Sherman, L. Zhong, C. Auffray, F. Coudert, R. Zoorob, and M. M. Miller. 2001. At least one class I gene in restriction fragment pattern-Y (Rfp-Y), the second MHC gene cluster in the chicken, is transcribed, polymorphic, and shows divergent specialization in antigen binding region. J. Immunol. 166:3324-3333.

Aliakbarpour, H. R., M. Chamani, G. Rahimi, A. A. Sadeghi, and D. Qujeq. 2012. The *bacillus subtilis* and lactic acid bacteria probiotics influences intestinal mucin gene expression, histomorphology and growth performance in broilers. Asian-Aust. J. Anim. Sci. 25:1285-1293.

Applegate, T. J., and M. S. Lilburn. 2002. Growth of the femur and tibia of a commercial broiler line. Poult. Sci. 81:1289-1294.

Anderson, P. H., P. D. O'Loughlin, B. K. May, and H. A. Morris. 2005. Modulation of CYP27B1 and CYP24 mRNA expression in bone is independent of circulating $1,25(OH)_2D_3$ levels. Bone. 36:654– 662.

Anders, S., P. T . Pyl, and W. Huber. 2015. HTSeq—a python framework to work with high-throughput sequencing data. Bioinformatics. 31:166-169.

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.

Angel, R., W. W. Saylor, A. D. Mitchell, W. Powers, and T. J. Applegate. 2006. Effect of dietary phosphorus, phytase, and 25-hydroxycholecalciferol on broiler chicken bone mineralization, litter phosphorus, and processing yields. Poult. Sci. 85:1200-1211.

Angelo, L. S., and R. Kurzrock. 2007. Vascular endothelial growth factor and its relationship to inflammatory mediators. Clin. Cancer. Res. 13:2825-2830.

Asasi, K., A. Mohammadi, Z. Boroomand, S. A. Hosseinian, and S Nazifi. 2013. Changes of several acute phase factors in broiler chickens in response to infectious bronchitis virus infection. Poult. Sci. 92:1989-1996.

Armand, O., A. M. Boutineau, A. Mauger, M. P. Pautou, and M. Kieny. 1983. Origin of satellite cells in avian skeletal muscles. Arch. Anat. Microsc. Morphol. Exp. 72:163-181.

Arnold, H. H., and B. Winter. 1998. Muscle differentiation: more complexity to the network of myogenic regulators. Curr. Opin. Genet. Dev. 8:539-544.

Atencio, A., G. M. Pesti, and H. M. Edwards. 2005. Twenty-five hydroxycholecalciferol as a cholecalciferol substitute in broiler breeder hen diets and its effect on the performance and general health of the progeny. Poult. Sci. 84:1277-1285.

Aviagen. 2009. Ross Broiler Management Manual. Aviagen Ltd., Newbridge, UK.

Awad, W. A., K. Chareeb, S. Abdel-Raheem and J. Bohm. 2009. Effects of dietary inclusion of probiotic and symbiotic on growth performance, organ weight and intestinal histomorphology of broiler chickens. Poult Sci. 88:49-56.

Balasubramanian, M. N., E. A. Butterworth, and M. S. Kilberg. 2013. Asparagine synthetase: regulation by cell stress and involvement in tumor biology. Am. J. Physiol. Endocrinol. Metab. 304:E789-E799.

Bale, T. L., F. J. Giordano, R. P. Hickey, Y. Huang, A. K. Nath, K. L. Peterson, W. W. Vale, and K. F. Lee. 2012. Corticotropin-releasing factor receptor 2 is a tonic suppressor of vascularization. Proc. Natl. Acad. Sci. USA. 99:7734-7739.

Baldi, C., G. Vazquez, and R. Boland. 2002. Characterization of a 1,25$(OH)_2$-vitamin D_3-responsive capacitative Ca^{2+} entry pathway in rat osteoblast-like cells. J. Cell. Biochem. 86:678-687.

Baumgartner, M. R., C. A. Hu, S. Almashanu, G. Steel, C. Obie, B. Aral, D. Rabier, P. Kamoun, J. M. Saudubray, and D. Valle. 2000. Hyperammonemia with reduced ornithine, citrulline, arginine and proline: a new inborn error caused by a mutation in the gene encoding delta (1)-pyrroline-5-carboxylate synthase. Hum. Mol. Genet. 9:2853-2858.

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, A., V. Razaphkovsky, E. Vax, and I. Plavnik. 2003. Performance and bone development in broiler chickens given 25-hydroxycholecalciferol. Br. Poult. Sci. 44:224-233.

Barthel, T. K., D. R. Mathern, G. K Whitfield. C. A. Haussler, H. A. Hopper, J. C. Hsieh, S. A. Slater, G. Hsieh, M. Kaczmarska, P. W. Jurutka, O. I. Kolek, F. K. Ghishan, and M. R. Haussler. 2007. 1,25-dihydroxyvitamin D_3/VDR-mediated induction of FGF23 as well as transcriptional control of other bone anabolic and catabolic genes that orchestrate the

regulation of phosphate and calcium mineral metabolism. J. Steroid. Biochem. Mol. Biol. 103:381-388.

Beal, R. K., P. Wigley, C. Powers, S. D. Hulme, P. A. Barrow, and A. L. Smith. 2004. Age at primary infection with *Salmonella enterica* serovar Typhimurium in the chicken influences persistence of infection and subsequent immunity to re-challenge. Vet. Immunol. Immunopathol. 100:151–164.

Bello, A., P. Y. Hester, P. D. Gerard, W. Zhai, and E. D. Peebles. 2014. Effects of commercial in ovo injection of 25-hydroxycholecalciferol on bone development and mineralization in male and female broilers. Poult. Sci. 93:2734-2739.

Berdeaux, R., and R. Stewart. 2012. cAMP signaling in skeletal muscle adaptation: hypertrophy, metabolism, and regeneration. Am. J. Physiol. Endocrinol. Metab. 303:E1-E17.

Berlanga-Acosta, J., R. J. Playford, N. Mandir, R. A. Goodlad. 2001. Gastrointestinal cell proliferation and crypt fission are separate but complementary means of increasing tissue mass following infusion of epidermal growth factor in rats. Gut. 48:803-807.

Billerbeck, E., J. A. Horwitz, R. N. Labitt, B. M. Donovan, K. Vega, W. C. Budell, G. C. Koo, C. M. Rice, and A. Ploss. 2013. Characterization of human antiviral adaptive immune responses during hepatotropic virus infection in HLA-transgenic human immune system mice. J. Immunol. 191:1753-1764.

Bradley, J. R. 2008. TNF-mediated inflammatory disease. J. Pathol. 214:149-160.

Biressi, S., C. R. Bjornson, P. M. Carlig, K. Nishijo, C. Keller, and T. A. Rando. 2013. Myf5 expression during fetal myogenesis defines the developmental progenitors of adult satellite cells. Dev. Biol. 379:195-207.

Bolan, N. S., A. A. Szogi, T. Chuasavathi, B. Seshadri, M. J. Rothrock, and P. Panneerselvam. 2010. Uses and management of poultry litter. World's Poult. Sci. J. 66:673-698.

Boland, R., A. R. de Boland, M. J. Marinissen, G. Santillan, G. Vazquez, and S. Zanello. 1995. Avian muscle cells as targets for the secosteroid hormone 1,25-dihydroxy-vitamin D_3. Mol. Cell. Endocrinol. 114:1-8.

Boland, R. L. 2011. VDR activation of intracellular signaling pathways in skeletal muscle. Mol. Cell. Endocrinol. 347:11-6.

Borel, P., P. Grolier, M. Armand, A. Partier, H. Lafont, D. Lairon, and V. Azais-Braesco. 1996. Carotenoids in biological emulsions: solubility, surface-to-core distribution, and release from lipid droplets. J. Lipid. Res. 37:250-261.

Bordbar, A., and B. O. Palsson. 2012. Using the reconstructed genome-scale human metabolic network to study physiology and pathology. J. Intern. Med. 271:131–141.

