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THE EFFECTS OF ACTIGEN® AND THREONINE SUPPLEMENTATION ON GROWTH PARAMETERS, IMMUNE FUNCTION, AND INTESTINAL HEALTH IN MONOGASTRICS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture
at the University of Kentucky

By
Lindsay R. Good
Lexington, KY

Director: Dr. Anthony J. Pescatore, Extension Professor of Animal and Food Sciences
Lexington, Kentucky
2013

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ABSTRACT OF THESIS

THE EFFECTS OF ACTIGEN® AND THREONINE SUPPLEMENTATION ON GROWTH PARAMETERS, IMMUNE FUNCTION, AND INTESTINAL HEALTH IN MONOGASTRICS

The objectives of these experiments were to evaluate the main effects and interactive effects between dietary supplementation of a mannan oligosaccharide based product, Actigen® (ACT), and different levels of threonine in monogastrics, as evidenced by growth parameters, immune function, and intestinal health characteristics.

In nursery piglets, ACT supplementation decreased average daily feed intake (P=0.04), but had no effect on body weight or feed conversion ratio. There were no noted differences between threonine levels on performance traits. There were no consistent differences in complete blood count or cytokine gene expression profiles in the blood. The highest level of threonine, 77% true ileal digestible threonine:lysine (Thr:Lys), increased villus height (P=0.007) and villus height:crypt depth (P=0.01). The lowest level, 57% Thr:Lys, decreased villus surface area (P=0.04) and goblet cell density (P=0.04). Supplementation with ACT increased total goblet cell area (P=0.02) and density (P=0.05). There were no interactions observed between ACT and Thr:Lys levels.

In broiler chicks, feeding a diet containing 0.56% threonine decreased body weight (P<0.0001) and feed consumption (P<0.0001) and increased the feed to gain ratio (P<0.0001). Supplementation with ACT tended to increase bird body weight (P=0.07). On d 7, birds supplemented with ACT had heavier spleens as a percentage of body weight (P=0.01) compared to no ACT. When adjusted for body weight, the 0.56% threonine fed birds had smaller spleens (P=0.05) on d 7 when compared to the other threonine levels. Humerus (P<0.0001) and tibia (P<0.0001) from chicks fed 0.56% threonine for 21 d required less force to break than the other threonine levels. Birds fed 0.80% threonine had a higher concentration of phosphorus (P=0.04) and birds fed 0.56% threonine had a higher concentration of potassium (P<0.0001) in humerus when compared to the other threonine levels. The ileum of birds fed 0.56% threonine, contained shorter villi (P=0.03) and few goblet cells (P=0.04) on d 7 when compared to the other threonine levels. In d 21 jejunum, supplementation with ACT reduced apical

width (P=0.03) and surface area (P=0.02). An interaction was observed between ACT and threonine level in the jejunum on d 21 on basal width (P=0.03) and surface area (P=0.02), indicating that in diets lacking ACT, excess threonine increased villus size.

Overall, ACT and threonine acted primarily independently to modulate the intestinal architecture of both nursery piglets and broiler chicks. However, in broiler chicks ACT and threonine interacted to alter villus size. These results indicate that ACT and threonine have direct effects on the intestines of monogastrics.

KEY WORDS: Threonine, mannan oligosaccharides, weanling pigs, broiler chicks, performance

Lindsay R. Good

October 19, 2013

THE EFFECTS OF ACTIGEN® AND THREONINE SUPPLEMENTATION ON GROWTH PARAMETERS, IMMUNE FUNCTION, AND INTESTINAL HEALTH IN MONOGASTRICS

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This thesis is dedicated to my parents, Amy and Bruce Good, who have always encouraged me to be a better person.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS……iii
TABLE OF CONTENTS……...……vi
LIST OF TABLES……..ix
LIST OF FIGURES……..xiii
LIST OF FILES……xiv
CHAPTER 1. LITERATURE REVIEW……...…..1
1.1 Introduction…….……..……1
1.2 Antibiotics in the Food Animal Industry……...……..……..1
1.2.1 History of antibiotics in food animal industry…....……....1
1.2.2 Ban of antibiotics as growth promoters…….……....2
1.3 Review of Animal Gastrointestinal Physiology…….…….……..…….....5
1.3.1 Pig Gastrointestinal Physiology……..……5
1.3.2 Chicken Gastrointestinal Tract…….……..……7
1.3.3 Intestinal structure and cellular physiology…..……..…….....8
1.4 Threonine…….……..…….…….12
1.4.1 Threonine biochemistry…..……..…..12
1.4.2 Threonine requirements in broilers……...…….12
1.4.3 Threonine requirements in weanling pigs…….……..…..13
1.4.4 Importance of threonine on intestinal health…….……..…13
1.5 Mannan oligosaccharides……...……..……....15
1.5.1 Mode of action…..……..……..…15
1.5.2 Research using MOS products…….…16
1.6 Conclusion……..…..…..……..17
CHAPTER 2. Effects of Actigen® and dietary threonine levels on intestinal histomorphology, immune status, and growth performance of nursery piglets…….……18
2.1 Introduction…….…….……18
2.2 Materials and Methods…….……19
2.2.1 Pigs and study design…..……...19
2.2.2 Diets…….……20
2.2.3 Sample collection…..……20
2.2.4 Sample preparation-Quantitative real time-PCR…….…….……21
2.2.5 Sample preparation-Histomorphology of jejunum…..…….……21
2.2.6 Quantification of cytokine genes……..……21
2.2.7 Observation of histomorphology of jejunum…….……...22
2.2.8 Statistical analysis…….……22
2.3 Results…..……23
2.3.1 Performance…….……23
2.3.2 Complete blood count profiles…….……23
2.3.3 qRT-PCR…….……24

2.3.4 Histomorphology of jejunal tissue..……24
2.4 Discussion…….……...24
2.5 Conclusion……...26
CHAPTER 3. The interaction between Actigen® and threonine supplementation on intestinal histomorphology and growth parameters in broiler chicks……42
3.1 Introduction…..……42
3.2 Materials and Methods…….……....43
3.2.1 Animals and Treatments…….……..…....43
3.2.2 Measurement of Performance Characteristics.……44
3.2.3 Sample collection…….……44
3.2.4 Histomorphological analysis…….……....44
3.2.5 Bone Breaking Strength and Mineral Analysis…….……...45
3.2.6 Statistical Analysis…….……...45
3.3 Results…….……45
3.3.1 Performance…..……45
3.3.2 Organ weights…….……..46
3.3.3 Bone breaking strength and mineral composition…….……...46
3.3.4 Histomorphology…….……46
3.4 Discussion…….……...47
3.5 Conclusion…….……..49
Appendices……70
Appendix 1. Example of simple monogastric digestive tract……70
Appendix 2. Diagram of modern chicken gastrointestinal tract……71
Appendix 3. Diagram of intestinal wall layers……..72
Appendix 4. Diagram of the ways the small intestine increases its surface area……...73
Appendix 5. Diagram of the cell types of the intestinal epithelia……..74
Appendix 6. Diagram of the structure of the mucin glycoprotein……75
Appendix 7. Diagram of the chemical structure of threonine……76
Appendix 8. Histomorphology protocol for Chapter 2 and Chapter 3……..77
Appendix 9. Interaction effects between Actigen® (ACT) and threonine concentration on nursery piglets performance characteristics……...79
Appendix 10. Interaction effects of Actigen® (ACT) and threonine concentration on jejunal histomorphology…….……...80
Appendix 11. Aflatoxin analysis of the peanut meal used in Chapter 3…..……..81
Appendix 12. Average organ weights of d 7 broiler chicks……..82
Appendix 13. Average organ weights of d 21 broiler chicks……....83
Appendix 14. Interaction between Actigen® (ACT) and threonine on performance characteristics of broiler chicks……84
Appendix 15. Interaction between Actigen® (ACT) and threonine on breaking strength of tibia and humerus from d 21 broiler chicks……...85

Appendix 16. Interaction between Actigen® (ACT) and threonine on d 7 duodenum intestinal histomorphology from broiler chicks…….86
Appendix 17. Interaction between Actigen® (ACT) and threonine on d 7 jejunum intestinal histomorphology from broiler chicks…….87
Appendix 18. Interaction between Actigen® (ACT) and threonine on d 7 ileum intestinal histomorphology from broiler chicks……88
Appendix 19. Interaction between Actigen® (ACT) and threonine on d 21 duodenum intestinal histomorphology from broiler chicks…….89
Appendix 20. Interaction between Actigen® (ACT) and threonine on d 21 jejunum intestinal histomorphology from broiler chicks…….90
Appendix 21. Interaction between Actigen® (ACT) and threonine on d 21 ileum intestinal histomorphology from broiler chicks…….91
References……..92
Vita..……...99

LIST OF TABLES

Table 2.1 Phase I weaning diets (0-7 d) based on National Research Council, 1998 requirements for 3-5 kg pigs (% as fed)……..…...27

Table 2.2 Phase II weaning diets (7-21 d) based National Research Council, 1998 requirements for 5-10 kg pigs (% as fed)……..…….28

Table 2.3 Phase III weaning diets (21-35 d) based on National Research Council, 1998 requirements for 10-20 kg pigs (% as fed)…….29

Table 2.4 Diets, Calculated nutrient content based on National Research Council, 1998 requirements (% as-fed)…….30

Table 2.5 Proximate analysis and analyzed amino acid composition of nursery piglet diets……31

Table 2.6 Main effects of dietary threonine concentration (Thr:Lys) and Actigen® (ACT) on body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and gain to feed conversion ratio (G:F) of piglets……..……32

Table 2.7 Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 0 d post weaning piglets……...…33

Table 2.8 Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 7 d post weaning piglets……...…34

Table 2.9 Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 14 d post weaning piglets……..…35

Table 2.10 Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 21 d post weaning piglets……..…36

Table 2.11 Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 28 d post weaning piglets……..…37

Table 2.12 Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 35 d post weaning piglets……..…38

Table 2.13 Main effects of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on villus height (μm), apical length (μm), basal length (μm), villus surface area (μm^2), crypt cell depth (μm), villus height:crypt cell depth, goblet cell count, goblet cell area (μm2), average goblet cell area (μm^2), and goblet cell density of piglets…..……...39

Table 2.14 Main effects of Actigen® (ACT) supplementation on 14 d post weaning cytokine gene expression of piglets……...…40

Table 2.15 Main effects of Actigen® (ACT) supplementation on 35 d post weaning cytokine gene expression of piglets……..……..……41

Table 3.1 Broiler chick (d 1-d 21) starter diet composition (% as-fed)…….……50

Table 3.2 Broiler chick (d 1-d 21) starter diet calculated nutrient content (% of diet)…..51

Table 3.3 Proximate analysis and analyzed amino acid composition of broiler chick (d 1-d 21) starter diet and ingredients…...……..52

Table 3.4 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on average body weights (ABW) of broiler chicks…….……...……53

Table 3.5 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on average daily feed intake (ADFI) of broiler chicks……...……54

Table 3.6 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on feed to gain ratio (F:G) of broiler chicks……...…55

Table 3.7 Average organ weights as a percentage of body weight of d 7 broiler chicks fed diets containing varying threonine (Thr) concentrations and Actigen® (ACT)……...…56

Table 3.8 Average organ weights as a percentage of body weight of d 21 broiler chicks fed diets containing varying threonine (Thr) concentrations and Actigen® (ACT)……57

Table 3.9 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on average breaking strength of tibia and humerus of broiler chicks...……..58

Table 3.10 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on average ash content of tibia and humerus of broiler chicks…...……58

Table 3.11 Main effects and interaction of dietary threonine (Thr) concentration and Actigen® (ACT) on mineral composition of tibia from broiler chicks..…….……..59

Table 3.12 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on mineral composition of humerus from broiler chicks……60

Table 3.13 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the duodenum of 7 day old broiler chicks.…61

Table 3.14 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the jejunum of 7 day old broiler chicks…..…62

Table 3.15 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the ileum of 7 day old broiler chicks.……63

Table 3.16 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the duodenum of 21 day old broiler chicks...64

Table 3.17 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the jejunum of 21 day old broiler chicks.…..65

Table 3.18 Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the ileum of 21 day old broiler chicks.……..66

Table A.2.1 Interaction of dietary threonine concentration (Thr:Lys) and Actigen® (ACT) on body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and gain to feed conversion ratio (G:F) of nursery piglets……...……...79

Table A.2.2 Interaction of dietary threonine concentration (Thr:Lys) and Actigen® (ACT) on jejunum histology in nursery piglets…….80

Table A.3.1. Aflatoxin analysis of peanut meal……...…….81

Table A.3.2 Average organ weights of d 7 broiler chicks fed diets containing varying threonine (Thr) concentrations and Actigen® (ACT)……..….82

Table A.3.3 Average organ weights of d 21 broiler chicks fed diets containing varying threonine (Thr) concentrations and Actigen® (ACT)……..…...…..83

Table A.3.4 Interaction of dietary threonine (Thr) concentrations and Actigen® (ACT) on body weight (BW), average daily feed intake (ADFI), and gain to feed conversion ratio (G:F) of broiler chicks……...84

Table A.3.5 Interaction of dietary threonine (Thr) concentrations and Actigen® (ACT) on average breaking strength of tibia and humerus of broiler chicks…….85

Table A.3.6 Interaction of dietary threonine (Thr) concentrations and Actigen® (ACT) on intestinal morphology for duodenum of 7 day old broiler chicks…….86

Table A.3.7 Interaction of dietary threonine (Thr) concentrations and Actigen® (ACT) on intestinal morphology for jejunum of 7 day old broiler chicks…….87

Table A.3.8 Interaction of dietary threonine (Thr) concentrations and Actigen® (ACT) on intestinal morphology for ileum of 7 day old broiler chicks…….88

Table A.3.9 Interaction of dietary threonine (Thr) concentrations and Actigen® (ACT) on intestinal morphology for duodenum of 21 day old broiler chicks……89

Table A.3.10 Interaction of dietary threonine (Thr) concentrations and Actigen® (ACT) on intestinal morphology for jejunum of 21 day old broiler chicks……..…...90

Table A.3.11 Interaction of dietary threonine (Thr) concentrations and Actigen® (ACT) on intestinal morphology for ileum of 21 day old broiler chicks……..……...91

LIST OF FIGURES

Figure 3.1 Interaction (*P*=0.03) between Actigen® (ACT) and threonine (Thr) concentration on basal width (μm) of jejunum villi in d21 broiler chicks……..…67

Figure 3.2 Interaction (*P*=0.02) between Actigen® (ACT) and threonine (Thr) concentration on surface area (mm^2) of jejunum villi in d21 broiler chicks…….68

Figure 3.3 Histomorphological characteristics of jejunum tissue supplemented with or without Actigen®…….……..69

LIST OF FILES

GoodLindsayThesis.pdf

CHAPTER 1. Literature Review

1.1 Introduction

A growing world population is expanding the need for a safe and sufficient food supply. Current production practices may result in animal performance below genetic potential, stemming primarily from bacteria that are present in these production environments. For many years these bacterial problems were alleviated with the inclusion of antibiotics in the animals' feed. An increase in antibiotic resistant organisms in hospitals as well as consumer concern about antibiotic residues in their food supply have led to government bans of the use of antibiotics as growth promoters. Therefore alternative strategies that promote animal health without negatively affecting the world's food supply are required.

1.2 Antibiotics in the Food Animal Industry

1.2.1 History of antibiotics in food animal industry

Antibiotics have long been found to make marked improvements in food animal performance (Dibner and Richards, 2005). The practice of including antibiotics in feed at sub-therapeutic levels, known as antimicrobial growth promoters (AGP), has been observed in animal agriculture since the mid-1900's (Dibner and Richards, 2005; Jukes et al., 1950; Moore et al., 1946). The success of AGP has been attributed to multiple mechanisms, although their antibacterial properties are recognized as the primary modes of action. In 2010, Huyghebaert et al. reported that AGP improved performance of animals through four different mechanisms: alleviating and reducing incidence of

subclinical bacterial infections in the gut of the animal (Brennan et al., 2003; George et al., 1982); reducing microbial competition for nutrients (Snyder and Wostmann, 1987); reduction of intestinal wall width, resulting in improved absorptive capacity and decreased production of Gram positive bacteria metabolites that are known to negatively affect growth (Feighner and Dashkevics, 1987; Huyghebaert et al., 2011).

Although AGP have provided a useful function in the food animal industry, due to governmental regulations and public concern, producers are either eliminating or limiting the use of AGP worldwide. Much of the debate about AGP center around the transfer of bacterial resistance from the animal microbiota populations to the human microbiota populations (Dibner and Richards, 2005). In 1969, Swann proposed to the British Parliament that sub-therapeutic antibiotics should be eliminated from the food supply in response to human pathogen resistance observed in hospitals (Soulsby, 2007).

1.2.2 Ban of antibiotics as growth promoters

Swann (1969) may have proposed the idea in the United Kingdom first, but Sweden was the first country to issue a ban on AGP in 1986 (Aarestrup, 2003). Denmark followed in 1995 banning the use of avoparcin. The European Union (EU) extended to all of their member countries (Dibner and Richards, 2005). The ban in the EU was revised to include all AGP in 2006. Although there are currently no such bans in the United States, the FDA has instituted a voluntary program to encourage producers to administer antibiotics on an as-need basis (FDA, 2012). Consumers increasingly desire antibiotic free (ABF) meat in the United States particularly from the poultry industry (Dibner and Richards, 2005). In addition to consumer demands, the ban in the EU means

that imports from the United States must not contain products from animals fed AGP as well.

There are conflicting views on the results of banning AGP in the EU. According to Casewell et al. (2003), before the full ban had been instituted, Europe had failed to see a reduction in antibiotic resistance genes in humans, although there had been a slight decrease in animals (Casewell et al., 2003), indicating that the primary medical concern of antibiotic resistance was not affected by the ban, which left some wondering if the ban was worth it (Casewell et al., 2003). This was in stark contrast to the report written by Wierup (2001) about Sweden's ban on AGP, where the author discussed the consequences of the ban, but concluded that the WHO and EU should continue to ban AGP.

The health and welfare of the animals suffered immediately following the ban, as evidenced particularly in Sweden. Prior to the ban, virginimycin was administered to broilers at a sub-therapeutic level of 10 ppm to prevent necrotic enteritis. By the second year after the ban was instituted, 100% of the birds in Sweden were receiving a therapeutic dose of virginimycin of 20 ppm. When the birds were treated production was relatively unaffected. As an industry though, Sweden's poultry producers worked to institute management practices that reduce contributing factors to necrotic enteritis (Wierup, 2001). It should be noted that Sweden has a relatively small broiler population, allowing these changes to be made without a huge financial backlash.

Although the broiler industry was negatively impacted, it was really the pork producers that were influenced the most when AGP were banned. Post-weaning, many piglets experience an enteritis related diarrhea, which was previously prevented with

olaquindox or mecadox. Following the ban there was a marked increase in the mortality of weaned piglets. Weaning often results in a period of anorexia and diarrhea in piglets (van Beers-Schreurs et al., 1992). It is thought that the stress of separation from their dam, a change from a liquid to solid diet, and change in environment and population all contribute to these conditions (Lalles et al., 2007). These stresses all contribute to a reduction in small intestine function, as evidenced by villus atrophy and apoptosis of enterocytes (Wijtten et al., 2011), which results in decreased absorption, as well as leaving the intestine prone to infection. *E. coli* has been noted as the pathogenic bacteria that is often present in weanling pigs, often being cited as the source of the diarrhea (van Beers-Schreurs et al., 1992). Increased mortality, coupled with the fact that the wean to finish phase increased by 2 days, resulted in a significant cost to pork producers (Wierup, 2001). Unlike the broiler industry which recovered by implementing good management practices, the pork industry in Sweden has yet to recover (Callesen, 2002). The ban in Denmark resulted in a rise in enteric infections in both weaned and finisher pigs (Verner Wheelock and Foster, 2002). These reports indicate that banning AGP without a plan to promote animal health has serious industry-wide implications

Although the health of the animals has suffered due to the ban on AGP, the WHO is still working to institute a worldwide ban on AGP. As noted earlier, good management practices can counteract a number of these negative effects. However, the level at which food animal production occurs in other countries may make this an unrealistic option. In order to please the consumer, who is the center of this movement, producers need to grow a product that is comparably priced and meets the expectations of the buyer. Instituting major changes in the management practices of an entire industry, much less multiple

industries, is time consuming and costs money. A cost-effective solution that can be used within our current management practices is the most ideal way to overcome the ban of AGP. The issue at hand, particularly with broilers and weaned piglets in an ABF production system, is a challenged gastrointestinal system. Many solutions being researched are based upon this principle and include, but are not limited to, enhanced amino acid profiles, mannan oligosaccharide (MOS) products, enzymes, and other minerals (Pettigrew, 2006). All of the potential solutions are aimed at improving the integrity of the gastrointestinal system.

1.3 Review of Animal Gastrointestinal Physiology

1.3.1 Pig Gastrointestinal Physiology

To understand how to improve an animal's gastrointestinal system, it is important to understand how it functions. The purpose of the alimentary canal is to protect, secrete, and absorb nutrients. Although there are many differences among the gastrointestinal tract (GIT) of different animals, for the purpose of this thesis we will focus on the simple monogastric tract and some of the variations in it. The pig gastrointestinal tract is considered the basic monogastric tract and, therefore, will be the basis of the background information presented (Figure A.1.1).

The digestive tract begins at the mouth. A pig uses their lips to obtain feed, which then undergoes mastication, the chewing of food particles to mechanically reduce their size. Food particles undergo salivation, which lubricates the feed, allowing for aided travel down the esophagus. The esophagus delivers food to the glandular stomach where protein digestion occurs. The glands in the stomach secrete hydrochloric acid and

pepsinogen. The hydrochloric acid activates the pepsinogen to become pepsin. Pepsin is involved in proteolysis to breakdown protein molecules to dipeptides and amino acids. The process of digesting food particles with enzymes is known as chemical digestion. There is also an amount of mechanical digestion that occurs in the stomach, through muscle contractions of the stomach wall (Moran, 1982). The breaking down of the food molecules into smaller particles aids in absorption of nutrients further down the tract.

Digesta passes from the stomach into the small intestine. The digesta is moved through the intestines by way of peristalsis, the rhythmic contractions of the intestinal wall in a wave pattern from the stomach to the rectum. The small intestine is considered as three sections: the duodenum, jejunum, and ileum. Each section has a specific purpose. The duodenum receives enzymes and bile salts produced in the pancreas and gallbladder, respectively, that continue to aid in the chemical break down of digesta. In addition to the pancreas and gallbladder, the intestine itself secretes specific enzymes that further aid in digestion. The jejunum is where initial absorption occurs. Digesta then continues to the final portion of the small intestine, the ileum. The ileum absorbs much of the remaining digesta (Moran, 1982).

From the ileum, digesta travels into a blind-ended sac called the cecum, where fiber fermentation occurs. Because monogastrics consume a low fiber diet when compared to ruminants or hindgut fermenters the cecum is relatively unadvanced (Nickel et al., 1973). In the cecum, volatile fatty acids (VFA) are produced from the microbes that break down the fiber. These VFAs can be used for energy (Kass et al., 1980). In the large intestine, VFAs, water, vitamins and minerals are absorbed (Moran, 1982). The remainder of this entire process is passed to the rectum and is finally excreted as feces.

1.3.2 Chicken Gastrointestinal Tract

Chickens have many physiological differences when compared to the pig (Figure A.1.2). The first difference is birds possess a beak instead of a mouth. They use this to obtain food, and then use their tongue to funnel food and water into their esophagus. There is little mechanical digestion in the bird before the esophagus. Because birds do not produce nearly the amount of saliva that other animals do, the esophagus produces a large amount of mucus to aid in passage. Nearly 2/3 down the esophagus there is an out pocket known as the crop. The primary functions of the crop are storage and moistening of food particles. Food particles travel from the crop to the proventriculus via the remaining portion of the esophagus. The proventriculus functions as the glandular stomach, secreting hydrochloric acid and pepsinogen (Moran, 1982). However, the mechanical mixing of digesta and gastric secretions occurs in the gizzard, a highly muscularized organ. The gizzard possesses a keratin lining protecting its muscle component from the gastric acids secreted in the proventriculus (Hill, 1971) as well as mechanical damage. Digesta is ground into fine particles in the gizzard before passing into the small intestine.

The small intestine of the bird has very similar functions to that of the pig. However, when digesta continues through the gastrointestinal tract, it comes in contact with some distinct differences. First, the bird possesses two ceca. Chickens rely very little on fiber fermentation, so it is unknown what purpose these ceca serve to the modern bird and it can be assumed that these ceca are a carryover of an evolutionary adaptation, much like a human's appendix (Hodges, 1974). Second, the chicken has a relatively short large intestine, again attributed to its lack of fiber fermentation. After the large

intestine, digesta travels to the cloaca, where urine waste is also emptied. It then exits a common passage known as the vent (Moran, 1982).

1.3.3 Intestinal structure and cellular physiology

The duodenum is considered the proximal 1/5 of the small intestine, beginning at the pyloric sphincter of the stomach. The pancreatic duct and common biliary duct enter the duodenum after the inferior duodenal flexure (Moran, 1982).

The jejunum is the major site of absorption in the midgut and considered to be 2/5 of the length of the small intestine (Calhoun, 1954). As digesta is digested by pancreatic enzymes and bile salts, enterocytes containing transport pathways absorb the peptides, amino acids, carbohydrates, and free fats and transport them to the capillary system to allow these nutrients to be available to the body (Winne, 1972). It is recognized in chickens that the jejunum meets the ileum at Meckel's diverticulum which is the location of the attachment of the remainder of the yolk sac (Moran, 1982). The ileum is the distal 2/5 of the small intestine and is noted to continue in absorption of nutrients.

The intestinal wall consists of four distinct layers that each serves their own purpose (Figure A.1.3). The first layer is the mucosal layer. The mucosal layer includes the epithelium, which aids in transporting nutrients across first layer and into the fenestrated capillaries. The mucosal layer also secretes the mucus, which in turn offers protection to the intestinal wall. The second layer is the submucosa, which contains the blood vessels, submucosa plexii, and the submucosal glands which are found only in the duodenum. The third layer, the muscularis layer, contains both the longitudinal and circular muscle that allow for the peristaltic action. The final and outermost layer, the serosa, is primarily connective tissue (Sloss, 1954).

The small intestine has a few adaptations that allow for an increase in surface area allowing for increased secretory and absorptive capacities, making the system more efficient. In the intestines of pigs, the increase in surface area begins with the circular folds (Figure A.1.4). Along the lining of these circular folds are villi, long finger-like projections that greatly increase surface area. Surface area is increased even more by microvilli, which line the villi. The intestines of chickens are very similar to those of pigs with the exception of circular folds; chickens lack circular folds, resulting in the villi lining the wall of the intestine (Moran, 1982).

To further understand how the intestines work, a review of cell types present along the epithelial layer is necessary (Figure 1.5). The crypt contains undifferentiated cells at the base of each villus. These undifferentiated cells mature into the other cells as they ascend up the villus (Smith and Jarvis, 1977). The enteroendocrine cells are known to secrete a variety of hormones, which regulate a multitude of different processes in the intestines (Allen and Porter, 1973). The absorptive cells absorb nutrients and have highly structured microvilli to increase their specific surface area. The goblet cells are a group of secretory cells that produce mucus, which binds together to form the mucosal covering of the intestines (Deplancke and Gaskins, 2001). There is relatively little known about the other cells of the villus, including paneth, cup, and caveolated cells (Moran, 1982).

A ratio of the villus height to its respective crypt depth is known as an indicator of gut cell turnover. A larger ratio indicates a longer villus with a small amount of cell turnover in the crypt-an ideal situation in a healthy adult animal. A smaller ratio indicates a shorter villus with a larger amount of cell turnover in the crypt, representing a challenged gut that is not performing at its maximum potential (Moran, 1982). Once

cells reach the apical point of the villus, they are sloughed off into the lumen of the intestine.

Goblet cells are columnar, epithelial cells (Deplancke and Gaskins, 2001) responsible for the production and secretion of mucin granules which form the mucosal layer. The mucosal layer is important as both a physical and chemical barrier (Specian and Oliver, 1991) protecting the intestinal epithelial lining from dehydration, infection, or injury (Perez-Vilar and Hill, 1999). There are morphological changes that occur in the goblet cell as it leaves the crypt and matures towards the tip of the villus, resulting in changes in the mucins present in the cell.

As an immature cell, the goblet cell begins to produce and secrete mucin granules and continues to do so as it matures. As the goblet cell matures it begins to restructure, ridding itself of organelles and cytoplasm when it secretes the early mucin granules (Specian and Oliver, 1991). The cell matures to look like a pyramid, with a small basal side, while the apical portion of the cell is packed with secretory vesicles full of mucin granules (Radwan et al., 1990). Also, immature goblet cells produce primarily neutral mucins, but as they mature contain more sialic acid (Filipe, 1979). It has been noted that the percentage of goblet cells increases aborally from the duodenum to the distal ileum (LaMont, 1992).

There are two ways in which mucin granules are secreted from the goblet cell. The first is baseline secretion. Baseline secretion is characterized by a continuous secretion of single mucin granules (Neutra et al., 1977; Neutra and LeBlond, 1966; Specian and Oliver, 1991). The second way in which mucin is secreted is accelerated secretion. Accelerated secretion occurs in response a challenge in the gut environment.

These stimuli have been known to include intestinal anaphylaxis (Lake et al., 1980), cholinergic challenge (Specian and Neutra, 1980, 1982, 1981), and chemical and physical injury (Neutra et al., 1982). During accelerated secretion, the mucin granules are fused and secreted, quickly emptying the goblet cell (Specian and Oliver, 1991). The goblet cell is, however, known to recover from this release, thanks to its network of keratin fibers that holds its shape (Specian and Neutra, 1984).

The structure of mucin follows very closely with the functions of the mucosal layer. The basic structure is a peptide core with oligosaccharide branches extending from the backbone in a radial fashion, like the head of a toilet brush. These oligosaccharide branches bind bacteria and toxins, while the backbone increases viscosity, allowing for a gel-like layer to form (LaMont, 1992).

The mucin structure (Figure A.1.6) includes a glycosylated region where the oligosaccharides attach and a non-glycosylated region, which lacks the oligosaccharide branches. The glycosylated regions of the peptide backbone are known to be high in threonine and serine residue. The hydroxyl groups on these residues allow for O-glycosidic linkages with the oligosaccharide branches (Perez-Vilar and Hill, 1999).

The peptide backbone is formed in the endoplasmic reticulum (ER) through disulfide bonds along the cysteine residues (Kim and Ho, 2010). The peptide backbone then travels from the ER to the Golgi apparatus and undergoes multiple reactions that result in the addition of the oligosaccharide branches attached via the O-glycosylation to the threonine and serine residues (Godl et al., 2002; Lidell et al., 2003). The oligosaccharide chains are made from sugar residues in either linear or branched formations in the Golgi apparatus (Specian and Oliver, 1991).

There are up to 20 mucin (MUC) genes that have been identified (Kim and Ho, 2010)-although the MUC2 gene stands to be the most researched mucin gene structure, especially in regards to intestinal mucus production. It is currently thought that expression of the mucin genes is regulated primarily through epigenetics or transcription (Andrianifahanana et al., 2006; Thai et al., 2008; Theodoropoulos and Carraway, 2007).

1.4 Threonine

1.4.1 Threonine biochemistry

Threonine is considered an essential amino acid in animal diets, meaning it must be supplied in the diet because it is not synthesized in adequate amounts in the body. Furthermore, threonine is considered the second limiting amino acid in pigs, after lysine, and the third limiting amino acid in chickens, after the sulfur containing amino acids and lysine in most commercial diets (Kidd et al., 1997).

Another name for threonine is α-amino β-hydroxybutyric acid based on its structure (Figure A.1.7). Threonine is involved in both gluconeogenesis and ketogenesis, making it both glucogenic and ketogenic.

1.4.2 Threonine requirements in broilers

In broilers the threonine requirement was debated until the early 1990's (Rangel-Lugo et al., 1994). Many of the studies conducted in broilers to determine amino acid requirements have based them on the ideal amino acid balance, relating threonine as a ratio to lysine. Studies that look at the threonine to lysine requirement for optimum weight gain varies in broilers due to bird age as well as the crude protein level of the diet (Kidd et al., 2004). However, the NRC established that the recommendation for

threonine for birds 0-3 weeks of age was 0.80% as a percentage of the diet (NRC, 1994). In 1997, Kidd et al. found that threonine levels ranging from 0.68% to 0.86%, as a percentage of diet, resulted in no differences in performance in birds 1 to 18 d of age, which contradicted earlier research, which substantiated the NRC recommendation of 0.80% (Kidd et al., 1996; Smith and Waldroup, 1988). In the same study though, it was found that an ideal total threonine:lysine ratio of 70% resulted in increased breast fillet yields (Kidd et al., 1997). Since then, it has been noted that although a threonine level of 0.72% of the diet resulted in greater carcass yields, when corrected for differences in body weights between treatment groups, there were no treatment differences (Barkley and Wallis, 2001). In conclusion, these studies show that the threonine requirement in broiler chicks during the starter phase has not been solidly established and further research needs to be conducted to substantiate the current literature.

1.4.3 Threonine requirements in weanling pigs

Many of the same conclusions have been made about the threonine requirement in pigs. Lewis and Peo (1986) noted that throughout the 1970's and 1980's, threonine recommendations greatly differed for young weaned pigs. Protein synthesis rates in the muscle has been found to be decreased when threonine was deficient as well as fed in excess to 25 d old pigs (Wang et al., 2007), indicating that the requirement was 0.74% total ileal digestible threonine (TIDT).

1.4.4 Importance of threonine on intestinal health

Although thorough research has been conducted on threonine requirements for performance parameters, a majority of the threonine research has been done in regards to threonine's role in intestinal health. It is known that all of the amino acids administered

enterally do not appear in the portal vein (Ebner et al., 1994; Rerat et al., 1988; Rerat et al., 1992). This is not surprising, as it is well established that the portal drained viscera (PDV), which includes the intestine, pancreas, spleen, and stomach, accounts for a high rate of protein synthesis (approximately 20-50%) (Burrin et al., 1990; Hoerr et al., 1993, 1991; Lobley et al., 1980; McNurlan and Garlick, 1980; Schaart et al., 2005; van der Schoor et al., 2002). A large percentage of threonine, between 60% and 80% of ingested threonine is retained in the PDV (Stoll et al., 1998a; Stoll et al., 1998b; van Goudoever et al., 2000). Likewise, it has been found that when fed parentally, the threonine requirements are reduced by nearly 60% (Bertolo et al., 1998). These findings indicate that threonine plays an important role in gastrointestinal health and optimizing the threonine requirement may offer protection from bacterial infections in the gut.

The high requirement of threonine utilization in the gut is expected due to the high percentage of threonine in the mucin. We have already established that threonine is incorporated in the mucin structure. It turns out that threonine represents nearly 30% of the total amino acid content in the mucin (Faure et al., 2002). The ability for the small intestine to synthesize these mucins is based on threonine availability (Faure et al., 2006; Faure et al., 2005; Law et al., 2007; Nichols and Bertolo, 2008; Puiman et al., 2011). It has been found in rats that diets deficient in threonine results in a decrease in mucin protein synthesis (Faure et al., 2005) and that healthy diet that supplies the recommended threonine level is not sufficient when the animal experiences a gut challenge because the requirement for gastrointestinal health is often higher than the threonine requirement for growth (Faure et al., 2006).

Although it is well established that threonine plays an important role in mucin production, other changes have been noted in intestinal structure when threonine is out of balance in a diet. Diets both deficient and excess in threonine have been found to result in shorter villus height and decreased villus height to crypt depth ratio (Chee et al., 2010a; Law et al., 2007; Wang et al., 2010), indicating that levels below or above the optimum level of threonine negatively affects the gut environment. Alternatively, there are studies that have shown no differences in villus height regardless of differences in threonine supplementation (Azzam et al., 2012). Threonine obviously plays an important role in protecting the gut health of the animal, and may very well play a part in solving the issues of banning AGP.

1.5 Mannan oligosaccharides

1.5.1 Mode of action

Mannan oligosaccharides (MOS) are a feed additive derived from the mannan fraction of the cell wall of the *Saccharomyces cerevisiae* species of yeast. The mannose portion of the MOS mimics the mannose residues in the mucin glycoprotein in the intestinal mucin (Ofek et al., 1977). Pathogenic bacteria that contain a Type I fimbriae, including both *Escherichia coli* and *Salmonella sp.*, attach to these mannose residues and colonize along the intestinal wall, causing disease in the animal. Instead, MOS products offer mannose residues for these bacteria to attach to, resulting in them being passed through the GIT and eventually excreted, causing no harm to the animal (Ofek et al., 1977). It has been observed that *in vitro* MOS inhibits *E. coli* from binding to broiler gut mucosa (Peuranen et al., 2006).

1.5.2 Research using MOS products

Multiple studies with MOS have examined intestinal health parameters and have found that the inclusion of MOS resulted in smaller crypt depths in piglets (Castillo et al., 2008), increased villus height to crypt depth ratio (Chee et al., 2010a), and increased mucosal thickness in broilers (Chee et al., 2010b). It also has been seen *in vivo* that MOS positively alters intestinal bacterial populations (Castillo et al., 2008; Geier et al., 2009; Yang et al., 2008; Zhao et al., 2012). Furthermore, and most importantly to the food animal industry, the addition of MOS improves growth characteristics likely due to the improved intestinal environment. Feed conversion ratios (Castillo et al., 2008; Yang et al., 2008) and average daily gain (Rozeboom et al., 2005; Zhao et al., 2012) have been observed to improve with the inclusion of MOS in both swine and poultry diets. Interestingly, there are some trials that have noted no improvements compared to the control on any measured parameter when MOS is added (Geier et al., 2009; LeMieux et al., 2003; Yitbarek et al., 2012) which some attribute to a lack of a gut challenge.

Actigen® (ACT, Alltech Inc., Nicholasville, KY) is a concentrated MOS product derived from the mannan fraction of a specific strain of *Saccharomyces cerevisae*. Actigen® supplementation also results in growth and intestinal improvements. On birds raised on dirty litter, the inclusion of ACT resulted in improved villus height, villus height to crypt depth ratio, goblet cell counts (Collett et al., 2011) and villus surface area (Barasch et al., 2011). Most interesting though are the effects ACT seems to have on the immune response. In weaned pigs infected with porcine reproductive and respiratory virus (PRRSV), ACT treatments improved antibody titers (Che et al., 2012). Likewise, in broilers, Actigen® has been shown to modulate gene expression in jejunal tissue (Xiao et

al., 2011). Because of the regulation of the intestinal environment, MOS products are being looked at closely as an alternative to antibiotics. Mannan oligosaccharides may provide protection to the gut through their mucin activation properties.

1.6 Conclusion

Finding a natural feed-thru solution to alleviate some of the negative effects associated with an ABF production environment is necessary to ensure animal health as well as answer the concerns of consumers. The inclusion of ACT has been shown to activate mucin production genes (Xiao et al., 2011), indicating that animal supplemented with ACT may have an increase in mucin production. An increase in mucin production would provide for a greater level of protection in the GIT. This is, in fact, seen in animal supplemented with ACT, as it has been found that these animals do have improved GIT parameters (Barasch et al., 2011; Collett et al., 2011). However, this increase in mucin production would increase the threonine requirement. It has been found that MOS supplementation alleviates negative effects associated with excess levels of threonine in broilers (Chee et al., 2010a, b). Research examining the interaction between ACT, specifically, and threonine has not been conducted previously. Understanding how these two factors interact may allow for an optimization of GIT health, which may be an answer in combatting the negative effects seen in an ABF industry.

CHAPTER 2. Effects of Actigen® and dietary threonine levels on intestinal histomorphology, immune status, and growth performance of nursery piglets

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2.1 Introduction

Weaning in piglets is known to be linked with a period of anorexia and diarrhea (van Beers-Schreurs et al., 1992), resulting in death or decreased production, causing a financial loss to producers (Svendsen, 1974). Weaning is linked with villus atrophy and apoptosis of enterocytes (Wijtten et al., 2011), which leads to decreased intestinal protection, leaving the intestine prone to infection. *E. coli* has been noted as the pathogenic bacteria that is often present in weanling pigs often being cited as the source of diarrhea (van Beers-Schreurs et al., 1992). Other sources include the separation from their dam, a change from a liquid to solid diet, and a change in environment and population (Lalles et al., 2007).