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

Canadian Council on Animal Care. 2009. A guide to the care and the use of experimental animals. CCAC, Ottawa, ON, Canada.

Chamanza, R., L. Van Veen, M.T. Tivapasi and M.J. Toussaint. 1999. Acute phase proteins in the domestic fowls. World's Poult Sci J. 55:61-71.

Chaudhry, F. A., R. J. Reimer, D. Krizaj, D. Barber, J. Storm-Mathisen, D. R. Copenhagen, R. H. Edwards. 1999. Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. Cell. 99:769-780.

Cheema, M. A., Qureshi, M. A., and G. B. Havenstein. 2003. A comparison of the immune response of a 2001 commercial broiler with a 1957 random bred broiler strain when fed representative 1957 and 2001 broiler diets. Poult. Sci. 82:1519-1529.

Chen, Y., H. Gao, Q. Yin, L. Chen, P. Dong, X. Zhang, and J. Kang. 2013. ER stress activating ATF4/CHOP-TNF-α signaling pathway contributes to alcohol-induced disruption of osteogenic lineage of multipotential mesenchymal stem cell. Cell. Physiol. Biochem.

Chen, J., G. Tellez, J. D. Richards, and J. Escobar. 2015. Identification of potential biomarkers for gut Barrier Failure in Broiler Chickens. Front. Vet. Sci. 2:14.

Chichlowski, M., W. J. Croom, F. W. Edens, B. W. McBride, R. Qiu, C. C. Chiang, L. R. Daniel, G. B. Havenstein, and M. D. Koci. 2007. Microarchitecture and spatial relationship between bacteria and ileal, cecal, and colonic epithelium in chicks fed a direct-fed microbial, PrimaLac, and salinomycin. Poult. Sci. 86:1121-1132.

Chinivasagam, H. N., M. Redding, G. Runge, and P. J. Blackall. 2010. Presence and incidence of food-borne pathogens in Australian chicken litter. Br. Poult. Sci. 51:311-318.

Choct, M. 2009. Managing gut health through nutrition. Br. Poult. Sci. 50:9–15.

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.

Christakos, S., D. V. Ajibade, P. Dhawan, A. J. Fechner, and L. J. Mady. 2010. Vitamin D: metabolism. Endocrinol. Metab. Clin. North. Am. 39:243–253.

32:743-754.

Cipolletta, E., S. Monaco, A. S. Maione, L. Vitiello, P. Campiglia, L. Pastore, C. Franchini, E. Novellino, V. Limongelli, K. U. Bayer, A. R. Means, G. Rossi, B. Trimarco, G. Iaccarino,

and M. Illario. 2010. Calmodulin-dependent kinase II mediates vascular smooth muscle cell proliferation and is potentiated by extracellular signal regulated kinase. Endocrinology. 151:2747-2759.

Clark, D. L., C. S. Coy, G. M. Strasburg, K. M. Reed, and S. G. Velleman. 2016. Temperature effect on proliferation and differentiation of satellite cells from turkeys with different growth rates. Pout. Sci. 95:934–947.

Cloonan, N., N. Cloonan, A. R. R. Forrest, G. Kolle, B. B. A. Gardiner, G. J. Faulkner, M. K. Brown, D. F. Taylor, A. L. Steptoe, S. Wani, G. Bethel, A. J. Robertson, A. C. Perkins, S. J. Bruce, C. C. Lee, S. S. Ranade, H. E. Peckham, J. M. Manning, K. J. McKernan, and S. M Grimmond. 2008. Stem cell transcriptome profiling via massive-scale mRNA sequencing. Nat. Methods. 5:613-619.

Coffey, J. D., E. A. Hines, J. D. Starkey, C. W. Starkey, and T. K. Chung. 2012. Feeding 25-hydroxycholecalciferol improves gilt reproductive performance and fetal vitamin D status. J. Anim. Sci. 11:3783-3788.

Corthesy, B., H. R. Gaskins, and A. Mercenier. 2007. Cross-talk between probiotic bacteria and the host immune system. J. Nutr. 137:781S-790S.

Coufal, C. D., C. Chavez, P. R. Niemeyer, and J. B. Carey. 2006. Effects of top-dressing recycled broiler litter on litter production, litter characteristics, and nitrogen mass balance. Poult. Sci. 85:392-397.

Cressman, M. D., Z. Yu, M. C. Nelson, S. J. Moeller, M. S. Lilburn, and H. N. Zerby. 2010. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. Appl. Environ. Microbiol.76:6572-6582.

Crhanova, M., H. Hradecka, M. Faldynova, M. Matulova, H. Havlickova, F. Sisak, and I. Rychlik. 2011. Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella enterica serovar enteritidis* infection. Infect. Immun. 79:2755-2763.

De Matos, R. 2008. Calcium metabolism in birds. Vet. Clin. North. Am. Exot. Anim. Pract. 11:59-82.

De Beer, M., and C. N. Coon. 2007. The effect of different feed restriction programs on reproductive performance, efficiency, frame size, and uniformity in broiler breeder hens. Poult. Sci. 86:1927-1939.

Deeb, K. K., D. L. Trump, and C. S. Johnson. 2007. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat. Rev. Cancer. 7:684-700.

Deng, B., M. Wehling-Henricks, S. A. Villalta, Y. Wang, and J. G. Tidball. 2012. IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. J. Immunol. 189:3669-3680.

Deng, B., M. Wehling-Henricks, S. A. Villalta, Y. Wang, and J. G . Tidball. 2012. IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. J. Immunol. 189:3669-3680.

Denkberg, G., C. J. Cohen, D. Segal, A. F. Kirkin, and Y. Reiter. 2000. Recombinant human single-chain MHC-peptide complexes made from *E. coli* by in vitro refolding: functional single-chain MHC-peptide complexes and tetramers with tumor associated antigens. Eur. J. Immunol. 30:3522-3532.

Diarra, M. S. and F. Malouin. 2014. Antibiotics in Canadian poultry productions and anticipated alternatives. Front. Microbiol. 5:282-297.

Dibaji, S. M., A. Seidavi, L. Asadpour, and F. M. da Silva. 2014. Effect of a synbiotic on the intestinal microflora of chickens. J. Appl. Poult. Res. 23:1-6.

Dibner, J. J., J. D. Richards, and C. D. 2008. Microbial imprinting in gut development and health. J. Appl. Poult. Res. 17:174–188.

Driver, J. P., G. M. Pesti, R. I. Bakalli, and H. M. Jr. Edwards. 2005. Calcium requirements of the modern broiler chicken as influenced by dietary protein and age. Poult. Sci. 84:1629-1639.

Dixit, S. S., T. Wang, E. J. Manzano, S. Yoo, J. Lee, D. Y. Chiang, N. Ryan, J. L. Respress, V. K. Yechoor, and X. H. Wehrens. 2013. Effects of CaMKII-mediated phosphorylation of ryanodine receptor type 2 on islet calcium handling, insulin secretion, and glucose tolerance. Plos. One. 8: e58655- e58667.

Dozier, W. A., M. T. Kidd, and A. Corzo. 2004. Dietary amino acid responses of broiler chickens. J. Appl. Poult. Res. 17:157-167.

Dusso, A. S., A. J. Brown, and E. Slatopolsky. 2005. Vitamin D. Am. J. Physiol. Renal Physiol. 289:8-28.

Fasina, Y. O., F. J. Hoerr, S. R. McKee, and Conner. D. E. 2010. Influence of *Salmonella enterica* serovar Typhimurium infection on intestinal goblet cells and villous morphology in broiler chicks. Avian. Dis. 54:841-847.

Fei, Y. J., M. Sugawara, T. Nakanishi, W. Huang, H. Wang, P. D. Prasad, F. H. Leibach, and V. Ganapathy. 2000. Primary structure, genomic organization, and functional and electrogenic characteristics of human system N 1, a Na+- and H+-coupled glutamine transporter. J. Biol. Chem. 275:23707-23717.