Threonine (Thr) is an important component of the mucin glycoprotein (Faure et al., 2002), which comprises the mucosal layer in the intestines. These mucins are secreted by goblet cells on the surface of the villi of the intestines. The mucosal layer offers a protective barrier in the gut, offering protection from dehydration, infection, and

injury (Perez-Vilar and Hill, 1999). An inadequate or excess level of threonine has been known to result in decreased mucin protein synthesis (Faure et al., 2006; Faure et al., 2005; Law et al., 2007) as well as shorter villus height and decreased villus height to crypt depth ratio (Chee et al., 2010a; Law et al., 2007; Wang et al., 2010).

Actigen® ™ (ACT; Alltech Inc., Nicholasville, KY) is a second generation mannan oligosaccharide (MOS) product derived from the cell wall of a specific strain of *Saccharomyces cerevisiae*. Supplementation with MOS results in an increase in villus height and increased goblet cell counts in broilers (Collett et al., 2011), providing for a healthier intestinal environment, thereby resulting in growth improvements (Rozeboom et al., 2005; Zhao et al., 2012). In addition, MOS products have been shown to decrease adhesion of *E. coli* to enterocytes (Castillo et al., 2008).

Both threonine and ACT provide for a healthier intestinal environment, which may combat some of the consequences of weaning of piglets. Therefore, we hypothesized that ACT and an optimal level of threonine would result in better growth and a healthier gastrointestinal environment in weaned piglets.

2.2 Materials and Methods

This experiment was approved by The Ohio State University Institutional Animal Care and Use Committee.

2.2.1 Pigs and study design

Piglets were progeny of Yorkshire x Landrace sows crossed with PIC (line 280) boars (PIC, Hendersonville, TN). All pigs were housed at The Ohio State University Swine Center. Pigs were weaned at 17 d of age and those with an initial weight of 6.3 ±

0.8 kg (mixed sex) were assigned to six dietary treatments. The experiment used a randomized complete block design with six replicates per treatment. Blocks were based on body weight (BW) and date of weaning with each nursery pen housing five piglets. Experimental diets were started at 0 d post weaning. Water and feed were provided *ad libitum* for the duration of the study.

2.2.2 Diets

Corn-soybean meal based diets were designed for three phases as the weanling pigs gained more weight (Table 2.1-2.3). A 2x3 factorial design with three levels of true ileal digestible threonine to lysine ratio (Thr:Lys) were used: 57% (low), 67% (recommended), and 77% (high) and two levels of ACT: 0 g/T and 400 g/T. Within each growth phase, lysine (Lys) was held constant for all experimental diets. Calculated Lys concentrations were 1.34%, 1.19%, and 1.02% on a true ileal digestible (TID) basis for Phase I (0-7 d), Phase II (7-21 d), and Phase III (21-35 d), respectively. A total of six treatments were used in a randomized complete block design. Diets were based on NRC (1998) recommendations and were isocaloric in nature (Table 2.4). A sample from each diet was analyzed for proximate analysis and amino acid composition (Table 2.5).

2.2.3 Sample collection

Starting at weaning, blood was collected weekly from two pigs per pen in heparinized blood tubes and sent to The Ohio State Veterinary Hospital Diagnostic Lab for complete blood cell counts (CBC). Beginning 14 d post weaning, blood samples were taken weekly from one pig per pen in PAXgene blood RNA tubes (Qiagen, Venlo, Limberg), frozen at -20°C for 24 hours, and then stored at -80°C until further processing. At 35±2 d post weaning one piglet per pen was sedated with an intramuscular injection of

ketamine and xylazine, and then euthanized by electrocution followed by exsanguination. A sample of jejunum (15 mm) was taken, rinsed with distilled H_2O and stored in 10% neutral buffered formalin.

2.2.4 Sample preparation-Quantitative real-time PCR

Total RNA was extracted from the blood collected in the PAXgene blood RNA tubes using the PAXgene Blood RNA kit (Qiagen, Limberg, Netherlands). The RNA integrity number (RIN) was determined using RNA quality chips (Agilent RNA 6000 Nanochip, Agilent 2100 Bioanalyzer, Santa Clara, CA). Concentration (ng/µl) was measured using the Nanodrop spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA). The total RNA was reverse transcribed (Life Technologies, Carlsbad, CA). Briefly, RNA was diluted to a concentration of 500 ng per 10 µl and added to a solution containing 2 µl 10X RT Buffer, 0.8 µl 25X dNTP mix, 2 µl 10X RT Random Primer, 1 µl Multiscribe, and 4.2 µl RNase-free H_2O. The cycle conditions for the reverse transcription (RT) procedure were as follows: one cycle at 25°C for 10 m, one cycle at 37°C for 120 m, one cycle at 85°C for 5 m, and then held at 4°C until removal. The resulting products were stored at -20°C until quantification.

2.2.5 Sample preparation- Histomorphology of the jejunum

Tissue fixed in formalin (24 h) was cleared, infiltrated and embedded in paraffin wax. Paraffin wax sections (5 µm thick) were fixed to slides and stained with Alcian Blue-Periodic Acid Schiff (Gafney 1994, Appendix 8).

2.2.6 Quantification of cytokine genes

Four cytokine genes were examined using commercially available primers (Life Technologies, Carlsbad, CA). The genes included interleukin-10 (IL-10,

Ss03382372_u1), interleukin-1β (IL-1β, Ss03393802_m1), interferon-γ (IFN-γ, Ss03391052_m1), and tumor necrosis factor-α (TNF-α, Ss03391317_g1). Glucuronidase beta (GUSB, Ss03387751_u1) was used as the housekeeping gene. A no template control was used as well as a pooled control of Thr:Lys 67% + NO ACT for each plate. Real-time PCR was carried out on 7500 Fast Real-time PCR System (Applied Biosystems, Carlsbad, CA) using the following parameters: 20 s at 95.0°C and 40 cycles of 95.0°C for 3 s an(Livak and Schmittgen, 2001)d 60.0°C for 30 s. Data was normalized to the housekeeping gene GUSB and to the control samples. Relative quantification (RQ) values were calculated using the $^{\Delta\Delta}$Ct method . The RQ for the control was set to 1.0 and differences were reported as RQ greater than or less than the control.

2.2.7 Observation of histomorphology of the jejunum

The sections were observed using a Nikon Eclipse E400 (Nikon, Tokyo, Japan) microscope and photomicrograph images were captured using a SPOT Flex 15.2 64 Mp Shifting Pixel camera (SPOT Imaging Solutions, Sterling Heights, MI). Image analysis was performed using Image J software (NIH, Bethesda, MD).

2.2.8 Statistical analysis

Data was analyzed using the Generalized Linear Mixed Models procedure (PROC GLIMMIX) of SAS Statistical Software (SAS 9.1, Cary, NC). An outlier test was conducted and data values that were more than three standard deviations about the average were removed. Statistical differences were declared at $P \leq 0.05$ and statistical tendency was considered when $0.05 < P < 0.10$.

2.3 Results

2.3.1 Performance

There were no differences in body weight (BW, kg). Supplementation with ACT tended to reduce average daily gain (ADG, g) from 0 d to 7 d post weaning (P=0.09) and from 7 d to 21 d post weaning (P=0.08). Supplementation of ACT resulted in a decrease in average daily feed intake (ADFI, g) from 0 to 7 d post weaning (P=0.04) and overall (P=0.04). Although ACT tended to increase the gain to feed ratio from 7 d to 21 d post weaning (P=0.08) and a threonine level in excess of 67% Thr:Lys tended to increase the gain to feed ratio from 21 d to 35 d post weaning (P=0.09), overall there were no differences in the gain to feed ratio (Table 2.6).

2.3.2 Complete blood count profiles

The piglets all began at 0 d post weaning with similar CBC (Table 2.7), with the exception of nucleated red blood cells (NRBC), which were higher in the ACT fed pigs (P=0.03). On 7 d post weaning (Table 2.8), piglets fed 77% Thr:Lys had lower (P=0.04) red blood cell counts (RBC) and greater mean corpuscular volume (P=0.007, MCV) and mean corpuscular concentration (P=0.04, MCHC). Inclusion of ACT reduced eosinophil levels on 7 d post weaning (P=0.02). At 14 d post weaning, 77% Thr:Lys increased eosinophil levels (P=0.008, Table 2.9). Basophils (P=0.002) and plasma protein (P=0.002) were increased in 77% Thr:Lys fed piglets on 21 d post weaning (Table 2.10). At 35 d post weaning (Table 2.12), RBC numbers were decreased (P=0.03) at the 77% Thr:Lys level, when compared to piglets fed 67% Thr:Lys; 57% Thr:Lys was intermediate and was not different.

2.3.3 qRT-PCR

There were no differences for TNF-α, IL-10, IL-1β, or IFN-γ mRNA levels for 14 d and 35 d posts weaning among treatments (Table 2.14 and Table 2.15).

2.3.4 Histomorphology of jejunal tissue

Piglets that were fed 77% Thr:Lys diets had increased villus height (*P*=0.007, Table 2.13). Pigs fed 57% Thr:Lys had the lowest villus surface area (*P*=0.04) as well as the lowest goblet cell density (*P*=0.04). Villi height to crypt cell depth ratio (VH:CD) was highest for 77% Thr:Lys (*P*=0.01). Total goblet cell area (*P*=0.02) and goblet cell density (*P*=0.05) were highest for ACT treatments.

2.4 Discussion

The reduction in ADFI in response to ACT supplementation is not commonly seen. This could be attributed to a palatability issue, although ACT was added at 400 g/T, per the manufacturer's instructions, and to our knowledge, no palatability issues have been noted in response to MOS products at that level. Although feed was mixed on different days, there was no noticeable relationship between day feed was mixed and a decrease in ADFI. It has been found that MOS products tend to improve performance (Che et al., 2012; Corcao et al., 2011; Nollet, 2012), however present results do not support these findings.

The most recent Nutrient Requirements for Swine (NRC, 2012) was released after this study was conducted. NRC (2012) increased the threonine requirement for growing/finishing pigs to 78% Thr:Lys TID. Because we failed to reach that level, even in our excess level, we cannot make conclusions about an optimal threonine level.

It was expected that the CBC would be similar among the piglets when the trial began. However, NRBC were higher for ACT for which we do not have an explanation for. A study examining the effect of Thr on RBC characteristics concluded that there was little evidence linking threonine and RBC indices (Le Floc'h et al., 2000). Our results conflict with those findings, indicating that at 7 d post weaning, threonine affects RBC indices. We found that 77% Thr:Lys decreased average red blood cell size (MCV) and the amount of hemoglobin in those cells (MCHC) while increasing the number of red blood cells. While significant differences were found among treatments for eosinophil and basophil levels, the relatively large standard error of the means (SEM) leads to the question of the relevance of these differences.

Piglets fed 77% Thr:Lys diets had increased villi height and VH:CD than the other Thr:Lys levels, corroborating the findings of Che et al. (2011). When comparing it to the other Thr:Lys levels, pigs fed 57% Thr:Lys have a significantly lower villus surface area. This indicates that a dietary level of 67% Thr:Lys or greater is necessary for optimum villus surface area which is an important factor for absorptive capacity. Including ACT positively affected both total goblet cell area and density, indicating that ACT supplementation results in more goblet cells to produce more mucin. Additional goblet cells should produce more mucin, which could lead to a protected gut environment with a thicker mucus layer. It has previously been found that the inclusion of ACT alters mucin production, which from our findings, may be due to an increase in the number of goblet cells.

This experiment was limited to histomorphology measurements from the jejunum. Sampling ileal tissue, in addition to jejunal, for histomorphology would have provided a

direct comparison with previous findings in other studies. Wang et al. (2010) found that diets containing both a deficiency as well as an excess level of threonine had decreased villus surface area in the ileum, but saw no differences in the jejunum. Furthermore, they found that ileal mucin synthesis was decreased when threonine was not fed at the recommended level (Wang et al., 2010).

Weaning is often viewed as one of the most stressful stages in a piglet's life. The combination of a change in environment as well as diet can stress the young animal, causing them to become susceptible to diseases. However, we did not observe effects of differences between dietary threonine levels on body weight or cytokine gene expression levels in this experiment. Examining the cytokine mRNA levels in the intestine might have shown a more complete picture, as ACT works in the intestinal environment, as it is ingested and passed through the gastrointestinal tract (Barasch et al., 2011; Collett et al., 2011).

2.5 Conclusion

The results from this study indicate that threonine and ACT work independently to alter intestinal morphology. However, no significant main effect or interactive effect on growth performance, immunity, and blood count profile were observed. Further research needs to be done looking at a possible interactive effect between threonine and ACT.

Table 2.1 *Phase I weaning diets (0-7 d) based on National Research Council, 1998 requirements for 3-5 kg pigs (% as fed)*

	Treatment					
Thr:Lys	57%	57%	67%	67%	77%	77%
Actigen®	+	-	+	-	+	-
Ingredient, % as fed						
Corn	62.0	62.0	62.0	62.0	62.0	62.0
Corn Oil	2.08	2.08	2.08	2.08	2.08	2.08
Soybean Meal	10.0	10.0	10.0	10.0	10.0	10.0
Soybean, protein conc	8.00	8.00	8.00	8.00	8.00	8.00
Dried Whey	4.00	4.00	4.00	4.00	4.00	4.00
Dried Plasma	3.30	3.30	3.30	3.30	3.30	3.30
Lactose	5.80	5.80	5.80	5.80	5.80	5.80
Lysine	0.40	0.40	0.40	0.40	0.40	0.40
Methionine	0.13	0.13	0.13	0.13	0.13	0.13
Tryptophan	0.03	0.03	0.03	0.03	0.03	0.03
Threonine	0.00	0.00	0.14	0.14	0.28	0.28
Trace Mineral Mix[1]	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin Mix[2]	0.25	0.25	0.25	0.25	0.25	0.25
Celite	0.42	0.47	0.28	0.33	0.14	0.19
Dicalcium Phosphate	2.21	2.21	2.21	2.21	2.21	2.21
Limestone	0.78	0.78	0.78	0.78	0.78	0.78
NaCl	0.25	0.25	0.25	0.25	0.25	0.25
Sel-Plex®	0.05	0.05	0.05	0.05	0.05	0.05
Actigen®	0.05	-	0.05	-	0.05	-

[1]The NRC (1998) requirement per kilogram of diet during the experiment was provided from Bioplex: 6 mg of Cu, 100 mg of Fe, 4 mg of Mn, and 100 mg of Zn, whereas 0.30 mg of Se was a yeast product (Sel-Plex, Alltech Inc., Nicholasville, KY).

[2]Supplied per kilogram of diet: 2,450 IU of vitamin A (acetate); 245 IU of vitamin D_3 (cholcalciferol); 0.6 mg of vitamin K (menadione Na bisulfate); 18 IU of vitamin E (DL-α tocopheryl acetate); 4.5 mg of riboflavin; 13.5 mg of D-pantothenic acid; 22.3 mg of niacin; 0.3 mg of folacin; 1.7 mg of thiamine; 2.8 mg of pyridoxine; 0.1 mg of D-biotin; 22.3 µg of vitamin B_{12}; 0.70 g of choline; and 66 mg of butylated hydroxytoluene as an antioxidant.

Table 2.2 *Phase II weaning diets (7-21 d) based on National Research Council, 1998 requirements for 5-10 kg pigs (% as fed)*

	Treatment					
Thr:Lys	57%	57%	67%	67%	77%	77%
Actigen®	+	-	+	-	+	-
Ingredient, % as fed						
Corn	59.0	59.0	59.0	59.0	59.0	59.0
Corn Oil	2.00	2.00	2.00	2.00	2.00	2.00
Soybean Meal	20.0	20.0	20.0	20.0	20.0	20.0
Dried Whey	4.00	4.00	4.00	4.00	4.00	4.00
Dried Plasma	2.80	2.80	2.80	2.80	2.80	2.80
Lactose	8.08	8.08	8.08	8.08	8.08	8.08
Lysine	0.35	0.35	0.35	0.35	0.35	0.35
Methionine	0.10	0.10	0.10	0.10	0.10	0.10
Tryptophan	0.03	0.03	0.03	0.03	0.03	0.03
Threonine	0.00	0.00	0.12	0.12	0.24	0.24
Trace Mineral Mix[1]	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin Mix[2]	0.25	0.25	0.25	0.25	0.25	0.25
Celite	0.42	0.47	0.3	0.35	0.18	0.23
Dicalcium Phosphate	1.40	1.40	1.40	1.40	1.40	1.40
Limestone	0.97	0.97	0.97	0.97	0.97	0.97
NaCl	0.25	0.25	0.25	0.25	0.25	0.25
Sel-Plex®	0.05	0.05	0.05	0.05	0.05	0.05
Actigen®	0.05	-	0.05	-	0.05	-

[1]The NRC (1998) requirement per kilogram of diet during the experiment was provided from Bioplex: 6 mg of Cu, 100 mg of Fe, 4 mg of Mn, and 100 mg of Zn, whereas 0.30 mg of Se was a yeast product (Sel-Plex, Alltech Inc., Nicholasville, KY).

[2]Supplied per kilogram of diet: 2,450 IU of vitamin A (acetate); 245 IU of vitamin D_3 (cholcalciferol); 0.6 mg of vitamin K (menadione Na bisulfate); 18 IU of vitamin E (DL-α tocopheryl acetate); 4.5 mg of riboflavin; 13.5 mg of D-pantothenic acid; 22.3 mg of niacin; 0.3 mg of folacin; 1.7 mg of thiamine; 2.8 mg of pyridoxine; 0.1 mg of D-biotin; 22.3 μg of vitamin B_{12}; 0.70 g of choline; and 66 mg of butylated hydroxytoluene as an antioxidant.

Table 2.3 *Phase III weaning diets (21-35 d) based on National Research Council, 1998 requirements for 10-20 kg pigs (% as fed)*

	Treatment					
Thr:Lys	57%	57%	67%	67%	77%	77%
Actigen®	+	-	+	-	+	-
Ingredient, % as fed						
Corn	60.3	60.3	60.3	60.3	60.3	60.3
Corn Starch	8.49	8.49	8.49	8.49	8.49	8.49
Corn Oil	2.00	2.00	2.00	2.00	2.00	2.00
Soybean Meal	21.5	21.5	21.5	21.5	21.5	21.5
Dried Whey	4.00	4.00	4.00	4.00	4.00	4.00
Lysine	0.31	0.31	0.31	0.31	0.31	0.31
Methionine	0.07	0.07	0.07	0.07	0.07	0.07
Tryptophan	0.03	0.03	0.03	0.03	0.03	0.03
Threonine	0.00	0.00	0.10	0.10	0.21	0.21
Trace Mineral Mix[1]	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin Mix[2]	0.25	0.25	0.25	0.25	0.25	0.25
Celite	0.42	0.47	0.32	0.37	0.21	0.26
Dicalcium Phosphate	1.22	1.22	1.22	1.22	1.22	1.22
Limestone	0.81	0.81	0.81	0.81	0.81	0.81
NaCl	0.25	0.25	0.25	0.25	0.25	0.25
Sel-Plex®	0.05	0.05	0.05	0.05	0.05	0.05
Actigen®	0.05	-	0.05	-	0.05	-

[1]The NRC (1998) requirement per kilogram of diet during the experiment was provided from Bioplex: 6 mg of Cu, 100 mg of Fe, 4 mg of Mn, and 100 mg of Zn, whereas 0.30 mg of Se was a yeast product (Sel-Plex, Alltech Inc., Nicholasville, KY).

[2]Supplied per kilogram of diet: 2,450 IU of vitamin A (acetate); 245 IU of vitamin D_3 (cholcalciferol); 0.6 mg of vitamin K (menadione Na bisulfate); 18 IU of vitamin E (DL-α tocopheryl acetate); 4.5 mg of riboflavin; 13.5 mg of D-pantothenic acid; 22.3 mg of niacin; 0.3 mg of folacin; 1.7 mg of thiamine; 2.8 mg of pyridoxine; 0.1 mg of D-biotin; 22.3 µg of vitamin B_{12}; 0.70 g of choline; and 66 mg of butylated hydroxytoluene as an antioxidant.