Fenech, M., A. El-Sohemy, L. Cahill, L. R. Ferguson, T. A. French, E. S. Tai, J. Milner, W. P. Koh, L. Xie, M. Zucker, M. Buckley, L. Cosgrove, T. Lockett, K. Y. Fung, and R. Head.2011. Nutrigenetics and nutrigenomics: viewpoints on the current status and applications in nutrition research and practice. J. Nutrigenet. Nutrigenomics. 4:69-89.

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.

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.

Fritts, C. A., and P. W. Waldroup. 2005. Comparison of cholecalciferol and 25-hydroxycholecalciferol in broiler diets designed to minimize phosphorus excretion. J. Appl. Poult. Res. 14:156-166.

Fulton, J. E., E. L. Thacker, L. D. Bacon, and H. D. Hunt. 1995. Functional analysis of avian class I (BFIV) glycoproteins by epitope tagging and mutagenesis in vitro. Eur. J. Immunol. 25:2069-2076.

Ehrhardt, M., A. Gerber, F. Hannemann, and R .Bernhardt. 2016. Expression of human CYP27A1 in B. megaterium for the efficient hydroxylation of cholesterol, vitamin D_3 and 7-dehydrocholesterol. J. Biotechnol. 218:34-40.

Easom R. A., N. R. Filler, E. M. Ings, J. Tarpley, and M. Landt. 1997. Correlation of the activation of Ca2+/calmodulin-dependent protein kinase II with the initiation of insulin secretion from perifused pancreatic islets. Endocrinology. 138:2359-2364.

Easom, R., 1999. CaM kinase II: a protein kinase with extraordinary talents germane to insulin exocytosis. Diabetes. 48:675-684.

Edmondson, D. G., and E. N. A. Olson. 1989. Gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes. Dev. 3:628-640.

Erickson, K., and N. E. Hubbard. 2000. Probiotic immunomodulation in health and disease. J. Nutr. 130:403S-409S.

Evstafieva, A. G., A. A. Garaeva, A. A. Khutornenko, A. V. Klepikova, M. D. Logacheva, A. A. Penin, G. E. Novakovsky, I. E. Kovaleva, and P. M. Chumakov. 2014. A sustained deficiency of mitochondrial respiratory complex III induces an apoptotic cell death through the p53-mediated inhibition of pro-survival activities of the activating transcription factor 4. Cell. Death Dis. 5:e1511-e1521.

Ewald, S. J., and E. J. 2004. Livant+ distinctive polymorphism of chicken B-FI (major histocompatibility complex class I) molecules. Poult. Sci. 83:600-605.

Galdeano, M. C., A. de Moreno de LeBlanc, G. Vinderola, M. E. Bibas Bonet, and G. Perdigón. 2007. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. Clin. Vaccine. Immunol. 14:485-492.

Gao, H., G. Wu, T. E. Spencer, G. A. Johnson, F. W. Bazer. 2009. Select nutrients in the ovine uterine lumen. IV. Expression of neutral and acidic amino acid transporters in ovine uteri and peri-implantation conceptuses. Biol. Reprod. 80:1196-1208.

Garrett, R. L., and R. J. Young. 1975. Effect of micelle formation on the absorption of neutral fat and fatty acids by the chicken. J. Nutr. 105:827-838.

Genxi Z., T. Ying, Z. Tao, W. Jinyu, and W. Yongjuan. 2014. Expression profiles and association analysis with growth traits of the MyoG and Myf5 genes in the Jinghai yellow chicken. Mol. Biol. Rep. 11:7331-7338.

Girgis, C. M., R. J. Clifton-Bligh, M. W. Hamrick, M. F. Holick, and J. E. Gunton. 2013. The roles of vitamin D in skeletal muscle: Form, function, and metabolism. Endocr. Rev. 34:33-83.

Gödl, I., C. Müller, H. Wolf, and M. M. Eibl. 1990. Acute phase reaction in acute viral hepatitis. Padiatr. Padol. 25:147-155.

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

Gomaa, M. H., D. Yoo, D. Ojkic, and J. R. Barta. 2009. Infection with a pathogenic turkey coronavirus isolate negatively affects growth performance and intestinal morphology of young turkey poults in Canada. Avian. Pathol. 38:279-286

Gómez-Verduzco. G., R. Morales-López, and E. Avila-Gozàlez. 2013. Use of 25 hydroxycholecalciferol in diets of broiler chickens: effects on growth performance, immunity and bone calcification J. Poult. Sci. 50: 60-64.

Gotoh, K., M. Inoue, T. Masaki, S. Chiba, T. Shimasaki, H. Ando, K. Fujiwara, I. Katsuragi, T. Kakuma, M. Seike, T. Sakata, and H. Yoshimatsu. 2012. A novel anti-inflammatory role for spleen-derived interleukin-10 in obesity-induced inflammation in white adipose tissue and liver. Diabetes. 61:1994-2003.

Gu, S., H. L. Roderick, P. Camacho, and J. X. Jiang. 2000. Identification and characterization of an amino acid transporter expressed differentially in liver. Proc. Natl. Acad. Sci. USA. 97:3230-3235.

Guernec A, C. Berri, B. Chevalier, N. Wacrenier-Ceré, E. Le Bihan-Duval, and M. J. Duclos. 2003. Muscle development, insulin-like growth factor-I and myostatin mRNA levels in chickens selected for increased breast muscle yield. Growth. Horm. IGF. Res. 13:8-18.

Gunal, M., G. Yayli, O. Kaya, N. Karahan, O. Sulak. 2006. The effects of antibiotic growth promoter, probiotic or organic acid supplementation on performance, intestinal microflora and tissue of broilers. Int. J. Poult. Sci. 5:149-55.

Haddad, F., and G. R. Adams. 2004. Inhibition of MAP/ERK kinase prevents IGF-I-induced hypertrophy in rat muscles. J. Appl. Physiol. 96:203-210.

Halbrook, E. R., A. R. Winter, and T. S. Sutton. 1950. The effect of management on the vitamin B_{12} content of poultry house litter: I. as measured by chick growth. Poult. Sci. 29:672-678.

Haldar, M., G. Karan, P. Tvrdik, and M. R. Capecchi. 2008. Two cell lineages, Myf5 and Myf5-independent, participate in mouse skeletall myogenesis. Dev. Cell. 14:437-445.

Han, J., S. H. Back, J. Hur, Y. H. Lin, R. Gildersleeve, J. Shan, C. L. Yuan, D. Krokowski, S. Wang, M. Hatzoglou, M. S. Kilberg, M. A. Sartor, R. J. Kaufman. 2013. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. Nat. Cell. Biol. 15:481-490.

Havenstein, G. B., Ferket, P. R., and M. A. Qureshi. 2003. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. Poult. Sci. 82:1500-1508.

Haussler, M. R., P. W. Jurutka, J. C. Hsieh, P. D. Thompson, S. H. Selznick, C. A. Haussler, and G. K. Whitfield. 1995. New understanding of the molecular mechanism of receptor-mediated genomic actions of the vitamin D hormone. Bone. 17:33S-38S.

He, L., K. Vasiliou, and D. W . Nebert. 2009. Analysis and update of the human solute carrier (SLC) gene superfamily. Hum. Genomics. 2:195-206.

Healy, K. D., J. B. Zella, M. J. Prahl, and H. F. De Luca. 2003. Regulation of the murine renal vitamin D receptor by 1,25-dihydroxyvitamin D_3 and calcium. Proc. Natl. Acad. Sci. 100:9733-9737.

Higgins, S. E., A. D. Wolfenden, G. Tellez, B. M. Hargis, and T. E. Porter. 2011. Transcriptional profiling of cecal gene expression in probiotic and *Salmonella*-challenged neonatal chicks. Poult. Sci. 90:901-913.

Hosokawa, H., T. Sawamura, S. Kobayashi, H. Ninomiya, S. Miwa, and T. Masaki. 1997. Cloning and characterization of a brain-specific cationic amino acid transporter. J. Biol. Chem. 272:8717-8722.