Table 2.4 *Diets, Calculated Nutrient Content based on National Research Council, 1998 requirements (% as-fed)*

	Thr:Lys		
	57%	67%	77%
Phase I			
Nutrient			
ME kcal/kg	3093	3093	3093
CP, %	18.2	18.3	18.4
Lys, %	1.34	1.34	1.34
Met+Cys, %	0.76	0.76	0.76
Thr, %	0.76	0.9	1.04
Trp, %	0.25	0.25	0.25
Ca, %	0.90	0.90	0.90
P, %, avail	0.55	0.55	0.55
Na, %	0.26	0.26	0.26
Cl, %	0.30	0.30	0.30
Phase II			
Nutrient			
ME kcal/kg	3100	3100	3100
CP, %	16.7	16.8	16.9
Lys, %	1.19	1.19	1.19
Met+Cys, %	0.68	0.68	0.68
Thr, %	0.68	0.8	0.92
Trp, %	0.23	0.23	0.23
Ca, %	0.80	0.80	0.80
P, %, avail	0.40	0.40	0.40
Na, %	0.24	0.24	0.24
Cl, %	0.29	0.29	0.29
Phase III			
Nutrient			
ME kcal/kg	3253	3253	3253
CP, %	15.2	15.3	15.4
Lys, %	1.02	1.02	1.02
Met+Cys, %	0.58	0.58	0.58
Thr, %	0.58	0.67	0.78
Trp, %	0.20	0.20	0.20
Ca, %	0.70	0.70	0.70
P, %, avail	0.32	0.32	0.32
Na, %	0.15	0.15	0.15
Cl, %	0.25	0.25	0.25

Table 2.5 *Proximate analysis and analyzed amino acid composition of nursery piglet diets*

	Thr:Lys	57%	57%	67%	67%	77%	77%
Nutrient	Actigen®	+	-	+	-	+	-
			Phase I				
Crude Protein		18.0	17.8	17.7	18.2	18.4	17.4
Crude Fat		3.36	3.37	3.3	3.21	3.37	3.39
Crude Fiber		1.40	1.92	1.63	1.49	1.16	1.67
Threonine		0.79	0.74	0.81	0.79	0.81	0.83
Lysine		1.35	1.36	1.40	1.34	1.37	1.41
			Phase II				
Crude Protein		15.5	16.6	16.9	16.2	16.2	15.7
Crude Fat		3.43	3.58	3.74	3.69	3.52	3.54
Crude Fiber		1.85	1.86	1.82	1.89	1.66	1.90
Threonine		0.66	0.65	0.76	0.73	0.87	0.88
Lysine		1.23	1.18	1.18	1.18	1.28	1.32
			Phase III				
Crude Protein		14.9	14.2	15.1	15.4	15.7	15.9
Crude Fat		3.62	3.49	3.38	3.81	3.36	3.77
Crude Fiber		2.33	2.14	1.96	1.97	2.08	2.02
Threonine		0.6	0.54	0.66	0.68	0.72	0.72
Lysine		1.05	1.00	1.11	1.08	1.05	1.06

Table 2.6 *Main effects of dietary threonine concentration (Thr:Lys)and Actigen® (ACT) on body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and gain to feed conversion ratio (G:F) of piglets*[1,2]

Factor	BW, kg				ADG, g				ADFI, g				G:F			
Thr:Lys	0 d	7 d	21 d	35 d	0-7 d	7-21 d	21-35 d	0-35 d	0-7 d	7-21 d	21-35 d	0-35 d	0-7 d	7-21 d	21-35 d	0-35 d
57%	6.3	6.3	10	16	-3.8	288	435	288	114	400	798	501	-0.10	0.72	0.54	0.57
67%	6.3	6.1	9.9	16	-29.9	272	435	276	91.0	368	749	463	-0.30	0.74	0.58	0.59
77%	6.3	6.3	10	16	-7.9	282	441	287	107	383	771	482	-0.10	0.74	0.57	0.6
SEM	0.3	0.2	0.3	0.5	10.7	11.9	20.6	17.0	8.8	15.7	27.0	16.5	0.12	0.02	0.01	0.02
Factor	BW, kg				ADG, g				ADFI, g				G:F			
ACT	0 d	7 d	21 d	35 d	0-7 d	7-21 d	21-35 d	0-35 d	0-7 d	7-21 d	21-35 d	0-35 d	0-7 d	7-21 d	21-35 d	0-35 d
+	6.3	6.2	9.9	16	-24.4	268	425	271	93.0^{B}	359	753	462^{B}	-0.30	0.75	0.57	0.59
-	6.3	6.3	10	17	-3.3	293	449	295	115^{A}	409	792	502^{A}	-0.10	0.72	0.57	0.59
SEM	0.2	0.2	0.3	0.4	8.8	9.7	16.8	17.0	7.2	12.8	22.1	13.5	0.10	0.01	0.01	0.01
Source of variation																
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*P=0.09*	*P=0.08*	n.s.	*n.s.*	*P=0.035*	*P=0.08*	n.s.	*P=0.04*	*n.s.*	*P=0.08*	n.s.	n.s.
Thr:Lys	*n.s.*	*n.s.*	n.s.	n.s.	*n.s.*	n.s.	n.s	n.s.	*n.s.*	n.s.	n.s.	n.s.	*n.s.*	n.s.	*P=0.09*	n.s.

[1]Each value represents the LS Means of 6 replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 2.7 *Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 0 d post weaning piglets*[1,2]

Thr:Lys	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
57%	5.5	33.9	11.3	5.8	58.0	33.3	24.3	4.8	4.3	39.4	56.5	3.2	0.6	0.3	252.1
67%	5.6	33.9	11.3	5.8	57.7	33.5	23.1	5.4	5.9	35.9	60.4	2.6	1.0	0.3	251.1
77%	5.7	27.7	11.2	5.6	59.2	33.9	24.2	4.1	6.5	34.6	61.9	2.9	0.4	0.3	262.5
SEM	0.0	0.3	0.1	0.1	0.5	0.1	0.4	0.3	1.0	1.3	1.4	0.3	0.1	0.1	12.4
ACT	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
+	5.5	33.3	11.2	5.7	58.1	33.6	23.8	4.9	7.8^{A}	36.1	60.3	2.8	0.6	0.2	171.3
-	5.6	30.3	11.3	5.8	58.5	33.4	23.8	4.6	3.3^{B}	37.1	58.9	3.0	0.7	0.3	163.5
SEM	0.0	0.3	0.1	0.1	0.5	0.1	0.4	0.3	1.0	1.3	1.4	0.3	0.1	0.1	12.4
Source of variation															
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*P=0.03*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr:Lys	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
ACT x Thr:Lys	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 2.8 *Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 7 d post weaning piglets*[1,2]

Thr:Lys	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
57%	5.1	38	12	6.9^{A}	54.2^{B}	33.2^{A}	27.8	9.2	0.2	26.5	67.2	5.3	1.0	0.08	240
67%	5.2	37	12	6.9^{A}	53.8^{B}	32.8^{B}	27.5	9.9	0.3	26.6	66.7	5.2	1.5	0.08	222
77%	5.2	37	12	6.6^{B}	56.2^{A}	33.1^{A}	26.7	8.9	0.2	26.8	64.8	6.1	1.9	0.25	243
SEM	0.0	0.3	0.1	0.1	0.30	0.10	0.60	0.5	0.1	2.10	2.40	0.7	0.3	0.07	25.0

ACT	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
+	5.1	37	12	6.8	55.0	32.9	26.9	8.9	0.2	27.4	65.5	5.8	1.1^{B}	0.10	223
-	5.1	37	12	6.8	54.5	33.1	27.7	9.7	0.2	25.8	66.8	5.3	1.8^{A}	0.20	247
SEM	0.0	0.3	0.1	0.1	0.30	0.10	0.60	0.5	0.1	1.80	2.00	0.6	0.2	0.05	21.0

Source of variation															
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*P=0.02*	*n.s.*	*n.s.*
Thr:Lys	*n.s.*	*n.s.*	*n.s.*	*P=0.0*	*P=0.007*	*P=0.04*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
ACT x Thr:Lys	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 2.9 *Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 14 d post weaning piglets*[1,2]

Thr:Lys	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
57%	4.70	35.0	11.6	6.4	52.9	33.1	25.6	11.4	0.00	38.6	53.3	4.2	1.4^{B}	0.4	259
67%	4.70	34.0	11.2	6.4	53.3	32.9	24.5	10.7	0.05	39.8	53.5	5.3	1.1^{B}	0.4	256
77%	4.70	34.2	11.3	6.4	54.0	32.6	25.4	10.9	0.10	41.8	49.4	6.1	2.3^{A}	0.3	265
SEM	0.06	0.50	0.20	0.2	0.50	0.30	0.60	0.60	0.06	1.80	2.00	0.6	0.3	0.2	32.0
ACT	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
+	4.70	34.4	11.4	6.3	53.4	33.0	25.5	10.9	0.00	40.7	51.7	5.8	1.5	0.4	283
-	4.70	34.4	11.4	3.5	53.4	32.8	24.8	11.1	0.10	39.4	52.4	4.6	1.8	0.4	237
SEM	0.05	0.40	0.10	0.1	0.40	0.20	0.50	0.50	0.05	1.50	1.60	0.5	0.2	0.1	26.0
Source of variation															
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr:Lys	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*P=0.008*	*n.s.*	*n.s.*
ACT x Thr:Lys	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 2.10 *Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 21 d post weaning piglets*[1,2]

Thr:Lys	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
57%	4.50^{B}	31.8	10.6	6.0	52.6	33.2	25.4	11.7	0.5	39.3	55.8	3.0	1.8	0.1^{B}	301
67%	4.50^{B}	31.0	10.1	6.0	52.5	32.7	24.6	10.1	2.0	39.5	53.7	3.0	1.9	0.4^{AB}	268
77%	4.80^{A}	31.9	10.6	6.0	53.6	33.1	23.8	12.1	0.2	39.5	55.2	2.9	1.8	0.6^{A}	295
SEM	0.07	0.50	0.10	0.1	0.6	0.20	0.80	0.80	1.0	1.90	2.30	0.6	0.3	0.1	34.0
ACT	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
+	4.60	31.3	10.3	6.0	52.6	33.0	24.9	11.6	0.3	39.7	55.1	3.3	1.8	0.4	271
-	4.60	31.8	10.5	6.0	53.1	33.0	24.2	10.9	1.5	39.2	54.7	2.7	1.9	0.3	305
SEM	0.05	0.40	0.10	0.1	0.50	0.20	0.70	0.70	0.8	1.50	1.90	0.4	0.2	0.1	28.0
	Source of variation														
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr:Lys	*P=0.002*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*P=0.002*	*n.s.*
ACT x Thr:Lys	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.
[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 2.11 *Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 28 d post weaning piglets*[1,2]

Thr:Lys	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
57%	4.8	33.8	11.1	6.4	50.8	32.8	22.3	9.90	0.3	38.0	56.9	3.2	1.6	0.6	223
67%	4.9	33.6	10.9	6.3	53.2	32.8	21.8	10.5	0.3	36.3	56.5	4.2	2.0	0.8	248
77%	4.9	33.0	10.8	6.1	54.0	32.8	22.0	10.7	0.7	40.8	53.3	3.2	2.0	0.9	252
SEM	0.1	0.50	0.10	0.1	1.2.0	0.10	0.40	0.50	0.1	1.70	1.60	0.5	0.3	0.2	26.0
ACT	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
+	4.8	33.8	11.1	6.3	52.0	32.8	22.3	11.1[A]	0.4	39.0	54.7	3.9	1.8	0.6	240
-	4.9	33.1	10.8	6.2	53.3	32.8	21.9	9.60[B]	0.5	37.7	56.4	3.1	1.9	0.9	242
SEM	0.1	0.40	0.10	0.1	1.00	0.10	0.30	0.40	0.1	1.40	1.30	0.4	0.2	0.2	21.0
Source of variation															
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*P=0.02*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr:Lys	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
ACT x Thr:Lys	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 2.12 *Main effects and interaction of dietary threonine concentrations (Thr:Lys) and Actigen® (ACT) on complete blood count levels of plasma protein, packed cell volume (PCV), hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), nucleated cells, nucleated red blood cells (NRBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and platelets of 35 d post weaning piglets*[1,2]

Thr:Lys	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
57%	5.0	34.1	11.3	6.6^{AB}	52.5	32.9	20.8	12.6	0.1	42.0	51.7	5.7	1.9	0.4	150
67%	5.1	35.4	11.5	6.7^{A}	52.8	32.4	20.6	11.3	0.3	44.2	49.5	4.4	1.5	0.3	190
77%	5.2	34.5	11.2	6.3^{B}	53.8	32.6	21.5	11.5	0.0	41.4	51.4	4.5	2.0	0.6	163
SEM	0.1	0.60	0.20	0.2	0.50	0.20	0.40	0.60	0.1	1.80	2.20	0.6	0.3	0.1	25.0
ACT	Plasma protein	PCV	Hb	RBC	MCV	MCHC	RDW	Nucleated cells	NRBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
+	5.1	35.0	11.4	6.6	52.7	32.6	21.0	11.9	0.1	43.4	50.4	5.1	1.7	0.4	162
-	5.1	34.3	11.3	6.5	53.3	32.6	20.9	11.7	0.2	41.6	51.3	4.7	1.9	0.4	173
SEM	0.1	0.50	0.10	0.1	0.40	0.20	0.30	0.50	0.1	1.50	1.80	0.5	0.3	0.1	21.0
Source of variation															
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr:Lys	*n.s.*	*n.s.*	*n.s.*	*P=0.03*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
ACT x Thr:Lys	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 2.13 *Main effects of dietary threonine concentration (Thr:Lys) and Actigen® (ACT) on villus height (μm), apical length (μm), basal length (μm), villus surface area (mm^2), crypt depth (μm), villus height:crypt depth (VH:CD), goblet cell count, goblet cell area (μm2), average goblet cell area (μm^2), and goblet cell density of 35 d post weaning piglets*[1,2]

Thr:Lys	Villus height, μm	Apical length, μm	Basal length, μm	Villus surface area, mm^2	Crypt depth, μm	VH:CD	Goblet cell count	Total goblet cell area, μm^2	Average goblet cell area, μm^2	Goblet cell density, cells/μm^2
57%	359^{B}	115	142	47^{B}	190	2.1^{B}	14.0	1677	117	0.3^{A}
67%	394^{B}	130	156	58^{AB}	222	2.0^{B}	13.3	1933	134	0.2^{B}
77%	459^{A}	126	159	64^{A}	198	2.6^{A}	13.4	1680	132	0.2^{B}
SEM	13.4	3.0	3.5	2.8	6.9	0.1	0.7	168	7.7	0

ACT	Villus height, μm	Apical length, μm	Basal length, μm	Villus surface area, μm^2	Crypt cell depth, μm	Villus height:crypt cell depth	Goblet cell count	Total Goblet cell area, μm^2	Average Goblet cell area, μm^2	Goblet cell density, cells/μm^2
+	397	127	157	56	201	2.2	14.7	2133^{A}	140	0.3^{A}
-	411	121	148	57	205	2.2	12.3	1396^{B}	115	0.2^{B}
SEM	13.4	2.9	3.5	2.8	6.9	0.1	0.7	168.0	7.7	0.0
Source of variation										
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*P=0.02*	*n.s.*	*P=0.05*
Thr:Lys	*P=0.007*	*n.s.*	*n.s.*	*P=0.04*	*n.s.*	*P=0.01*	*n.s.*	*n.s.*	*n.s.*	*P=0.04*

[1] Each value represents the LS Means of 6 replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 2.14 *Main effects of Actigen® (ACT) supplementation on 14 d post weaning cytokine gene expression of piglets*[1]

Treatments	TNF-α		IL-10		IL-1β		IFN-γ	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Actigen®	0.93	0.12	0.83	0.08	1.02	0.20	1.46	0.19
No Actigen®	1.01	0.08	0.97	0.12	1.16	0.32	1.19	0.30
P-value	n.s.		n.s.		n.s.		n.s.	

[1] Each value represents the LS Means of 18 replicates for each Actigen® level.

Table 2.15 *Main effects of Actigen® (ACT) supplementation on 35 d post weaning cytokine gene expression of piglets*[1]

Treatments	TNF-α		IL-10		IL-1β		IFN-γ	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Actigen®	0.89	0.06	0.93	0.05	0.93	0.05	0.98	0.09
No Actigen®	0.84	0.10	0.79	0.08	0.79	0.08	0.74	0.15
P-value	n.s.		n.s.		n.s.		n.s.	

[1] Each value represents the LS Means of 18 replicates for each Actigen® level.

CHAPTER 3. The interaction of Actigen® and threonine supplementation on intestinal histology and growth performance in broiler chicks

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3.1 Introduction

The intestines are lined with a mucosal layer offering protection from dehydration, infection, and injury (Perez-Vilar and Hill, 1999). Threonine is an important component of the mucin glycoprotein (Faure et al., 2002), which is a component of the mucosal layer in the intestines. These mucins are secreted by goblet cells on the surface of the villi of the intestines. An inadequate or excess level of threonine has been shown to result in decreased mucin protein synthesis (Faure et al., 2006; Faure et al., 2005; Law et al., 2007) as well as shorter villus height and decreased villus height to crypt depth ratio (Chee et al., 2010a; Law et al., 2007; Wang et al., 2010).

Actigen® ™ (ACT, Alltech Inc., Nicholasville, KY) is a second generation mannan oligosaccharide (MOS) product derived from the cell wall of a specific strain of *Saccharomyces cerevisiae* yeast. It has been shown that MOS supplementation results in an increase in villus height and increased goblet cell counts in broilers (Collett et al., 2011), providing for a healthier intestinal environment, thereby resulting in growth improvements (Rozeboom et al., 2005; Zhao et al., 2012).

Both threonine and ACT, separately, provide for a healthier intestinal environment. Therefore, we hypothesized that ACT and an optimal level of threonine would result in better growth and a healthier gastrointestinal environment in broiler chicks.

3.2 Materials and Methods

This experiment was approved by the University of Kentucky Institutional Animal Care and Use Committee.

3.2.1 Animals and Treatments

A total of 360 Cobb500™ day old male broiler chicks (Cobb-Vantress, Monticello, KY) were allocated to 10 dietary treatments in a randomized complete block design utilizing a 2x5 factorial arrangement. Six chicks were placed in each of the 60 mesh wire-floored cages (61 cm x 51 cm x 36 cm). One replicate cage in each of six blocks, based on room location, was assigned to each dietary treatment.

The basal diet consisted of corn-peanut meal-soybean meal diet with a threonine level of 56 mg/kg (0.56%, Table 3.1). Dietary treatments consisted of the basal diet with or without added threonine (Ajinimoto Inc., Tokyo, Japan) and a MOS product (Actigen®, Alltech, Inc., Nicholasville, KY) in a factorial arrangement. Five levels of threonine were used: 0.56%, 0.64%, 0.80%, 0.96% and 1.04% of the diet and ACT was added either at 0 or 400 g/T. The basal diet (Table 3.2) was designed to meet the NRC (1994) recommendations for broiler chicks (0-3 weeks) for all nutrients with the exception of threonine. All diets were formulated to be isocaloric and isonitrogenous. Glutamic acid (Ajinimoto Inc., Tokyo, Japan) was added at the appropriate level to achieve isonitrogenous diets as threonine inclusion changed. A sample from each diet and feedstuff was analyzed for proximate analysis and amino acid composition (Table 3.3). Feed and water were provided *ad libitum*.

Peanut meal is known to often be contaminated with aflatoxins, so a sample was sent out to be tested for mycotoxin levels using the 37+ mycotoxin test (Alltech Inc., Nicholasville, KY). The peanut meal tested to containing high risk levels of aflatoxins (Table A.3.1), so a monitoring treatment containing 0.80% Thr + Integral (Alltech Inc., Nicholasville, KY) was added to the experiment.

3.2.2 Measurement of Performance Characteristics

Chicks were weighed at the time of placement and then weekly for 3 weeks on a pen basis. Feed intake was measured weekly and gain to feed ratio was calculated.