Hu, X. F., Y. M. Guo, B. Y. Huang, S. Bun, L. B. Zhang, J. H. Li, D. Liu, F. Y. Long, X. Yang, and P. Jiao. 2010. The effect of glucagon-like peptide 2 injection on performance, small intestinal morphology, and nutrient transporter expression of stressed broiler chickens. Poult Sci. 89:1967-1974.

Hutton, K. C., M. A. Vaughn, G. Litta, B. J. Turner, and J. D. Starkey. 2014. Effect of vitamin D status improvement with 25-hydroxycholecalciferol on skeletal muscle growth characteristics and satellite cell activity in broiler chickens. J. Anim. Sci. 92:3291-3299.

Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4:44-57.

Hystad, M. E., J. H. Myklebust, T. H. Bo, E. A. Sivertsen, E. Rian, L. Forfang, E. Munthe, A. Rosenwald, M. Chiorazzi, I. Jonassen, L. M. Staudt, and E. B. Smeland. 2007.

Characterization of early stages of human B cell development by gene expression profiling. J. Immunol. 179:3662–3671.

Inan, M. .S., V. Tolmacheva, Q. S. Wang, D. W. Rosenberg, and C. Giardina, 2000. Transcription factor NF-kappaB participates in regulation of epithelial cell turnover in the colon. Am. J. Physiol. Gastrointest. Liver Physiol. 279, G1282–G1291.

Ito, K., and M. Groudine. 1997. A new member of the cationic amino acid transporter family is preferentially expressed in adult mouse brain. J. Biol. Chem. 272:26780-26786.

Jain, S., V. Gautam, and S. Naseem. 2011. Acute phase proteins: as diagnostic tool. J. Pharm. Bioallied. Sci. 3:118-127.

Jendral, M. J., D. R. Korver, J. S. Church, and J. J. R. Feddes. 2008. Bone mineral density and breaking strength of white leghorns housed in conventional, modified, and commercially available colony battery cages. Poult. Sci. 87:828–837.

Juknat, A., M. Pietr, E. Kozela, N. Rimmerman, R. Levy, G. Coppola, D. Geschwind, and Z.Vogel. 2012. Differential transcriptional profiles mediated by exposure to the cannabinoids cannabidiol and Δ9-tetrahydrocannabinol in BV-2 microglial cells. Br. J. Pharmacol. 165:2512-2528.

Juul-Madsen, H. R., M. Munch, K. J. Handberg, P. Sørensen, A. A. Johnson, L. R. Norup, and P. H. Jørgensen. 2003. Serum levels of mannan-binding lectin (MBL) in chickens prior to and during experimental infection with avian infectious bronchitis virus (IBV). Poult. Sci. 82:235-241.

Kasim, S., B. L. Blake, and X. Fan. 2006. The role of dopamine receptors in the neurobehavioral syndrome provoked by activation of L-type calcium channels in rodents. Dev. Neurosci. 28:5055-17.

Kalita, K. P., R. Saikia, and J. D. Mahanta. 2012. Performance of commercial broilers raised on reused and mixed type of litters. Indian. J. Hill. Farm. 25:33-36.

Käppeli, S., E. Fröhlich, S. G. Gebhardt-Henrich, A. Pfulg, H. Schäublin, R. Zweifel, H. Wiedmer, and M. H. Stoffel. 2011. Effects of dietary supplementation with synthetic vitamin D_3 and 25-hydroxycholecalciferol on blood calcium and phosphate levels and performance in laying hens. Arch. Geflügelk. 75:175-184.

Kefal. S, and N. Y. Toker, 2006. Effects of probiotics on some acute phase proteins in broilers exposed to *Salmonella* Typhimurium lipopolysaccharides. Arch. Geflügelk. 70:270-277

Kettunen, H., K. Tiihonena, S. Peuranena, M. T. Saarinena, and J. C. Remusb. 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. Part A Mol. Integr. Physiol. 130:759–769.

Khan, S. H., R. Shahid, A. A. Mian, R. Sardar, and M. A. Anjum. 2010. Effect of the level of cholecalciferol supplementation of broiler diets on the performance and tibial dyschondroplasia. J. Anim. Physiol. Anim. Nutr. 94:584-593.

Kim, S., N. K. Shevde, and J. W. Pike. 2005. 1,25-Dihydroxyvitamin D_3 stimulates cyclic vitamin D receptor/retinoid X receptor DNA binding, co-activator recruitment, and histone acetylation in intact osteoblasts. J. Bone. Miner. Res. 20:305-317.

Kim, D., G. Pertea, C. Trapnell, H. Pimente, R. Kelley, and S. L. Salzberg. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome. Biol. 14:R36-R49.

Kokosharov, T. 2006. Changes in the protein profile in birds with experimental acute fowl typhoid. Bulg. J. Vet. Med. 9:189–192.

Kommadath, A., H. Bao, A. S. Arantes, G. S. Plastow, C. K. Tuggle, S. M. Bearson, L. Guan le, and P. Stothard. 2014. Gene co-expression network analysis identifies porcine genes associated with variation in Salmonella shedding. BMC Genomics. 15:452-467.

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. Poultry Science 77:1217-1227.

Korver, D. R., J. L. Saunders-Blades, and K. L. Nadeau. 2004. Assessing bone mineral density in vivo: quantitative computed tomography. Pult. Sci. 83:222-229.

Koutsos, E. A., and K. C. Klasing. 2001. The acute phase response in Japanese quail (*coturnix coturnix japonica*). Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 128:255-263.

Krämer. A., J. Green , J. Pollard, and S. Tugendreich. 2014. Causal analysis approaches in ingenuity pathway analysis (IPA). Bioinformatics. 30:523-530.

Kris-Etherton P. M., K. D. Hecker, A. Bonanome, S. M. Coval, A. E. Binkoski, K. F. Hilpert, A. E. Griel, and T. D. Etherton. 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am. J. Med. 113:71S-88S.

Kunita R., O. Nakabayashi, J. Y. Wu, Y. Hagiwara, M. Mizutani, M. Pennybacker, Y. T. Chen, and T. Kikuchi. 1997. Molecular cloning of acid a-glucosidase cDNA of Japanese quail (*Coturnix coturnix japonica*) and the lack of its mRNA in acid maltase deficient quails. Biochim. Biophys. Acta. 1362:269-278.

Kuznetsova, L. A., S. A. Plesneva, A. O. Shpakov, and T. S. Sharova. 2004. Dehydrogenase, and glycogen synthase in skeletal muscles of chick embryos and chickens. J. Evol. Biochem. Phys. 40:409-419.

Laplante, M, and D. M. Sabatini. 2012. mTOR signaling in growth control and disease. Cell. 149:274-293.

Lee, K. W., S. H. Lee, H. S. Lillehoj, G. X. Li, S. I. Jang, U. S. Babu, M. S. Park, D. K. Kim, E. P. Lillehoj, A. P. Neumann, T. G. Rehberger, and G. R. Siragusa. 2010. Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. Poult. Sci. 89:203-216.

Lee, K. W., H. S. Lillehoi, S. H. Lee, S. I. Jang, G. D. Ritter, D. A. Bautista, and E. P. Lillehoj. 2011. Impact of fresh or used litter on the posthatch immune system of commercial broilers. Avian. Dis. 55:539-544.

Lee, K. W., H. S. Lillehoi, S. H. Lee, S. I. Jang, D. A. Bautista, and G. R. Siragusa. 2013. Effect of bacillus subtilis-based direct-fed microbials on immune status in broiler chickens raised on fresh or used litter. Asian-Australas. J. Anim. Sci. 26: 1592-1597.

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

Leshem, Y., I. Gitelman, C. Ponzetto, and O. Halevy. 2002. Preferential binding of Grb2 or phosphatidylinositol 3-kinase to the met receptor has opposite effects on HGF-induced myoblast proliferation. Exp. Cell. Res. 274:288–298.

Lim, C. H., W. Jeong, W. Lim, J. Kim, G. Song, F. W. Bazer. 2012. Differential expression of select members of the SLC family of genes and regulation of expression by microRNAs in the chicken oviduct. Biol. Reprod. 87:145-166.

Liu, Q. C., X. H. Zha, H. Faralli, H. Yin, C. Louis-Jeune, E. Perdiguero, P. Muñoz-Cànoves, M. A. Rudnicki, M. Brand, C. Perez-Iratxeta, and F. J. Dilworth. 2012. Comparative expression

profiling identifies differential roles for MyoGenin and p38a MAPK signaling in myogenesis. J. Mol. Cell. Biol. 4:386-397.

Liu, J., S. Guo, Q. Li, L. Yang, Z. Xia, L. Zhang, Z. Huang, and N. Zhang. 2013. Phosphoglycerate dehydrogenase induces glioma cells proliferation and invasion by stabilizing forkhead box M1. J. Neurooncol. 11:245-255.

Loor, J. J., H. M. Dann, N. A. Guretzky, R. E. Everts, R. Oliveira, C. A. Green, N. B. Litherland, S. L. Rodriguez-Zas, H. A. Lewin, and J. K. Drackley. 2006. Plane of nutrition prepartum alters hepatic gene expression and function in dairy cows as assessed by longitudinal transcript and metabolic profiling. Physiol. Genomics 27:29-41.

Loor, J. J., K. M. Moyes, and M. Bionaz. 2011. Functional adaptations of the transcriptome to mastitis-causing pathogens: The mammary gland and beyond. J. Mammary Gland Biol. Neoplasia. 16:305-322.

Loor, J. J., M. Bionaz, and J. K. Drackley. 2013. Systems physiology in dairy cattle: Nutritional genomics and beyond. Annu. Rev. Anim. Biosci. 1:365-392.

Lorri, J. J., M. Vailati-Riboni, J. C. McCann, Z. Zhou, and M. Bionaz. 2015. Nutrigenomics in livestock: Systems biology meets nutrition. J. Anim. Sci. 93:5554-5574.

Lu, J., S. Sanchez., C. Hofacre, J. J. Maurer, B. G. Harmon, and M. D. Lee. 2003. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. Appl. Environ. Microbiol. 69:901-908.

Lysne, V., B. Bjørndal, R. Vik, J. E. Nordrehaug, J. Skorve, O. Nygård, and R. K. Berge. 2015. A protein extract from chicken reduces plasma homocysteine in rats. Nutrients. 7:4498-4511.

Ma, X. M., and J. Blenis. 2009. Molecular mechanisms of mTOR-mediated translational control. Nat. Rev. Mol. Cell. Biol. 10:307-318.

Malila Y., K. M. Carr, C. W. Ernst, S. G. Velleman, K. M. Reed, and G. M. Strasburg. 2014. Deep transcriptome sequencing reveals differences in global gene expression between normal and pale, soft, and exudative turkey meat. J. Anim. Sci. 92:1250-1260.

Martin, S. A., and M. A. McCann. 1998. Microbiological survey of Georgia poultry litter. J. Appl. Poult. Res. 7:90–98.

Marioni, J. C., C. E. Mason, S. M. Mane, M. Stephens and Y. Gilad. 2008. RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. Genome. Res. 18:1509-1517.

Mattar, A. F., D. H. Teitelbaum., R. A. Drongowski., F. Yongi., C. M. Harmon, and A. G. Coran. 2002. Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model. Pediatr. Surg. Int. 18:586–590.

McCollum. M.V. 1925. Studies on experimental rickets. J. Biol. Chem. 65:97.100.

Melton, L. J., B. S. Coan, and S. C. Lee. 1999. Prediction of the strength of the elderly proximal femur by bone mineral density and quantitative ultrasound measurements of the heel and tibia. Bone. 24:49-55.

Miraoui, H., K. Oudina, H. Petite, Y. Tanimoto, K. Moriyama, and P. J. Marie. 2009. Fibroblast growth factor receptor 2 promotes osteogenic differentiation in mesenchymal cells via ERK1/2 and protein kinase C signaling. J. Biol. Chem. 284:4897-4904.

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. 84:553-60.

Michalczuk, M., D. Pietrzak, J. Niemiec, 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.

Mok, G. F., R. H. Mohammed, and D Sweetman. 2015. Expression of myogenic regulatory factors in chicken embryos during somite and limb development. J. Anat. 227:352-60.

Moncaut, N., P. W. Rigby, and J. J. Carvajal. 2013. Dial M(RF) for myogenesis. FEBS. J. 280:3980-3990.

Montagne, L., J. R. Pluske, and D. J. Hampson. 2003. A review of interactions between dietary fiber and the intestinal mucosa, and their consequences on digestive health in young nonruminant animals. Anim. Feed Sci. Technol. 108:95–117.

Morales, A., F. Grageola, H. García, N. Arce, B. Araiza, J. Yáñez, and M. Cervantes. 2014. Performance, serum amino acid concentrations and expression of selected genes in pair-fed growing pigs exposed to high ambient temperatures. J. Anim. Physiol. Anim. Nutr. 98:928-35.

Morris, A., R. Shanmugasundaram. M. S. Lilburn, and R. K. Selvaraj. 2014. 25-Hydroxycholecalciferol supplementation improves growth performance and decreases inflammation during an experimental lipopolysaccharide injection. Poult. Sci. 93:1951-1956.

Morris, A., and R. K. Selvaraj. 2014. In vitro 25-hydroxycholecalciferol treatment of lipopolysaccharide-stimulated chicken macrophages increases nitric oxide production and mRNA of interleukin-1 beta and 10.

Mosaad, Y. M., R. E. Farag, M. M. Arafa, S. Eletreby, H. A. El-Alfy, B. S. Eldeek, and Z. M. Tawhid. 2010. Association of human leucocyte antigen class I (HLA-A and HLA-B) with chronic hepatitis C virus infection in egyptian patients. Scand. J. Immunol. 6:548-553.

Moss, F. P. 1968. The relationship between the dimensions of the fibers and the number of nuclei during normal growth of skeletal muscle in the domestic fowl. Am. J. Anat. 122:555–563.

Moss, F. P., and C. P. Leblond. 1971. Satellite cells as the source of nuclei in muscles of growing rats. Anat. Rec. 170:421–435.

Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer, and B. Wold. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods. 5:621-628.

Mutryn, M. F., E. M. Brannick, W. Fu, W. R. Lee, and B. Abasht. 2015. Characterization of a novel chicken muscle disorder through differential gene expression and pathway analysis using RNA-sequencing. BMC Genomics. 16:399-418.

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.

Nazifi, S., A. Rezakhani, M. Koohimoghadam, M. Ansari-Lari, and Z. Esmailnezhad. 2008. Valuation of serum haptoglobin in clinically healthy cattle and cattle with inflammatory diseases in Shiraz, a tropical area in southern Iran. Bul. J. Vet. Med. 11:95-101.

Nakamura, A., Y. Ota, A. Mizukami, T. Ito, Y. B. Ngwai, and Y. Adachi. 2002. Evaluation of aviguard, a commercial competitive exclusion product for efficacy and after-effect on the antibody response of chicks to *Salmonella*. Poult Sci. 8:1653-1660.

Neibergs, H. L., and K. A. Johnson. 2012. Nutrition and the genome. J. Anim. Sci. 90:2308-2316.

Ng, S. C, A. L. Hart, M. A. Kamm, A. J. Stagg, and S. C. Knight. 2009. Mechanisms of action of probiotics: Recent advances. Inflamm. Bowel. Dis. 15:300-310.

Nguyen, A., P. Chen, and H. Cai. 2004. Role of CaMKII in hydrogen peroxide activation of ERK1/2, p38 MAPK, HSP27 and actin reorganization in endothelial cells. FEBS. Lett. 572:307-313.