3.2.3 Sample Collection

On d 7 and d 21, one chick from each pen was randomly chosen and euthanized by asphyxiation using argon gas. The thymus, spleen, bursa, pancreas and liver were removed from the bird and weighed. The intestine was then divided into the duodenum (from the posterior gizzard outlet to the end of the duodenal loop), the jejunum (from the duodenal loop to Meckel's diverticulum), and the ileum (Meckel's diverticulum to the ileocecal junction). Each region was opened longitudinally and rinsed with distilled water. Sections (2 cm) were taken from each region and fixed in 10% neutral buffered formalin for 24 h.

On d 21, the tibia and humerus of the bird was excised then analyzed for breaking strength. On d 22, one chick from each pen was randomly chosen and euthanized by asphyxiation using argon gas and cervical dislocation. The tibia and humerus of the bird were excised then boiled in de-ionized water for 15 minutes and then cleared of any remaining soft tissue. The bones were then placed in a drying oven at 60°C for 72 h. The bones were then stored at -20°C for mineral analysis.

3.2.4 Histomorphological analysis

Fixed tissues were transferred to a 70% ethanol solution. The tissue samples were then dehydrated, cleared, and embedded in paraffin wax. Sections (5 μm) were cut, floated in a water bath, and placed on charged glass slides. The tissue sections were then stained with an Alcian Blue (pH 2.5)-Periodic Acid Schiff stain (Appendix 8, Gafney, 1994) and viewed on a Nikon Eclipse E400 (Nikon, Tokyo, Japan) microscope and photomicrograph images were captured using a SPOT Flex 15.2 64 Mp shifting pixel camera (SPOT Imaging Solutions, Sterling Heights, MI). Measurements of villus length, villus width, crypt cell depth, as well a count of the number

of goblet cells per villus were performed using image analysis software (Image J, NIH, Bethesda, MD).

3.2.5 Bone Breaking Strength and Mineral Analysis

The birds' tibia and humerus were removed, cleaned, and broken using an Instron 4301 machine using a three point breaking procedure (Cantor et al., 1980). Previously dried bones were defatted in petroleum ether for 72 h, dried at 105°C for 12 h, and then ashed at 600°C in a muffle furnace. Weights were recorded for defatted dry samples, as well as ashed samples, which were used to calculate percentage ash and total bone mineral content. The ashed samples were crushed to a powder and then microwave digested with HNO_3 before undergoing analysis (ICP-OES, Varian Analytical Instruments, Walnut Creek, CA).

3.2.6 Statistical Analysis

Data was analyzed using the Generalized Linear Mixed Models procedure (PROC GLIMMIX) of SAS Statistical Software (SAS 9.1, Cary, NC). Statistical differences were declared at $P \leq 0.05$ and statistical tendency was considered when $0.05 < P < 0.10$.

3.3 Results

3.3.1 Performance

Birds fed 0.56% threonine had lower body weights each week (Table 3.4) than the other threonine levels ($P<0.0001$). Overall, chicks fed the 0.56% threonine level had the lowest feed consumption (Table 3.5, $P<0.0001$) when compared to birds fed the other threonine levels. Also, 0.56% threonine fed birds had the highest feed to gain conversion ratio when compared to the other threonine levels (Table 3.6, $P<0.0001$). There were no interactions between ACT and threonine levels on body weight, feed intake, and feed to gain ratio. There were no differences between the 0.80%+No ACT and the 0.80%+Integral diet.

3.3.2 Organ weights

On d 7, 0.56% threonine fed birds resulted in decreased liver (*P*=0.001), spleen (*P*=0.0005), and pancreas (*P*=0.0003) weights from birds fed the other threonine levels (Table A.3.1). However, when expressed as a percentage of body weight, only a decrease in spleen weight persisted between 0.56% threonine and all other threonine levels (*P*=0.05, Table 3.7). Supplementation with ACT resulted in greater spleen weight as a percentage of body weight when compared to no ACT (*P*=0.01, Table 3.7). No other differences on organ weights were observed on d 7 (Table A.3.1, 3.7).

On d 21, 0.64% threonine level increased absolute pancreas weight (*P*=0.003, Table 3.8) when compared to other threonine levels. No other differences were observed on d 21 organ weights (Table A.3.2, 3.8).

3.3.3 Bone breaking strength and mineral composition

For humerus, bones from 0.80% threonine fed birds required a higher amount of force to break than bones from 0.56% threonine fed birds (*P*=0.0004, Table 3.9). Tibia from birds fed 0.64% threonine required more force to break than bones from 0.56% threonine fed birds (*P*<0.0001, Table 3.9).

There were no differences in either tibia or humerus bone ash (Table 3.10) or tibia mineral content (Table 3.11). Humerus from 0.80% threonine fed birds resulted in a greater concentration of phosphorous when compared with 0.56% threonine and 0.64% threonine fed birds (*P*=0.04, Table 3.12). Feeding 0.56% threonine resulted in a greater concentration of potassium compared with the other threonine levels (*P*<0.0001, Table 3.12).

3.3.4 Histomorphology

There were no differences for any of the parameters on d 7 in the duodenum (Table 3.13) or jejunum (Table 3.14) or on d 21 in the duodenum (Table 3.16) or ileum (Table 3.18). However, in the jejunum on d 21, we saw that supplementation with ACT reduced apical width (*P*=0.03, Table 3.17), surface area (*P*=0.03, Table 3.17), and a trend on basal width (*P*=0.09,

Table 3.17), with ACT supplementation resulting in thinner villi and less surface area. On d 21, an interaction between ACT and threonine in the jejunum indicated that basal width (*P*=0.03, Figure 3.1) and surface area (*P*=0.02, Figure 3.2) were increased by dietary threonine when diets did not contain ACT. No differences were observed with the 0.56%, 0.64%, and 0.80% threonine levels. There were no other differences seen for d 21 in the jejunum.

In the ileum on d 7, birds fed 0.64% threonine, 0.80% threonine, and 1.04% threonine had longer villi than 0.56% threonine fed birds (*P*=0.03, Table 3.15). Feeding 0.96% threonine had a shallower crypt depth than 0.64% threonine fed birds (*P*=0.04, Table 3.15). Goblet cell counts were affected by threonine on d 7 in the ileum (Table 3.15) where 0.56% threonine fed birds has fewer (*P*=0.01) goblet cells than 0.64% or 0.80% threonine fed birds. There were no other effects in the ileum on d 7.

3.4 Discussion

Average body weight, average daily feed intake, and feed to gain ratio were negatively affected by a deficient threonine level, a finding that closely follows that of others who have reported decreased growth in animals fed diets deficient in threonine (Chee et al., 2010b; Kidd et al., 2004; Rangel-Lugo et al., 1994). Likewise, although significance was not seen, it was expected that ACT would improve body weight (Rozeboom et al., 2005; Zhao et al., 2012). If the study had been extended, it is expected that we would have seen a significant weight difference due to ACT supplementation. However, this was designed to be a pilot study to gather preliminary data. There were no differences between the 0.80% threonine and 0.80% threonine+Integral™ indicating that there was no negative effects from the aflatoxin contaminated peanut meal.

Although there were multiple differences in absolute organ weights seen on d 7 and d 21, once adjusted for treatment effects on body weight, the only difference noted was d 7 spleen. Both ACT supplementation and threonine level, independently, had an effect on spleen weight on

d 7. The chicken spleen functions differently from its mammalian counterpart. Whereas the mammalian spleen acts to store erythrocytes, the avian spleen acts primarily as a systemic immunity organ. In the presence of ACT supplementation, spleen weight as a percentage of body weight was greatly increased. This substantiates other reports that have found that ACT has immunomodulatory effects (Che et al., 2012; Xiao et al., 2011), however without other further evidence we cannot make a claim that ACT affects the immune system in birds. Although there are no reports of a link between threonine and spleen weights that we know of, the diet deficient in threonine may have affected the animal's ability to mount an immune response, explaining these findings.

There is little literature establishing a relationship between threonine and bone mineralization. However, it has been established that threonine is present both in its natural form and in a phosphorylated form in the bone of chicken(Cohen-Solal et al., 1978). Our results indicate that a dietary level of 0.64% Thr is required for optimal tibial strength, but a level of 0.80% is required for optimal humeral strength. Because there were little mineral differences due to threonine, differences in breaking strength may be due to the threonine content in the bone protein matrix (Cohen-Solal et al., 1978).

It often takes the gastrointestinal tract in broiler chicks up to a week to fully develop, which may explain the lack of differences in the jejunum on d 7. However, we observed a difference in many characteristics in the ileum on d 7, all of which are results of a main effect of threonine. Many researchers have found that a deficiency in threonine negatively affects villus height, crypt depth, and goblet cell counts (Chee et al., 2010a; Law et al., 2007; Wang et al., 2010) as seen in this study.

A wider basal width may indicate the merging of villi, a response to an unhealthy gut environment. In this study, we observed that supplementation with ACT reduced basal width and surface area in birds fed excess of 0.80% threonine. It has previously been found that MOS alleviates negative effects of excess threonine in the gut of broilers (Chee et al., 2010b). In fact,

ACT supplemented villi actually embody the classic description of fingerlike projections (Figure 3.3, A), while birds that were not supplemented with ACT have villi that have merged and are continually sloughing off their tips (Figure 3.3, B). This study further substantiates current literature that has found that ACT and threonine interact to improve gastrointestinal health.

3.5 Conclusion

This study found that ACT and threonine work independently, as well as interactively, to improve the structural properties of the gut. Furthermore, these results indicate that the threonine level recommended by the NRC (1994) meets the requirements for growing broilers. However, further research needs to look at the interaction of ACT and threonine in an environment similar to those which are present in the food animal industry.

Table 3.1 *Broiler chick (d 1-d 21) starter diet composition (% as-fed)*

		Diet									
	Thr %	0.56	0.56	0.64	0.64	0.8	0.8	0.96	0.96	1.04	1.04
Ingredients	Actigen®	-	+	-	+	-	+	-	+	-	+
Corn		58.9	58.9	58.9	58.9	58.9	58.9	58.9	58.9	58.9	58.9
SBM-48		9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50	9.50
Peanut meal		19.0	19.0	19.0	19.0	19.0	19.0	19.0	19.0	19.0	19.0
Animal fat		6.14	6.14	6.14	6.14	6.14	6.14	6.14	6.14	6.14	6.14
Limestone		1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Dical Phos		1.89	1.89	1.89	1.89	1.89	1.89	1.89	1.89	1.89	1.89
Sodium bicarb		0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
Salt		0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28
Vitamin Mix[1]		0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mineral Mix[2]		0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
DL-Met		0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39
Lys HCl		0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51
Celite		0.05	0.00	0.05	0.00	0.05	0.00	0.05	0.00	0.07	0.02
L-Glutamate		1.07	1.07	1.00	1.00	0.84	0.84	0.68	0.68	0.57	0.57
Threonine		0.00	0.00	0.07	0.07	0.23	0.23	0.39	0.39	0.48	0.48
Actigen®		0.00	0.05	0.00	0.05	0.00	0.05	0.00	0.05	0.00	0.05
Total		100	100	100	100	100	100	100	100	100	100

[1]Supplied per kilogram of diet: 8,000 IU vitamin A (acetate), 3,000 IU vitamin D_3, 3.0 mg vitamin K (menadione dimethylpyrimidinol bisulfite), 2.0 mg thiamin, 10.0 mg riboflavin, 12.0 mg pantothenic acid, 40.0 mg niacin, 4.0 mg pyridoxine, 0.20 mg biotin, 0.75 mg folic acid, 0.015 mg vitamin B-12, 500 mg choline, and 125 mg ethoxyquin.

[2]Mineral Mix contained 0.96% $CuSo_4$, 0.04% KlO_3, 16.00% $FeSo_4$, 11.25% $MnSo_4$, and 3.33% ZnO.

Table 3.2 *Broiler chick (d 1-d 21) starter diet calculated nutrient content*

		Diet									
	Thr %	0.56	0.56	0.64	0.64	0.8	0.8	0.96	0.96	1.04	1.04
Nutrient	Actigen®	-	+	-	+	-	+	-	+	-	+
ME,Mcal/kg		3.17	3.17	3.17	3.17	3.17	3.17	3.17	3.17	3.17	3.17
Protein		20.1	20.9	20.9	20.9	20.9	20.9	20.9	20.9	20.9	20.9
Ca		1.06	1.06	1.06	1.06	1.06	1.06	1.06	1.06	1.06	1.06
P avail.		0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Na		0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Arg		1.53	1.53	1.53	1.53	1.53	1.53	1.53	1.53	1.53	1.53
Lys		1.10	1.10	1.10	1.10	1.10	1.10	1.10	1.10	1.10	1.10
Met		0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63
Met+Cys		0.92	0.92	0.92	0.92	0.92	0.92	0.92	0.92	0.92	0.92
Thr		0.57	0.57	0.64	0.64	0.80	0.80	0.96	0.96	1.04	1.04
Trp		0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
Linoleic		1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.38

Table 3.3 *Proximate analysis and analyzed amino acid composition of broiler chick (d 1-d 21) starter diet and ingredients*

		Diet												
	Thr %	0.56	0.56	0.64	0.64	0.8	0.8	0.96	0.96	1.04	1.04	Peanut meal	Corn	Soybean meal
Nutrient	Actigen®	-	+	-	+	-	+	-	+	-	+			
ME,Mcal/kg		4.27	4.26	4.28	4.27	4.28	4.23	4.25	4.24	4.25	4.28	4.33	4.11	3.65
Crude Protein		20.8	22.2	21.1	20.5	21.6	21.7	22.4	21.0	20.2	22.3	39.3	9.34	54.0
Crude Fat		10.4	10.8	11.2	10.5	11.0	10.3	10.4	10.6	10.4	10.7	11.0	3.36	7.90
Crude Fiber		2.53	3.40	3.00	3.03	3.36	2.97	3.66	3.01	3.01	3.49	5.78	2.76	3.75
Threonine		0.61	0.63	0.65	0.68	0.77	0.82	0.93	0.89	0.99	1.07	1.05	0.28	2.03
Lysine		1.17	1.24	1.27	1.13	1.11	1.25	1.21	1.35	1.15	1.29	1.44	0.25	3.46
Cysteine		0.27	0.28	0.26	0.27	0.26	0.27	0.27	0.26	0.29	0.27	0.44	0.17	0.77
Methionine		0.66	0.68	0.59	0.61	0.80	0.67	0.62	0.54	0.58	0.81	0.40	0.17	0.74
Arginine		1.61	1.77	1.68	1.52	1.53	1.64	1.65	1.58	1.69	1.75	4.71	0.38	3.84
Tryptophan		0.23	0.22	0.22	0.20	0.20	0.20	0.21	0.21	0.21	0.22	0.42	0.06	0.79

Table 3.4 *Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on average body weights (ABW) of broiler chicks*[1,2]

	ABW (g)		
Thr (% of diet)	*d 7*	*d 14*	*d 21*
0.56	117^{C}	295^{C}	582^{B}
0.64	136^{B}	396^{B}	833^{A}
0.80	143^{A}	415^{A}	858^{A}
0.96	141^{A}	405^{AB}	833^{A}
1.04	141^{AB}	407^{AB}	840^{A}
Pooled SEM	2.00	5.90	12.18
ACT	*d 7*	*d 14*	*d 21*
+	135	386	799
-	136	380	779
Pooled SEM	1.27	3.73	7.70
Source of variation			
ACT	*n.s.*	*n.s.*	*P=0.07*
Thr (% of diet)	*P<0.0001*	*P<0.0001*	*P<0.0001*

[1] Each value represents the LS Means for 6 replicates for each treatment group.
[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.5 *Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on average daily feed intake (ADFI) of broiler chicks*[1,2]

	ADFI (g/day)			
Thr (% of diet)	*d 1-d 7*	*d 7-d 14*	*d 14-d 21*	*d 1-d 21*
0.56	19.0^{B}	41.7^{B}	61.2^{B}	41.5^{B}
0.64	21.8^{A}	53.2^{A}	92.5^{A}	53.7^{A}
0.80	21.7^{A}	52.9^{A}	94.5^{A}	54.0^{A}
0.96	22.9^{A}	53.8^{A}	92.4^{A}	54.1^{A}
1.04	22.2^{A}	52.1^{A}	92.0^{A}	53.4^{A}
Pooled SEM	0.90	1.17	1.51	0.78
ACT	*d 1-d 7*	*d 7-d 14*	*d 14-d 21*	*d 1-d 21*
+	21.7	51.3	88.8	51.7
-	21.4	50.3	86.6	50.8
Pooled SEM	0.57	0.74	0.96	0.50
Source of variation				
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*P=0.04*	*P<0.0001*	*P<0.0001*	*P<0.0001*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.6 *Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on feed to gain ratio (F:G) of broiler chicks*[1,2]

	F:G (g of feed/g of gain)		
Thr (% of diet)	*d 7*	*d 14*	*d 21*
0.56	1.83	1.89[A]	1.70[A]
0.64	1.68	1.60[B]	1.48[B]
0.80	1.56	1.59[B]	1.50[B]
0.96	1.7	1.62[B]	1.51[B]
1.04	1.62	1.53[B]	1.49[B]
Pooled SEM	0.066	0.051	0.025
ACT	*d 7*	*d 14*	*d 21*
+	1.7	1.66	1.54
-	1.65	1.63	1.54
Pooled SEM	0.042	0.032	0.016
Source of variation			
ACT	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*P=0.06*	*P<0.0001*	*P<0.0001*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.7 *Average organ weights as a percent of body weight of d 7 broiler chicks fed diets containing varying threonine (Thr) concentrations and Actigen® (ACT)*[1,2]

	Organ weight (g/g%)				
Thr (% of diet)	*Liver*	*Spleen*	*Bursa*	*Thymus*	*Pancreas*
0.56	4.16	0.07[B]	0.134	0.287	0.47
0.64	4.73	0.10[A]	0.132	0.311	0.56
0.80	4.48	0.09[AB]	0.134	0.287	0.52
0.96	4.65	0.08[AB]	0.124	0.316	0.50
1.04	4.20	0.10[A]	0.131	0.295	0.54
Pooled SEM	0.196	0.006	0.008	0.021	0.023
ACT	*Liver*	*Spleen*	*Bursa*	*Thymus*	*Pancreas*
+	4.51	0.097[A]	0.137	0.312	0.519
-	4.38	0.081[B]	0.125	0.186	0.520
Pooled SEM	0.123	0.004	0.005	0.013	0.014
Source of variation					
ACT	*n.s.*	*P=0.009*	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*n.s.*	*P=0.03*	*n.s.*	*n.s.*	*P=0.06*
ACT x Thr	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.8 *Average organ weights as a percent of body weight of d21 broiler chicks fed diets containing varying threonine (Thr) concentrations and Actigen® (ACT)*[1,2]

	Organ weight (g/g%)				
Thr (% of diet)	*Liver*	*Spleen*	*Bursa*	*Thymus*	*Pancreas*
0.56	3.38	0.097	0.174	0.461	0.296
0.64	3.00	0.104	0.173	0.470	0.321
0.80	2.98	0.101	0.170	0.418	0.309
0.96	3.11	0.098	0.195	0.413	0.277
1.04	2.97	0.098	0.181	0.460	0.281
Pooled SEM	0.140	0.008	0.012	0.041	0.016
ACT	*Liver*	*Spleen*	*Bursa*	*Thymus*	*Pancreas*
+	3.06	0.10	0.17	0.43	0.29
-	3.12	0.10	0.18	0.47	0.30
Pooled SEM	0.089	0.005	0.007	0.027	0.011
Source of variation					
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
ACT x Thr	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.9 *Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on average breaking strength of tibia and humerus of broiler chicks*[1,2]

	Force required to break (kg force)	
Thr (% of diet)	*Tibia*	*Humerus*
0.56	1.51^{C}	1.23^{C}
0.64	2.23^{A}	1.57^{AB}
0.80	2.18^{AB}	1.61^{A}
0.96	1.93^{B}	1.40^{BC}
1.04	2.07^{AB}	1.51^{AB}
Pooled SEM	0.098	0.062
ACT	*Tibia*	*Humerus*
+	2.02	1.49
-	1.94	1.44
Pooled SEM	0.062	0.039
Source of variation		
ACT	*n.s.*	*n.s.*
Thr(% of diet)	*P<0.0001*	*P=0.0004*

[1] Each value represents the LS Means for 6 replicates for each treatment group.
[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.10 *Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on average ash content of tibia and humerus of broiler chicks*[1,2]

	% Ash	
Thr (% of diet)	*Tibia*	*Humerus*
0.56	53.62	55.74
0.64	54.59	56.88
0.80	54.15	56.43
0.96	54.42	56.35
1.04	54.08	56.59
Pooled SEM	0.098	0.339
ACT	*Tibia*	*Humerus*
+	54.04	56.44
-	54.3	56.34
Pooled SEM	0.212	0.214
Source of variation		
ACT	*n.s.*	*n.s.*
Thr (% of diet)	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.
[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.11 *Main effects and interaction of dietary threonine (Thr) concentration and Actigen® (ACT) on mineral composition of tibia from broiler chicks*[1,2]

	Concentration of mineral (ppm)						
Thr (% of diet)	*Phosphorous*	*Manganese*	*Iron*	*Zinc*	*Calcium*	*Potassium*	*Sodium*
0.56	182518	2.80	230.58	350.76	303069	11064	17620
0.64	181053	2.64	220.91	326.93	296883	10545	15360
0.80	181973	3.13	259.85	347.89	304189	11097	16151
0.96	182101	2.95	234.37	374.65	294274	10603	15176
1.04	182216	3.12	258.26	374.93	301148	10910	15737
Pooled SEM	1588.99	0.334	10.94	14.12	2282	171.11	1448.09
ACT	*Phosphorous*	*Manganese*	*Iron*	*Zinc*	*Calcium*	*Potassium*	*Sodium*
+	181913	2.98	248.58	351.12	299266	10882	16612
-	182031	2.87	233.00	358.94	300560	10805	15405
Pooled SEM	1004.97	0.211	6.92	8.93	2282	108.22	915.85
Source of variation							
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
ACTxThr	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.12 *Main effects and interaction of dietary threonine (Thr) concentration and Actigen® (ACT) on mineral composition of humerus from broiler chicks*[1,2]

	Concentration of mineral (ppm)						
Thr (% of diet)	*Phosphorous*	*Manganese*	*Iron*	*Zinc*	*Calcium*	*Potassium*	*Sodium*
0.56	185905^{AB}	1.52	124.45	236.35	321091	11429^{A}	12158
0.64	186942^{A}	1.68	108.14	218.4	323288	9777^{B}	13731
0.80	179846^{C}	2.41	123.07	231.61	306563	9596^{B}	11613
0.96	181133^{BC}	1.82	124.86	230.45	309685	9801^{B}	11852
1.04	182741^{ABC}	2.28	138.44	243.93	309924	9681^{B}	12974
Pooled SEM	1826.81	0.51	28.16	51.68	2142.72	228.83	1702.16
ACT	*Phosphorous*	*Manganese*	*Iron*	*Zinc*	*Calcium*	*Potassium*	*Sodium*
+	182040	1.89	127.77	232.53	312228	9913	12724
-	184586	2.00	119.81	231.77	315991	10200	12207
Pooled SEM	1155.38	0.337	17.81	32.68	2142.72	144.72	1076.54
Source of variation							
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*P=0.04*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*P<0.0001*	*n.s.*
ACT x Thr	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.13 *Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the duodenum of 7 day old broiler chicks*[1,2]

	Intestinal Morphology Characteristics							
Thr (% of diet)	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
0.56	1372	253.7	243.26	3492.79	301.94	4.87	87.23	0.03
0.64	1486	267.2	246.56	3965.50	295.27	5.43	100.64	0.03
0.80	1540	251.5	215.53	3743.52	288.76	5.88	88.46	0.03
0.96	1529	285.7	269.47	4440.29	330.51	5.14	91.22	0.03
1.04	1663	266.5	282.89	4686.77	343.62	5.14	102.12	0.03
Pooled SEM	87.1	27.6	21.70	517.53	27.94	0.34	6.84	0.003
ACT	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
+	1487	255.6	250.74	3928.26	314.42	5.17	88.77	0.03
-	1549	274.3	252.35	4203.29	309.62	5.41	99.09	0.03
Pooled SEM	56.2	17.8	13.99	333.80	18.02	0.22	4.41	0.001
Source of variation								
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1]Each value represents the LS Means for 6 replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.14 *Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the jejunum of 7 day old broiler chicks*[1,2]

	Intestinal Morphology Characteristics							
Thr (% of diet)	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
0.56	1135	312.69	294.08	3353.15	360.2	3.35	61.94	0.02
0.64	1274	275.42	270.37	3498.07	391.29	3.55	71.06	0.02
0.80	1247	294.63	285.15	3612.1	396.63	3.26	80.44	0.03
0.96	1216	320.13	278.25	3653.94	356.32	3.55	66.76	0.02
1.04	1188	292.99	285.40	3446.93	360.44	3.45	64.26	0.02
Pooled SEM	59.2	23.89	16.38	264.94	17.36	0.20	5.66	0.002
ACT	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
+	1994	305.41	284.31	3477.56	370.76	3.45	67.78	0.02
-	1229	292.93	280.99	3548.11	375.19	3.41	70.00	0.02
Pooled SEM	38.2	15.11	10.36	167.57	10.98	0.13	3.58	0.001
Source of variation								
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1]Each value represents the LS Means for 6 replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.15 *Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the ileum of 7 day old broiler chicks*[1,2]

	Intestinal Morphology Characteristics							
Thr (% of diet)	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
0.56	766^{B}	260.39	261.21	2019.04	302.39^{AB}	2.67	40.89^{C}	0.02
0.64	933^{A}	293.32	292.11	2760.39	327.47^{A}	2.93	51.37^{A}	0.02
0.80	909^{A}	252.23	246.97	2274.42	301.64^{AB}	3.06	48.28^{AB}	0.02
0.96	841^{AB}	267.23	234.92	2102.51	278.11^{B}	3.14	44.14^{BC}	0.02
1.04	881^{A}	276.84	278.89	2444.99	292.01^{B}	3.08	46.93^{AB}	0.02
Pooled SEM	37.9	18.88	19.67	187.29	10.74	0.12	2.08	0.001
ACT	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
+	867	266.53	250.81	2261.72	295.04	3.01	45.8	0.02
-	865	273.48	274.83	2378.83	305.61	2.94	46.84	0.02
Pooled SEM	23.7	11.83	12.33	117.37	6.73	0.08	1.31	0.001
Source of variation								
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*P=0.03*	*n.s.*	*n.s.*	*n.s.*	*P=0.04*	*n.s.*	*P=0.01*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.16 *Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the duodenum of 21 day old broiler chicks*[1,2]

	Intestinal Morphology Characteristics							
Thr (% of diet)	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
0.56	2532	354.18	295.08	8262.43	366.43	7.21	54.26	0.007
0.64	2447	288.58	276.67	7010.00	370.71	7.03	50.65	0.008
0.80	2389	373.40	397.30	9087.81	387.01	6.40	49.57	0.008
0.96	2444	360.78	310.36	8080.47	384.21	6.85	51.11	0.008
1.04	2567	323.55	297.49	8155.58	369.31	7.26	48.96	0.007
Pooled SEM	197.6	29.17	30.71	892.91	18.78	0.65	5.36	0.0009
ACT	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
+	2472	328.94	322.15	8068.87	367.78	7.11	50.17	0.008
-	2480	351.25	308.61	8169.64	383.78	6.79	51.65	0.007
Pooled SEM	120.7	17.82	18.76	545.48	11.47	0.42	3.27	0.0005
Source of variation								
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment.

[2] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.17 *Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the jejunum of 21 day old broiler chicks*[1,2]

	Intestinal Morphology Characteristics							
Thr (% of diet)	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
0.56	1986	388.32	382.44	7549.32	337.67	6.09	46.93	0.007
0.64	2017	377.63	347.63	7297.23	331.43	6.30	48.91	0.007
0.80	1993	353.28	318.17	6658.25	314.81	6.66	47.38	0.009
0.96	2043	412.04	387.89	8278.27	353.66	6.04	45.33	0.006
1.04	2023	449.83	406.76	8591.76	352.52	6.00	48.34	0.007
Pooled SEM	59.2	31.68	25.43	593.65	14.78	0.27	3.36	0.0007
ACT	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
+	1958	365.25^{B}	349.22	7080.58^{B}	331.82	6.18	45.52	0.008
-	2067	427.19^{A}	387.93	8269.36^{A}	344.21	6.25	49.23	0.007
Pooled SEM	58.0	19.48	15.63	382.89	9.02	0.17	2.06	0.0004
Source of variation								
ACT	*n.s.*	*P=0.03*	*n.s.*	*P=0.03*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 3.18 *Main effects of dietary threonine (Thr) concentration and Actigen® (ACT) on intestinal morphology characteristics from the ileum of 21 day old broiler chicks*[1,2]

	Intestinal Morphology Characteristics							
Thr (% of diet)	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
0.56	1433	321.09	302.32	4490.85	306.19	4.88	53.85	0.013
0.64	1408	354.62	307.59	4651.72	333.98	4.32	50.89	0.012
0.80	1401	375.48	340.39	4939.05	319.26	4.62	50.55	0.011
0.96	1312	422.09	368.23	5206.46	328.43	4.16	51.52	0.010
1.04	1425	347.78	305.24	4589.00	327.59	4.50	52.00	0.012
Pooled SEM	69.1	24.44	23.64	342.38	12.20	0.20	3.60	0.001
ACT	*Villus height (μm)*	*Apical width (μm)*	*Basal width (μm)*	*Villus surface area (mm^2)*	*Crypt Depth (μm)*	*Villus height: Crypt depth*	*No. of Goblet cells*	*Goblet cell density ($cells/mm^2$)*
+	1384	373.55	329.47	4857.91	326.27	4.43	50.60	0.11
-	1408	354.87	320.04	4692.93	319.91	4.57	52.92	0.12
Pooled SEM	44.6	15.76	15.24	220.83	7.87	0.13	2.32	0.0007
Source of variation								
ACT	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Thr (% of diet)	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Figure 3.1 Interaction (*P*=0.03) between Actigen (ACT) and threonine (Thr) concentration on basal width (µm) of jejunum villi in d21 broiler chicks

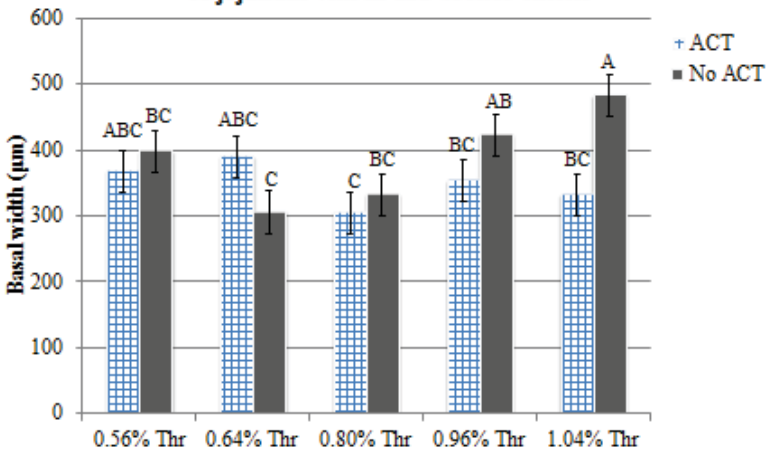
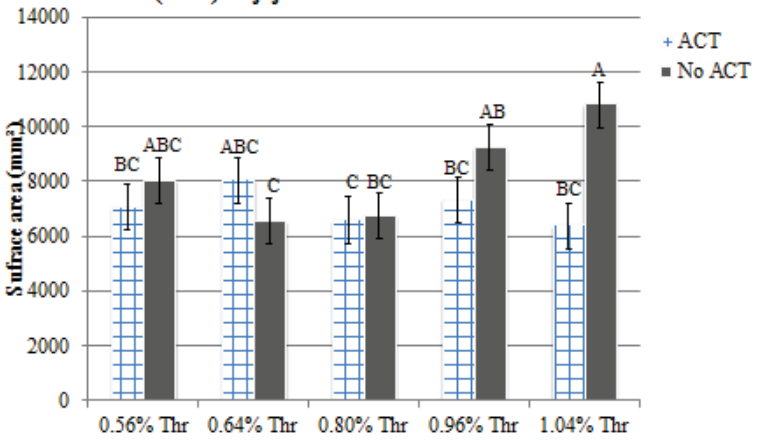


Figure 3.2 Interaction (*P*=0.02) between Actigen (ACT) and threonine (Thr) concentration on surface area (mm²) of jejunum villi in d21 broiler chicks



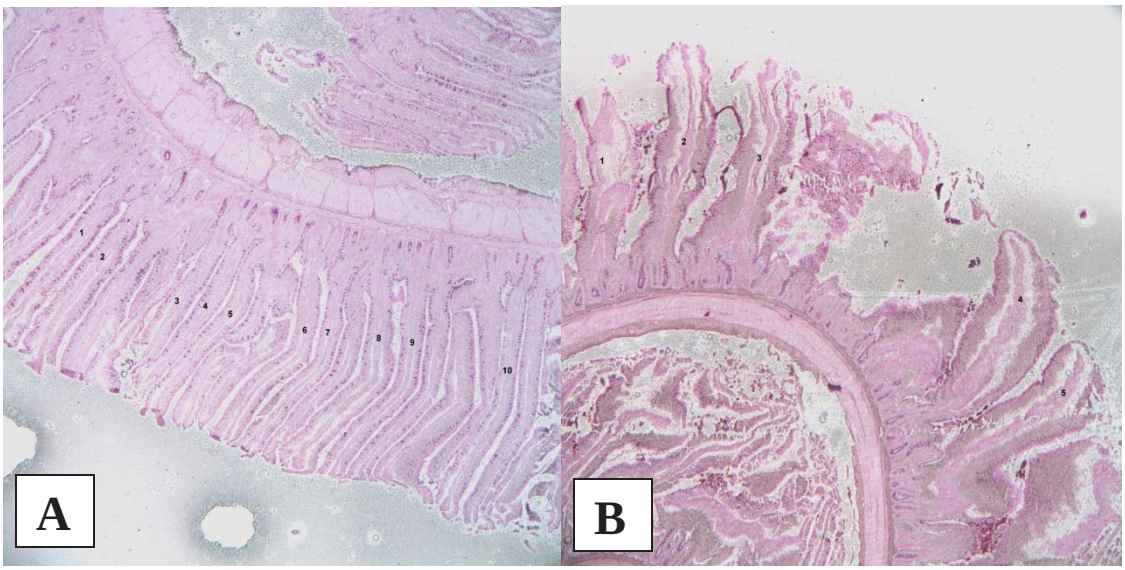
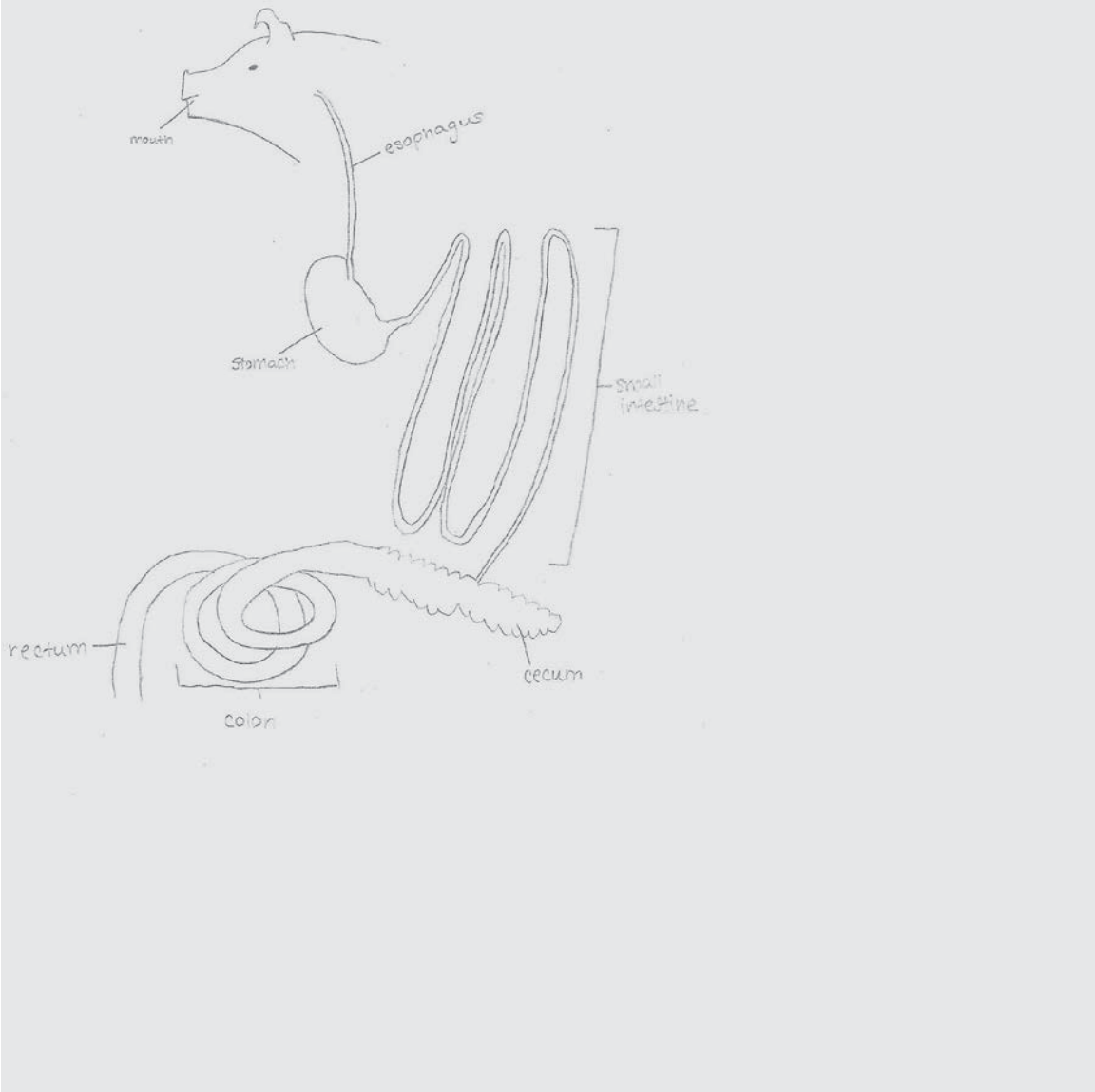
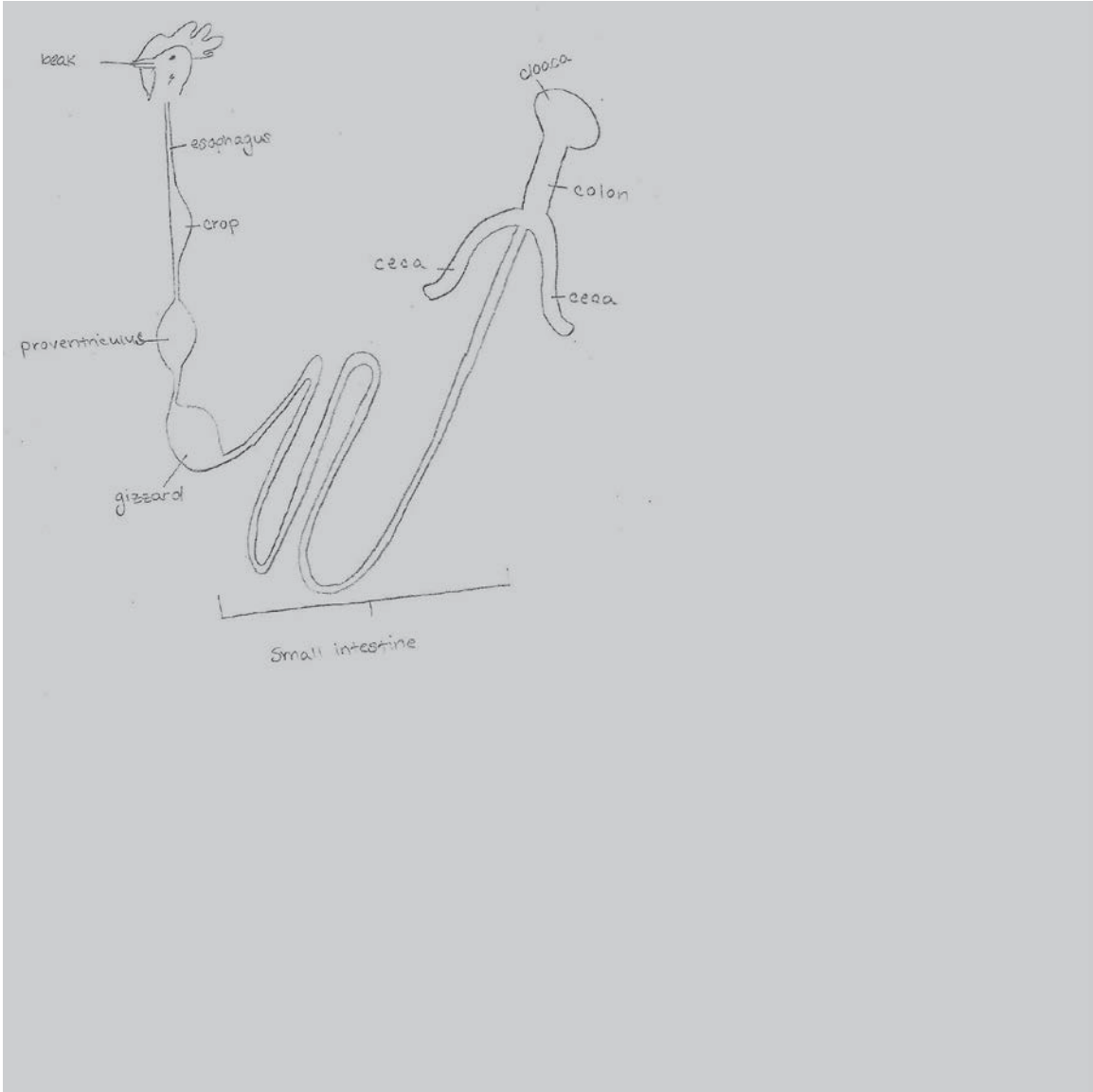