Nielsen, O. L., J. Jensenius, P. H. Jørgensen, and S. B. Laursen. 1999. Serum levels of chicken mannan-binding lectin (MBL) during virus infections: Indication that chicken MBL is an acute phase reactant. Vet. Immunol. Immunopathol. 70:309-316.

Nii, T.; Sonoda, Y, N. Isobe, and Y. Yoshimura. 2011. Effects of lipopolysaccharide on the expression of proinflammatory cytokines and chemokines and the subsequent recruitment of immunocompetent cells in the oviduct of laying and molting hens. Poult. Sci. 90:2332-2341.

Norman, A. W. 1987. Studies of the vitamin D endocrine system in the avian. J. Nutr. 117:797–807.

North, C. M., R. B. Crawford, H. Lu, and N. E Kaminski. 2009. Simultaneous in vivo time course and dose response evaluation for TCDD-induced impairment of the LPS-stimulated primary IgM response. Toxicol. Sci. 112:123-132.

Noy, Y., and D. Sklan, D. 1995. Digestion and absorption in the young chick. Poult. Sci. 74:366-373.

Nurmi, E., and M. Rantala. 1973. New aspects of *Salmonella* infection in broiler production. Nature. 241:210-211.

Omdahl, J. L., H. A. Morris, and B. K. May. 2002. Hydroxylase enzymes of the vitamin D pathway: expression, function, and regulation. Annu. Rev. Nutr. 22:139-166.

Onderci, M., N. Sahin, K. Sahin, G. Cikim, A. Aydìn, I. Ozercan, and S. Aydìn. 2006. Efficacy of supplementation of α-amylaseproducing bacterial culture on the performance, nutrient use, and gut morphology of broiler chickens fed a corn-based diet. Poult. Sci. 85:505–510.

Pan, D., and Z. Yu. 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut. Microbes. 5:108-119.

Papešová, L., A. Fučíková, M. Pípalová, P. Tupý. 2008. The synergic effect of vitamin D_3 and 25-hydroxycholecalciferol/calcidiol in broiler diet. SAB. 39: 273-277.

Parkinson, G. B., and P. H. Cransberg. 2004. Effect of casein phosphopeptide and 25-hydroxycholecalciferol on tibial dyschondroplasia in growing broiler chickens. Br. Poult. Sci. 45:802:806.

Peighambari, S. M., R. J. Julian, and C. L. Gyles. 2000. Experimental *Escherichia coli* respiratory infection in broilers. Avian. Dis. 44:759-769.

Peng, I. C., R. L. Adams, E. J. Furumoto, P. Y. Hester, J. E. Larsen, O. A. Pike, and W. J. Stadelman. 1985. Allometric growth patterns and meat yields of carcass parts of turkey toms as influenced by lighting programs and age. Poult. Sci. 64:871-876.

Pesti, G. M. 2009. Impact of dietary amino acid and crude protein levels in broiler feeds on biological performance. J. Appl. Poult. Res. 18:477-486.

Pestka, S., C. D. Krause, D. Sarkar, M. R. Walter, Y. Shi, and P. B. Fisher. 2004. Interleukin-10 and related cytokines and receptors. Annu. Rev. Immunol. 22:929–979.

Perić, L., N. Milosevic, D. Zikic, S. Bjecov, D. Cvetkovic, S. Markov, M. Mohnl, and T. Steiner. 2010. Effects of probiotic and phytogenic products on performance, gut morphology and cecal microflora of broiler chickens. Archiv. Anim. Breed. 53:350-359.

Perry, R. L., and M. A Rudnick. 2000. Molecular mechanisms regulating myogenic determination and differentiation. Front. Biosci. 5:750-767.

Phadnis, R., and I. Nemere, I. 2003. Direct, rapid effects of 25-hydroxyvitamin D_3 on isolated intestinal cells. J. Cell. Biochem. 90:287-293.

Piórkowska, K., K. Żukowski, J. Nowak, K. Połtowicz, K. Ropka-Molik, and A .Gurgul. 2016. Genome-wide RNA-Seq analysis of breast muscles of two broiler chicken groups differing in shear force. Anim. Genet. 47:68-80.

Rama-Rao, S. V., M.V. L. N. Raju, A. K. Panda, A. K., G. Shyam Sunder, and R. P. Sharma. 2006. Effect of high concentrations of cholecalciferol on growth, bone mineralization, and mineral retention in broiler chicks fed suboptimal concentrations of calcium and nonphytate phosphorus. J. Appl. Poult. Res. 15:493-501.

Rama-Rao, S. V., M.V. L. N. Raju, A. K. Panda, A. K., G. Shyam Sunder, and R. P. Sharma. 2009. Performance and bone mineralization in broiler chicks fed on diets with different concentrations of cholecalciferol at a constant ratio of calcium to non-phytate phosphorus. Br. Poult. Sci. 50:528-535.

Raman, K., H. Wang, M. J. Troncone, W. I Khan, G Pare, and J. Terry. 2015. Overlap chronic placental inflammation is associated with a unique gene expression pattern. PLoS One. 10:e0133738-e0133750.

Rath, N. C., G. R. Huff, W. E. Huff, and J. M. Balog. 2000. Factors regulating bone maturity and strength in poultry. Poult. Sci. 79:1029-1032.

Rennie, J. S., and C. C. Whitehead. 1996. Effectiveness of dietary 25 and 1 hydroxycholecalciferol in combating tibial dyschondroplasia in broiler chickens. Br. Poult. Sci. 37:413-421.

Roberson, K. D., M. F. Ledwaba, and R. A. Charbeneau. 2005. Studies on the efficacy of twenty-five-hydroxycholecalciferol to prevent tibial dyschondroplasia in Ross broilers fed marginal calcium to market age. Int. J. Poult. Sci. 4:85-90.

Robinson, M. D., D. J. McCarthy, and G. K. Smyth. 2010. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26:139-140.

Roll, V. F. B., M. A. Dai Prá, and A. P. Roll. 2011. Research on *Salmonella* in broiler litter reused for up to 14 consecutive flocks. Poult. Sci. 90:2257-2262.

Round, J. L., and S. K. Mazmanian. 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc. Natl. Acad. Sci. 107:12204-12209.

Roux, S., and P. Orcel. 2000. Bone loss: factors that regulate osteoclast differentiation: an update. Arthritis. Res. 2:451-456.

Rucker, M., T. J. Fraites, S. L. Porvasnik, M. A. Lewis, I. Zolotukhin, D. A. Cloutier, B. J. Byrne. 2004. Rescue of enzyme deficiency in embryonic diaphragm in a mouse model of metabolic myopathy: Pompe disease. Development.13:3007-3019.

Russell, J., A. Bar, L. M. Sherwood, and S. Hurwitz. 1993. Interaction between calcium and 1,25-dihydroxyvitamin D_3 in the regulation of preproparathyroid hormone and vitamin D receptor messenger ribonucleic acid in avian parathyroids. Endocrinology. 132:2639-2644.

Sabourin, L. A., A. Girgis-Gabardo, P. Seale, A. Asakura, and M. A. Rudnicki. 1999. Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. J. Cell. Biol. 144:631-643.

Santillan, G., S. Katz, C. Buitrago, and R. Boland. 2004. $1\alpha,25(OH)_2D_3$ induces capacitative calcium entry involving a trpc3 protein in skeletal muscle and osteoblastic cells. Biol. Res. 37: 647-651.

Samanya M., Yamauchi K., 2002. Histological alterations of intestinal villi in chickens fed dried *Bacillus subtilis var. natto*. Comp. Biochem. Physiol. 133:95-104.

Saunders-Blades, J. L., J. L. MacIsaac, D. R. Korver, and D. M. Anderson. 2009. The effect of calcium source and particle size on the production performance and bone quality of laying hens. Poult. Sci. 88:338–353.

Saunders-Blades, J., and, D. R. Korver. 2014. The effect of maternal vitamin D source on broiler hatching egg quality, hatchability, and progeny bone mineral density and performance. Poult Sci. 23:773-783.