Figure 3.3 Histomorphological characteristics of jejunum tissue supplemented with or without Actigen®. *(A) Jejunal villi from Actigen® (ACT) supplemented treatments from 21 day old chicks exhibit a healthier appearance, despite having a significantly smaller surface area, possessing the classic finger-like structure common of villi. (B) Comparatively, jejunal villi from 21 day old chicks not supplemented with ACT display merging of villi and extensive sloughing of villus tips, both signs of an unhealthy gut.*

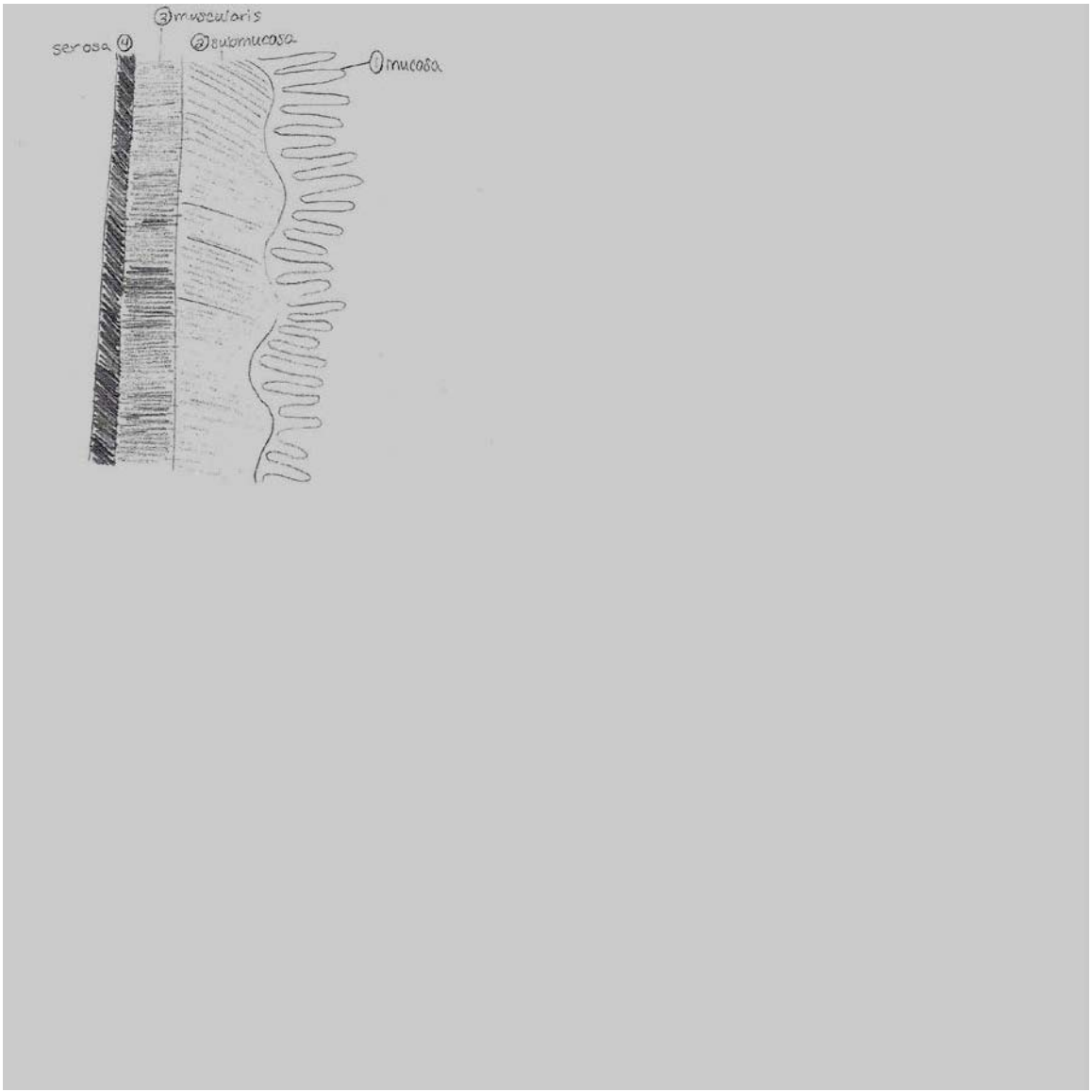
Appendix 1. Example of simple monogastric digestive tract



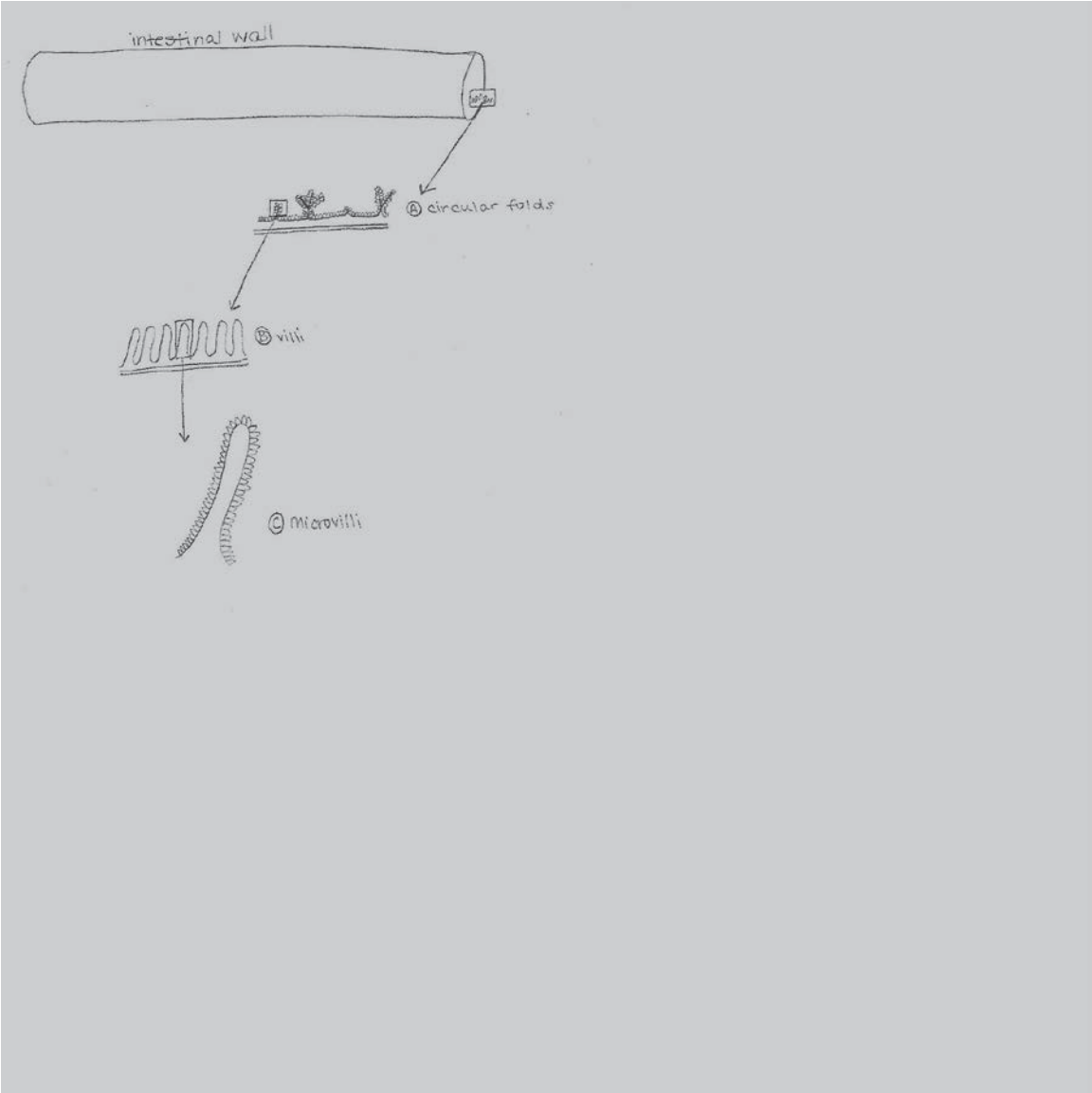
Appendix 2. Diagram of modern chicken gastrointestinal tract



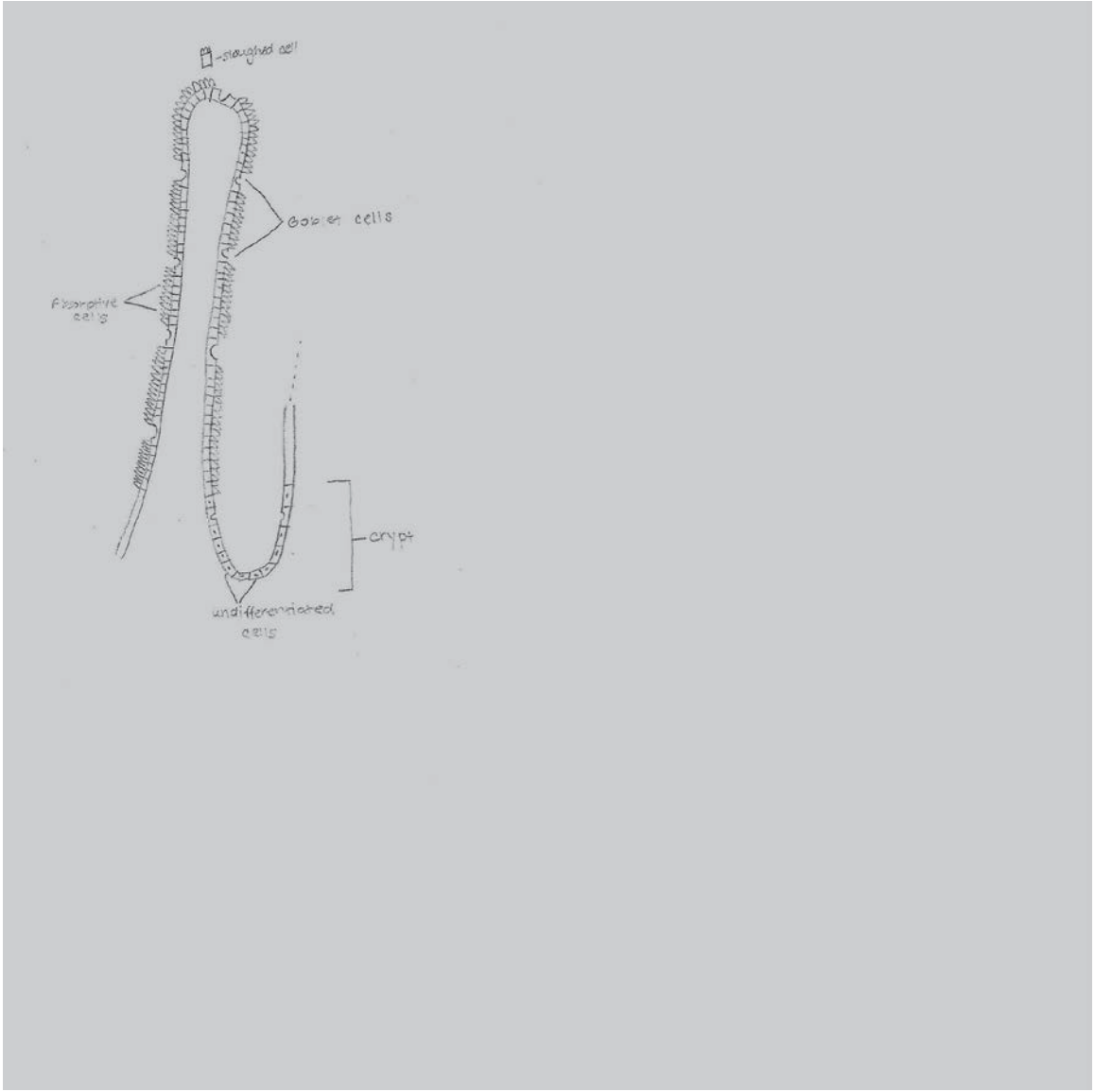
Appendix 3. Diagram of the intestinal wall layers



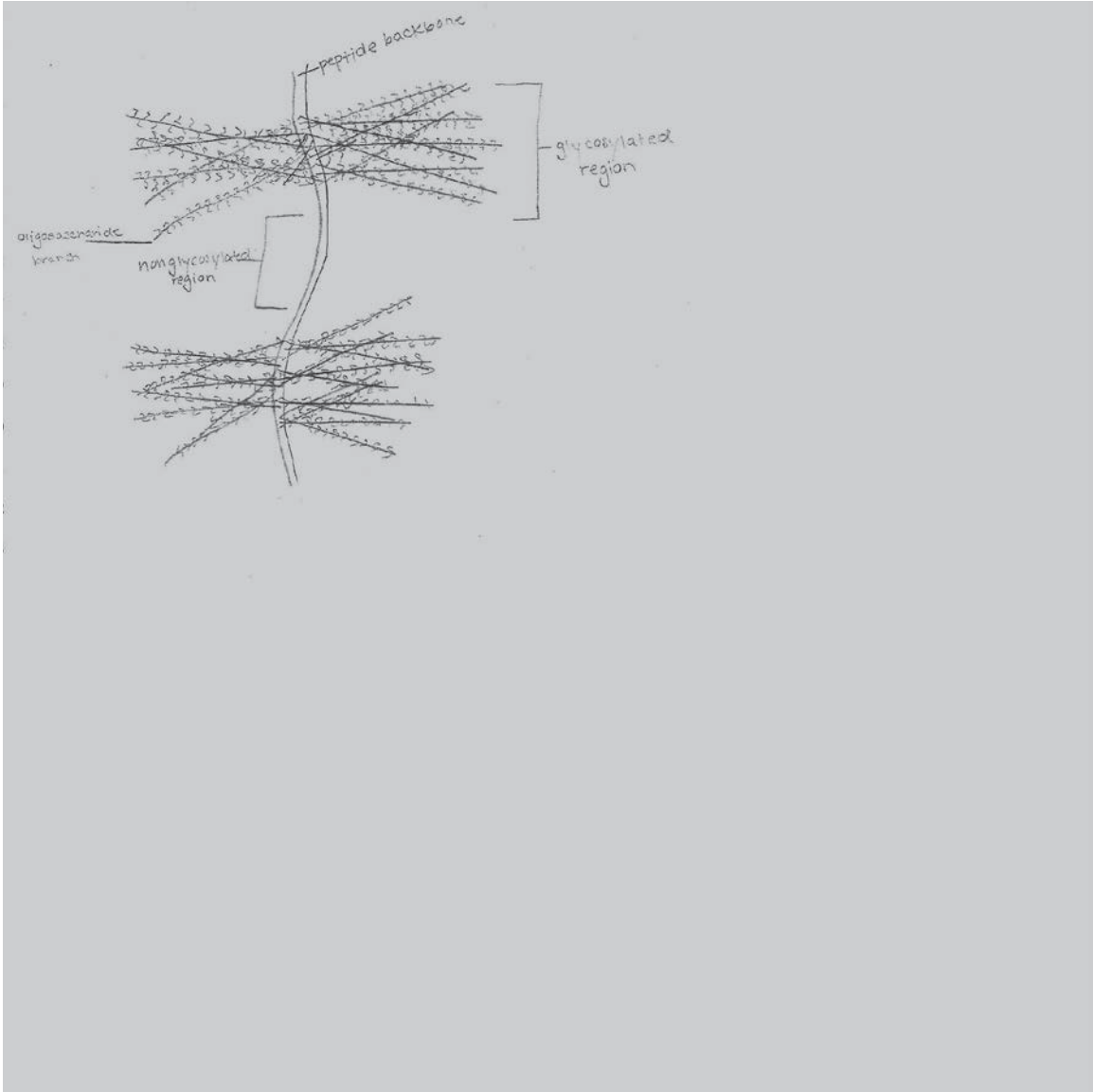
Appendix 4. Diagram of the ways the small intestine increases its surface area



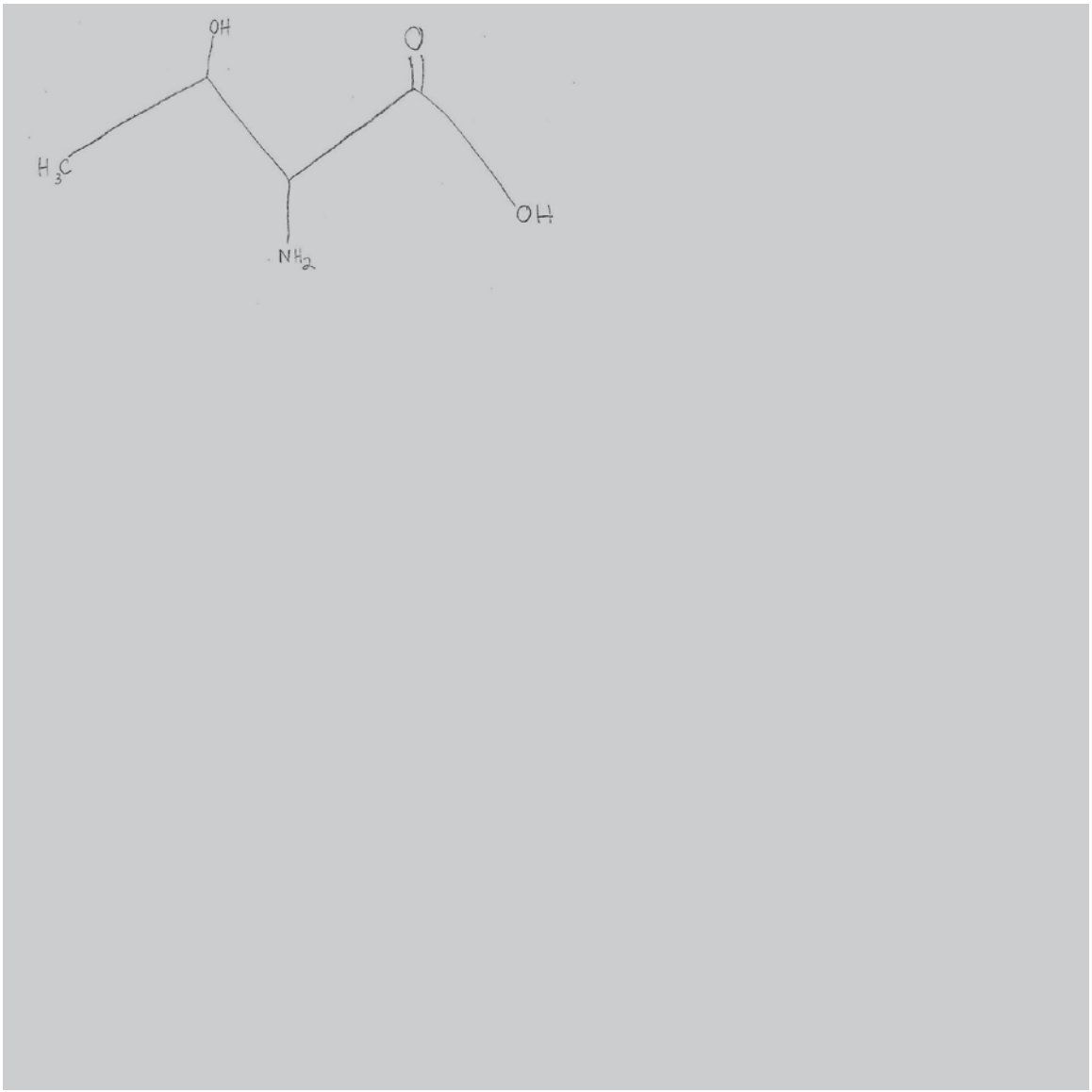
Appendix 5. Diagram of the cell types of the intestinal epithelia



Appendix 6. Diagram of the structure of the mucin glycoprotein



Appendix 7. Diagram of the chemical structure of threonine



Appendix 8. Histomorphology protocol for Chapter 2 and Chapter 3

Tissue Processing

Tissue is transferred to labeled cassettes and placed in an automated tissue process (Microm STP 120, Thermo Fisher Scientific, Waltham, MA). The tissues then undergo the following procedure.