Savage, D. C., J. E. Siegel, J. E. Snellen, and D. D. Whitt. 1981. Transit time of epithelial cells in the small intestines of germfree mice and ex-germfree mice associated with indigenous microorganisms. Appl. Environ. Microbiol. 42:996–1001.

Schmidt, C. J., M. E. Persia, E. Feierstein, B. Kingham, and W. W. Saylor. 2009. Comparison of a modern broiler line and a heritage line unselected since the 1950s. Poult. Sci. 88:2610-2619.

Schneider, K., S. Kothlow, P. Schneider, A. Tardivel, T. Göbel, B. Kaspers, and P. Staeheli. 2004. Chicken BAFF--a highly conserved cytokine that mediates B cell survival. Int. Immunol. 16:139-48.

Schneider, B. L., R. A. Renema, M. Betti, V. L. Carney , and M. J. Zuidhof. 2012. Effect of holding temperature, shackling, sex, and age on broiler breast meat quality. Poult. Sci. 91:468-477.

Seifert, H. A., A. A. Hall, C. B. Chapman, L. A. Collier, A. E. Willing, and K. R. Pennypacker. 2012. A transient decrease in spleen size following stroke corresponds to splenocyte release into systemic circulation. J. Neuroimmune. Pharmacol. 7:1017-1024.

Sedlackova, T., G. Repiska, P. Celec, T. Szemes, and G. Minarik. 2013. Fragmentation of DNA affects the accuracy of the DNA quantitation by the commonly used methods. Biological procedures online, fragmentation of DNA affects the accuracy of the DNA quantitation by the commonly used methods. Biol. Proced. Online. 15:5-13.

Selvaraj, R. K., R. Shanmugasundaram, and R. Rengasamy. 2016. The use and abuse of immune indices in nutritional immunology studies. J. Appl. Poult. Res. 00:1-8.

Shanmugasundaram, R., and R. K. Selvaraj. 2011. Regulatory T cell properties of chicken $CD4^+$ $CD25^+$ cells. J. Immunol. 186:1997-2002.

Shanmugasundaram, R., M. S. Lilburn, and R. K. Selvaraj. 2012. Effect of recycled litter on immune cells in the cecal tonsils of chickens. Poult. Sci. 91:95-100.

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

Shirpoor, A., B. Ilkhanizadeh, R. Saadatian, B. S. Darvari, F. Behtaj, M. Karimipour, F. Ghaderi-Pakdel, and E. Saboori. 2006. Effect of vitamin E on diabetes-induced changes in small intestine and plasma antioxidant capacity in rat. J. Physiol. Biochem. 62:171-177.

Sibut, V., C. Hennequet-Antier, E. Le Bihan-Duval, S. Marthey, M. J. Duclos, and C. Berri. 2011. Identification of differentially expressed genes in chickens differing in muscle glycogen content and meat quality. BMC Genomics. 12:112-125.

Sitrin, M. D., K. L. Pllack, M. J. Bolt, and I. H. Roseberg. 1982. Comparison of vitamin D and 25-hydroxyvitamin D absorption in the rat. Amer. J. Physiol. 242:326-332.

Simon, J. 1989. Chicken as a useful species for the comprehension of insulin action. Crit. Rev. Poult. Biol. 2:121-148.

Shafey, T. M., S. Al-Mufarej and, H. A. Al-Batshan. 2006. Effect of electric field during incubation of eggs on the immune responses of hatched chickens. Electromagn. Biol. Med. 25:163-75.

Shim, M. Y., A. B. Karnuah, A.D. Mitchell, N. B. Anthony, G. M. Pesti, and S. E. Aggrey. 2012. The effects of growth rate on leg morphology and tibia breaking strength, mineral density, mineral content, and bone ash in broilers. Poult. Sci. 91:1790-1795.

Singh, K., and F. J. Dilworth. 2013. Differential modulation of cell cycle progression distinguishes members of the myogenic regulatory factors. FEBS. J. 280:3991-4003.

Shinki, T., N. Takahashi, T. Kadofuku, T. Sato, and T. Suda. 1985. Induction of spermidine N1-acetyltransferase by 1α, 25-dihydroxyvitamin D_3 as an early common event in the target tissues of vitamin D. J. Biol. Chem. 260:2185–2190.

Shinki, T., N. Takahashi, C. Miyaura, K. Samejima, Y. Nishii, and T. Suda. 1981. Ornithine decarboxylase activity in chick duodenum induced by 1α,25-dihydroxycholecalciferol. Biochem. J. 195:685–690.

Shinki, T., H. Tanaka, J. Takito, A. Yamaguchi, Y. Nakamura, S. Yoshiki, and T. Suda. 1991. Putrescine is involved in the vitamin D action in chick intestine. Gastroenterology. 100:113–122.

Shirkey, T. W., R. H. Siggers, B. G. Goldade, J. K. Marshall, M. D. Drew, B. Laarveld, and Van A. G. Kessel. 2006. Effects of commensal bacteria on intestinal morphology and expression of proinflammatory cytokines in the gnotobiotic pig. Exp. Biol. Med. 231:1333-1345.

Shojadoost, B., A. R. Vince, and J. F Prescott. 2012. The successful experimental induction of necrotic enteritis in chickens by *Clostridium perfringens*: a critical review. Vet Res. 43:74-86.

Shojadoost, B., S. Behboudi, A. I. Villanueva, J. T. Brisbin, A. A. Ashkar, and S. Sharif. 2015. Vitamin D_3 modulates the function of chicken macrophages. Res. Vet. Sci.100:45-51.

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.

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

Song, J., K. Xiao, Y. L. Ke , L. F. Jiao, C. H. Hu , Q. Y. Diao, B. Shi, and X. T. Zou. 2014. Effect of a probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. Poult. Sci. 93:581–588.

Spratt, R. S., B. W. McBride, H. S. Bayley, and S. Leeson. 1990. Energy metabolism of broiler breeder hens. 2. Contribution of tissues to total heat production in fed and fasted hens. Poult. Sci. 69:1348–1356.

Sun, P., H. Enslen, P. Myung, and R. Maurer. 1994. Differential activation of CREB by Ca2+/calmodulin-dependent protein kinase type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev. 8:2527-2539.

Sun, Z. W., L. Yan, Y. Y. G, J. P. Zhao, H. Lin, and Y. M. Guo. 2013. Increasing dietary vitamin D_3 improves the walking ability and welfare status of broiler chickens reared at high stocking densities. Poult. Sci. 92:3071-3079.

Świątkiewicz, S, and J. Koreleski. 2005. The effect of particulate limestone and 25-hydroxy-cholecalciferol in broiler chicken diets on mechanical properties of bones. J. Anim. Food. Sci. 14:495-498.

Świątkiewicz, S., J. Koreleski, and J. Kopowski. 2006. Effect of phytase and 25-hydroxycholecalciferol on the performance and bone quality in broiler chickens. Med. Weter. 62:81-84.

Szpirer, J., C. Szpirer, M. Riviere, G. Levan, P. Marynen, J. J. Cassiman, R. Wiese, and H. F. De Luca. 1991. The Sp1 transcription factor gene (SP1) and the 1,25-dihydroxyvitamin D_3 receptor gene (VDR) are colocalized on human chromosome arm 12q and rat chromosome 7. Genomics. 11:168-73.

Tabor, C. W., and H. Tabor. 1984. Polyamines. Annu. Rev. Biochem. 53:749–790.

Taira, I., T. Fujinaga, and M. Okumura. 1992. Equine haptoglobin isolation, characterization and the effects of ageing, delivery, inflammation on the serum concentration. J. Vet. Med. Sci. 54:435-442.

Tam, S., M. S. Tsao, and J. D. McPherson. 2015. Optimization of miRNA-seq data preprocessing. Brief. Bioinform. 6:950-963.

Targowski, S. P., W. Klucinski, S. Babiker, and B. J. Nonnecke. 1986. Effect of ammonia on in vivo and in vitro immune responses. Infect. Immun. 43:289-293.

Thaxton, J. P., and S. Puvadolpirod. 2000. Model of physiological stress in chickens. 5. Qualitative evaluation. Poult. Sci. 79:391-395.

Tesseraud, S., M. Abbas, S. Duchene, K. Bigot, P. Vaudin, J. Dupont. 2006. Mechanisms involved in the nutritional regulation of mRNA translation: features of the avian model. Nutr. Res. Rev. 19:104-116.

Tickle, P. G., H. Paxton, J. W. Rankin, J. R. Hutchinson, and J. R. Codd. 2014. Anatomical and biomechanical traits of broiler chickens across ontogeny. Part I. Anatomy of the musculoskeletal respiratory apparatus and changes in organ size. Peer. J. 2:e432-e449.

Torok, V. A., R. J. Hughes, K. Ophel-Keller, M. Ali, and R. MacAlpine. 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. Poult. Sci. 88:2474–2481.

Uni, Z., Y. Noy, and D. Sklan. 1999. Posthatch development of small intestinal function in the poult. Poult. Sci. 78:215-22.

Vest, R. S., K. D. Davies, H. O'Leary, J. D. Port, and K. U. Bayer. 2007. Dual mechanism of a natural CaMKII inhibitor. Mol. Biol. Cell. 18: 5024–5033.

Vignale, K., J. V. Caldas, J. England, N. Boonsinchai, P. Sodsee, E. D. Pollock, and C. N. Coon. 2013. The effect of HyD on broiler breast meat yield. Poult. Sci. 92(Suppl. 1):120. (Abstr.).

Vignale, K., E. S. Greene, J. V. Caldas, J. England, N. Boonsinchai, P. Sodsee, E. D. Pollock, S. Dridi, and C. N. Coon. 2015. 25-Hydroxycholecalciferol enhances male broiler breast meat yield through the mTOR pathway. J. Nutr. 145:855-863.

16479.

Volkova, V. V., R. H. Bailey, and R. W. Wills. 2009. *Salmonella* in broiler litter and properties of soil at farm location. Plos. One. 4:e6403-e6409.

Von Essen M.R., M. Kongsbak, P Schjerling, , Klaus Olgaard, N. Ødum , C. Geisler. 2010. Vitamin D controls T cell antigen receptor signalingand activation of human T cells. Nat. Immunol. 11:344-349.

Von Maltzahn, J., A. E. Jones, R. J. Parks, and M. A. Rudnicki. 2013. Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. Proc. Natl. Acad. Sci. 110:16474-

Waititu, S. M., A. Yitbarek, E. Matini, H. Echeverry, E. Kiarie, J. C. Rodriguez-Lecompte, and C. M. Nyachoti. 2014. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poult. Sci. 93:625-635.

Wang, Z., M. Gerstein, and M. Snyder. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. 10:57-63.

Wang T. T., B. Dabbas, D. Laperriere, A. J. Bitton, H. Soualhine, L. E. Tavera-Mendoza, S. Dionne, M.J. Servant , A. Bitton, E. G. Seidman, S. Mader, M. A. Behr, J. H.White. 2010. Direct and indirect induction by 1,25-dihydroxyvitamin D_3 of the NOD2/CARD15-defensin beta2 innate immune pathway defective in Crohn disease. J. Biol. Chem. 4:2227-2231.

Wang, Y., B. Lupiani, S. M. Reddy, S. J. Lamont, and H. Zhou. 2014. RNA-seq analysis revealed novel genes and signaling pathway associated with disease resistance to avian influenza virus infection in chickens. Poult. Sci. 93:485-493.

Weissen-Plenz, G., Nitschke, Y. and Rutsch F. 2008. Mechanisms of arterial calcification: spotlight on the inhibitors. Adv. Clin. Chem. 46:263-293.

Wei, S., A. Gutek, M. Lilburn, and Yu Z. 2013. Abundance of pathogens in the gut and litter of broiler chickens as affected by bacitracin and litter management. Vet. Microbiol. 166:595-601.

Wong, N., G. Morahan, M. Stathopoulos, J. Proietto, and S. Andrikopoulos. 2013. A novel mechanism regulating insulin secretion involving HERPUD1 in mice. Diabetologia. 56:1569-1576.

Wouters, B. G., and M. Koritzinsky. 2008. Hypoxia signaling through mTOR and the unfolded protein response in cancer. Nature. 8:851-864.

Wu, Q. J., Y. M. Zhou, Y. N. Wu, and T. Wang. 2013. Intestinal development and function of broiler chickens on diets supplemented with clinoptilolite. Asian-Australas. J. Anim. Sci. 26:987-994.

Yablonka-Reuveni, Z. 1995a. Myogenesis in the chicken: the onset of differentiation of adult myoblasts is influenced by tissue factors. Basic. Appl. Myol. 5:33-41.

Yablonka-Reuveni, Z. 1995b. Development and postnatal regulation of adult myoblasts. Microsc. Res. Tech. 30:366-380.

Yablonka-Reuveni Z., and B. M. Paterson. 2001. MyoD and myogenin expression patterns in cultures of fetal and adult chicken myoblasts. J. Mol. Histol. 49:455-462.

Yang, Y., P. A. Iji, A. Kocher, L. L. Mikkelsen, and M. Choct. 2008. Effects of dietary mannanoligosaccharide on growth performance, nutrient digestibility, and gut development of broilers given different cereal-based diets. J. Anim. Physiol. Anim. Nutr. Berl. 92:650–659.

Xu, S., X. Liu, Y. Bao, X. Zhu, C. Han, P. Zhang, X. Zhang, W. Li, and X. Cao. 2012. Constitutive MHC class I molecules negatively regulate TLR-triggered inflammatory responses via the Fps-SHP-2 pathway. Nat. Immunol. 13:551-559.

Yablonka-Reuveni Z., and B. M. Paterson. 2001. MyoD and myogenin expression patterns in cultures of fetal and adult chicken myoblasts. J. Mol. Histol. 49:455-462.

Yason, C. V., B. A. Summers, and K. A. Schat. 1987. Pathogenesis of rotavirus infection in various age-groups of chickens and turkeys: pathology. Am. J. Vet. Res. 48:927–938.

Yarger, J.G., C.A., Sunders, 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.

Yarru, L. P., R. S. Settivari, E. Antoniou, D. R. Ledoux, and G. E. Rottinghaus. 2009. Toxicological and gene expression analysis of the impact of aflatoxin B_1 on hepatic function of male broiler chicks. Poult. Sci. 88:360-371.

Yu, B., C.C. Tsai, J.C. Hsu, and P.W.S. Chiou. 1998. Effect of different sources of dietary fiber on growth performance, the intestinal morphology and caecal carbohydrases of the domestic geese. Br. Poultry Sci. 39:560-567.

Zhang, G., L. Ma, and M.P. Doyle. 2007. Potential competitive exclusion bacteria from poultry inhibitory to *Campylobacter jejuni* and *Salmonella*. J. Food. Prot.70:867-873.

Zhang, J., D Upadhya, L. Lu, and L. W Reneker. 2015. Fibroblast growth factor receptor 2 (FGFR2) is required for corneal epithelial cell proliferation and differentiation during embryonic development. Plos. One. 10:e1371-e1389.

Zhao, S., W. P. Fung-Leung, A. Bittner, K. Ngo, and X. Liu. 2014. Comparison of RNA-seq and microarray in transcriptome profiling of activated T cells. PLoS One. 9:e78644-e78657.

Zhou, N., W. R. Lee, and B. Abasht. 2015. Messenger RNA sequencing and pathway analysis provide novel insights into the biological basis of chickens` feed efficiency. BMC. Genomics. 16:195-215.

Zuidhof, M. J., B. L. Schneider, V. L. Carney, D. R. Korver, and F. E. Robinson. 2014. Growth, efficiency, and yield of commercial broilers from 1957, 1978, and 2005. Poult. Sci. 93:2970–2982.