Bucket	Solution	Time	RPM	Temperature (°C)
1	70% EtOH	Hold 8 hours	n/a	room
2	95% EtOH	1	70	room
3	95% EtOH	1	70	room
4	100% EtOH	1	70	room
5	100% EtOH	1	70	room
6	Citrisolv	1	70	room
7	Citrisolv	1	60	room
8	Paraffin Wax	1	60	60
9	Paraffin Wax	1	60	60

Tissue embedding

Tissue is embedded (Microm EC 350, Thermo Fisher Scientific, Waltham, MA) with wax heated to 60°C and then are allowed to cool to room temperature before further processing.

Cutting of paraffin blocks to fix to slides

Tissue-paraffin blocks were cleaned, trimmed, and cut (Microm HM 340 E, Thermo Fisher Scientific, Waltham, MA) into 5 μm sections. These sections were floated in a water bath heated to 45°C, then fixed to polarized slides. These slides were allowed to dry overnight before staining.

Staining of slides

Slides were stained using the following procedure.

1. Place slides in 60°C oven for 2 minutes.
2. Hydration Step – immerse sections in solutions for appropriate amount of time

Solution	Time (min)
Histolene (Citrisolv)	10
100% Ethanol	2
80% Ethanol	2
30% Ethanol	2
Distilled H_2O	2

3. Alcian Blue

Solution	Time (min)
Alcian Blue pH 2.5	5
Distilled H_2O Rinse	

4. Periodic Acid-Schiff Reaction

Solution	Time (min)
Periodic Acid	10
Distilled H_2O Rinse	3
Schiff's Reagent	15

5. Dehydration Step – immerse sections in solutions for appropriate amount of time

Solution	Time (min)
Distilled H_2O Rinse	
70% Ethanol	1
80% Ethanol	1
100% Ethanol	2
Histolene (Citrisolv)	5

Appendix 9. Interaction effects between Actigen® (ACT) and threonine concentration on nursery pig performance characteristics

Table A.2.1 *Interaction of dietary threonine concentration (Thr:Lys) and Actigen® (ACT) on body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and gain to feed conversion ratio (G:F) of nursery piglets*[1,2]

Treatment		BW (kg)				ADG(g)				ADFI(g)				G:F			
ACT	Thr:Lys	0 d	7 d	21 d	35 d	0-7 d	7-21 d	21-35 d	0-35 d	0-7 d	7-21 d	21-35 d	0-35 d	0-7 d	7-21 d	21-35 d	0-35 d
-	57%	6.3	6.3	10.6	16.9	2.7	303.0	460.5	305.4	130.8	433.3	816.6	525.0	-0.1	0.7	0.6	0.6
-	67%	6.3	6.2	10.2	16.1	-11	286.2	426.4	281.9	104.3	396.9	758.8	481.6	-0.1	0.7	0.6	0.6
-	77%	6.3	6.1	10.4	16.7	-1.7	289.2	458.9	298.2	110.8	396.7	801.1	500.0	-0.2	0.7	0.6	0.6
+	57%	6.3	6.3	10.1	15.7	-10.4	272.1	408.5	269.4	97.00	367.1	778.7	476.3	-0.1	0.7	0.5	0.6
+	67%	6.3	5.9	9.5	15.7	-48.8	257.9	444.2	270.2	78.20	339.0	738.5	445.3	-0.5	0.8	0.6	0.6
+	77%	6.3	6.2	10.1	15.9	-14.0	274.2	422.5	275.2	103.7	369.8	740.5	463.8	-0.2	0.7	0.6	0.6
SEM		0.36	0.35	0.49	0.76	15.16	16.78	29.13	17.4	12.41	22.2	38.2	23.37	0.2	0.02	0.02	0.02
P-value		*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1]Each value represents the LS Means of 6 replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Appendix 10. Interaction between Actigen® (ACT) and threonine concentration levels on nursery piglet jejunal histomorphology

Table A.2.2 *Interaction of dietary threonine concentration supplemented with or without Actigen® (ACT) on villus height (μm), apical length (μm), basal length (μm), crypt depth (μm), total goblet cell area per villus (μm²), villus surface area (mm²), villus height: crypt depth ratio, goblet cell count, and goblet cell density (mm²) of nursery piglets*[1,2]

Treatment		Villus Height, μm	Apical Length, μm	Basal Length, μm	Crypt Depth, μm	Goblet Cell Area/per villus, μm^2	Villus surface area, mm^2	Villus Height: Crypt Depth	Goblet cell count	Goblet cell density, mm^2
ACT	Thr:Lys									
+	57%	336.64	118.48	145.12	169.63	1823.33	45.38	2.2	14.5	0.38
-	57%	381.23	111.64	139.16	210.08	5131.67	48.58	1.9	13.4	0.3
+	67%	404.18	132.3	163.11	228.41	2814.42	61.49	1.9	16.1	0.3
-	67%	384.42	1285.43	148.4	215.75	1050.81	55.35	2	10.4	0.23
+	77%	450.62	130.53	161.51	205.86	1762.36	60.81	2.4	13.6	0.26
-	77%	467.92	121.72	157.27	189.54	1597.56	67.38	2.7	13.1	0.24
SEM		29.57	6.81	8.35	16.14	375.5	6.44	0.19	1.6	0.033
P-value		*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1]Each value represents the LS Means of 6 replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Appendix 11. Aflatoxin analysis of the peanut meal used in broiler chick diets

Table A.3.1 *Aflatoxin analysis of peanut meal*

	Amount, ppb	Risk Category
Aflatoxin (B1)	21.545	High Risk
Aflatoxin (B1+B2+G1+G2)	29.206	Medium Risk

Appendix 12. Average organ weights of d 7 broiler chicks

Table A.3.2 *Average organ weights of d 7 broiler chicks fed diets containing varying threonine (Thr) concentrations and Actigen® (ACT)*[1,2]

	Organ weight (g)				
Thr (% of diet)	*Liver*	*Spleen*	*Bursa*	*Thymus*	*Pancreas*
0.56	5.37^{B}	0.0957^{B}	0.175	0.376	0.61^{B}
0.64	7.07^{A}	0.150^{A}	0.199	0.472	0.85^{A}
0.80	6.92^{A}	0.135^{A}	0.205	0.448	0.80^{A}
0.96	7.57^{A}	0.135^{A}	0.200	0.514	0.82^{A}
1.04	6.60^{A}	0.150^{A}	0.204	0.458	0.85^{A}
Pooled SEM	0.357	0.0088	0.013	0.036	0.04
ACT	*Liver*	*Spleen*	*Bursa*	*Thymus*	*Pancreas*
+	6.62	0.14	0.200	0.463	0.76
-	6.79	0.13	0.194	0.444	0.81
Pooled SEM	0.226	0.006	0.008	0.023	0.025
Source of variation					
Actigen®	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Threonine	*P=0.0001*	*P=0.0005*	*n.s.*	*n.s.*	*P=0.0003*
ACT x Thr	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[1] Each value represents the LS Means for 6 replicates for each treatment group.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Appendix 13. Average organ weights of d21 broiler chicks

Table A.3.3 *Average organ weights of d 21 broiler chicks fed diets containing varying threonine (Thr) concentrations supplemented with or without Actigen® (ACT)*[1,2]

	Organ weight (g)				
Thr (% of diet)	*Liver*	*Spleen*	*Bursa*	*Thymus*	*Pancreas*
0.56	25.08	0.715	1.31	3.44	2.21^{C}
0.64	27.58	0.956	1.58	4.54	2.94^{A}
0.80	27.07	0.920	1.53	3.77	2.78^{AB}
0.96	27.05	0.854	1.70	3.61	2.41^{BC}
1.04	26.98	0.895	1.65	4.19	2.55^{BC}
Pooled SEM	1.346	0.076	0.112	0.372	0.136
ACT	*Liver*	*Spleen*	*Bursa*	*Thymus*	*Pancreas*
+	26.77	0.86	1.53	3.76	2.56
-	26.74	0.88	1.58	4.05	2.59
Pooled SEM	0.85	0.048	0.071	0.24	0.085
Source of variation					
Actigen®	n.s.	n.s.	n.s.	n.s.	n.s.
Threonine	n.s.	n.s.	n.s.	n.s.	*P*=0.0032
ACT x Thr	n.s.	n.s.	n.s.	n.s.	n.s.

[1] Each value represents the LS Means for 6 replicates for each treatment.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Appendix 14. Interaction between Actigen® (ACT) and threonine on performance characteristics of broiler chicks

Table A.3.4 *Interaction of dietary threonine (Thr) concentration and Actigen® (ACT) on body weight (BW), average daily feed intake (ADFI) and gain to feed conversion ratio (G:F) of broiler chicks*[1,2]

		BW (g)	**ADFI (g)**	**G:F**
ACT	*Thr (% of diet)*	d 21	0-21 d	0-21 d
-	0.56	564	39.5	1.7
-	0.64	827	53.8	1.5
-	0.8	836	53.2	1.5
-	0.96	820	53.7	1.5
-	1.04	848	53.7	1.5
+	0.56	600	42.8	1.7
+	0.64	838	53.7	1.5
+	0.8	879	54.7	1.5
+	0.96	846	54.4	1.5
+	1.04	832	53.0	1.5
	SEM	17.2	1.11	0.03
	P-value	n.s.	n.s.	n.s.

[1] Each value represents the LS Means for 6 replicates for each treatment.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Appendix 15. Interaction between Actigen® (ACT) and threonine on breaking strength of tibia and humerus from d 21 broiler chicks

Table A.3.5 *Interaction of dietary threonine (Thr) concentration and Actigen® (ACT) on average breaking strength of tibia and humerus of broiler chicks* [1,2]

		Force required to break (kg force)	
ACT	*Thr (% of diet)*	Tibia	Humerus
-	0.56	1.47	1.18
-	0.64	2.19	1.54
-	0.8	2.23	1.61
-	0.96	1.89	1.31
-	1.04	1.92	1.55
+	0.56	1.54	1.28
+	0.64	2.28	1.6
+	0.8	2.12	1.6
+	0.96	1.96	1.49
+	1.04	2.22	1.47
SEM		0.14	0.09
P-value		n.s.	n.s.

[1] Each value represents the LS Means for 6 replicates for each treatment.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Appendix 16. Interaction between Actigen® (ACT) and threonine on d 7 duodenum intestinal morphology from broiler chicks

Table A.3.6 *Interaction between dietary threonine (Thr) and Actigen® (ACT) on intestinal morphology from the duodenum of 7 day old broiler chicks*[1,2]

		Intestinal Morphology Characteristics							
ACT	*Thr (% of diet)*	Villus height (µm)	Apical width (µm)	Basal width (µm)	Villus surface area (mm²)	Crypt depth (µm)	Villus height: Crypt depth	No. of goblet cells	Goblet cell density (cells/mm²)
-	0.56	1477	259	251	3863	315	5.13	97.7	0.03
-	0.64	1540	308	241	4191	285	5.65	110.7	0.03
-	0.8	1647	293	220	4472	297	5.84	88.7	0.03
-	0.96	1500	260	257	4005	321	5.33	88.4	0.03
-	1.04	1584	252	293	4484	330	5.12	110	0.03
+	0.56	1268	248	236	3122	289	4.62	76.8	0.03
+	0.64	1433	227	252	3740	306	5.21	90.6	0.03
+	0.8	1432	210	211	3015	280	5.92	88.3	0.03
+	0.96	1559	312	282	4875	340	4.95	94	0.02
+	1.04	1742	281	272	4889	357	5.16	94.2	0.02
SEM		123	39	31	732	40	0.48	9.7	0.004
P-value		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[1] Each value represents the LS Means for 6 replicates for each treatment.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Appendix 17. Interaction between Actigen® (ACT) and threonine on d 7 jejunum intestinal morphology from broiler

Table A.3.7 *Interaction between dietary threonine (Thr) and Actigen® (ACT) on intestinal morphology from the jejunum of 7 day old broiler chicks*[1,2]

		Intestinal Morphology Characteristics							
ACT	*Thr (% of diet)*	Villus height (μm)	Apical width (μm)	Basal width (μm)	Villus surface area (mm^2)	Crypt depth (μm)	Villus height: Crypt depth	No. of goblet cells	Goblet cell density (cells/mm^2)
-	0.56	1141	303	268	3233	352	3.42	54.9	0.02
-	0.64	1367	268	253	3626	381	3.67	74.5	0.02
-	0.8	1257	296	289	3683	402	3.25	83.8	0.03
-	0.96	1209	276	271	3382	357	3.51	67.5	0.02
-	1.04	1173	322	325	3815	383	3.19	69.4	0.02
+	0.56	1128	322	320	3473	368	3.28	69	0.02
+	0.64	1181	283	288	3370	401	3.42	67.6	0.02
+	0.8	1237	294	281	3541	392	3.27	77.1	0.03
+	0.96	1223	364	286	3925	355	3.58	66.1	0.02
+	1.04	1203	264	246	3078	338	3.71	59.1	0.02
SEM		84	34	23	375	25	0.28	8	0.003
P-value		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[1] Each value represents the LS Means for 6 replicates for each treatment.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Appendix 18. Interaction between Actigen® (ACT) and threonine on d 7 ileum intestinal morphology from broiler chicks

Table A.3.8 *Interaction between dietary threonine (Thr) and Actigen® (ACT) on intestinal morphology from the ileum of 7 day old broiler chick*[1,2]

		Intestinal Morphology Characteristics							
ACT	*Thr (% of diet)*	Villus height (μm)	Apical width (μm)	Basal width (μm)	Villus surface area (mm^2)	Crypt depth (μm)	Villus height: Crypt depth	No. of goblet cells	Goblet cell density (cells/mm^2)
-	0.56	795	277	290	2278	333	2.5	43.5	0.02
-	0.64	933	333	321	3045	333	2.9	53	0.02
-	0.8	883	247	245	2160	296	3.03	46.3	0.02
-	0.96	880	247	251	2178	279	3.29	48	0.02
-	1.04	835	263	268	2233	287	2.97	43.4	0.02
+	0.56	738	243	232	1760	271	2.84	38.2	0.02
+	0.64	934	254	263	2476	322	2.95	49.8	0.02
+	0.8	934	258	249	2389	307	3.09	50.3	0.02
+	0.96	802	287	219	2027	277	3	40.3	0.02
+	1.04	927	291	290	2657	297	3.19	50.4	0.02
SEM		51	25	27	253	14	0.17	2.8	0.002
P-value		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[1] Each value represents the LS Means for 6 replicates for each treatment.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Appendix 19. Interaction between Actigen® (ACT) and threonine on d 21 duodenum intestinal morphology from broiler chicks

Table A.3.9 *Interaction between dietary threonine (Thr) and Actigen® (ACT) on intestinal morphology from the duodenum of 21 day old broiler chick*[1,2]

		Intestinal Morphology Characteristics							
ACT	*Thr (% of diet)*	Villus height (μm)	Apical width (μm)	Basal width (μm)	Villus surface area (mm^2)	Crypt depth (μm)	Villus height: Crypt depth	No. of goblet cells	Goblet cell density ($cells/mm^2$)
-	0.56	2562	328	282	7867	342	7.71	48.9	0.01
-	0.64	2401	313	293	7291	389	6.5	46.5	0.01
-	0.8	2520	374	342	8569	412	6.33	55.3	0.01
-	0.96	2411	410	326	8838	405	6.2	56.1	0.01
-	1.04	2507	332	300	8283	369	7.2	51.5	0.01
+	0.56	2501	380	308	8658	391	6.71	59.6	0.01
+	0.64	2492	264	260	6729	353	7.56	54.8	0.01
+	0.8	2258	373	453	9606	362	6.47	43.9	0.01
+	0.96	2478	312	295	7323	363	7.5	46.2	0.01
+	1.04	2627	315	295	8028	370	7.31	46.4	0.01
SEM		255	38	40	1153	24	0.84	6.9	0.001
P-value		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[1] Each value represents the LS Means for 6 replicates for each treatment.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Appendix 20. Interaction between Actigen® (ACT) and threonine on d 21 jejunum intestinal morphology from broiler chicks

Table A.3.10 *Interaction between dietary threonine (Thr) and Actigen® (ACT) on intestinal morphology from the jejunum of 21 day old broiler chick*[1,2]

		Intestinal Morphology Characteristics							
ACT	*Thr (% of diet)*	Villus height (µm)	Apical width (µm)	Basal width (µm)	Villus surface area (mm²)	Crypt depth (µm)	Villus height: Crypt depth	No. of goblet cells	Goblet cell density (cells/mm²)
-	0.56	2082	405	397^{ABC}	8032^{BC}	339	6.31	48.2	0.01
-	0.64	2015	359	306^{C}	6545^{C}	334	6.29	50.1	0.01
-	0.8	1922	364	332^{BC}	6733^{C}	325	6.28	47.6	0.01
-	0.96	2121	458	423^{AB}	9231^{AB}	352	6.25	46.5	0.01
-	1.04	2195	549	482^{A}	10806^{A}	372	6.12	53.9	0.01
+	0.56	1891	372	368^{BC}	7067^{BC}	337	5.88	45.6	0.01
+	0.64	2019	396	390^{ABC}	8050^{BC}	329	6.3	47.8	0.01
+	0.8	2063	343	304^{C}	6584^{C}	305	7.04	47.2	0.01
+	0.96	1965	366	353^{BC}	7325^{BC}	356	5.82	44.2	0.01
+	1.04	1852	350	331^{BC}	6377^{C}	333	5.89	42.8	0.01
SEM		127	43	34	840	20	0.37	4.5	0.001
P-value		n.s.	n.s.	P=0.025	P=0.015	n.s.	n.s.	n.s.	n.s.

[1] Each value represents the LS Means for 6 replicates for each treatment.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

Appendix 21. Interaction between Actigen® (ACT) and threonine on d 21 ileum intestinal histology from broiler chicks

Table A.3.11 *Interaction between dietary threonine (Thr) and Actigen® (ACT) on intestinal morphology from the ileum of 21 day old broiler chick*[1,2]

		Intestinal Morphology Characteristics							
ACT	*Thr (% of diet)*	Villus height (µm)	Apical width (µm)	Basal width (µm)	Villus surface area (mm²)	Crypt depth (µm)	Villus height: Crypt depth	No. of goblet cells	Goblet cell density (cells/mm²)
-	0.56	1333	316	288	4096	267	4.8	48.1	0.01
-	0.64	1422	335	284	4311	327	4.44	50.2	0.01
-	0.8	1384	370	363	4918	308	4.65	53.5	0.01
-	0.96	1376	432	387	5657	349	4.12	52.5	0.01
-	1.04	1525	321	278	4483	328	4.81	60.3	0.01
+	0.56	1534	326	317	4886	326	4.96	59.6	0.01
+	0.64	1394	374	331	4992	341	4.21	51.5	0.01
+	0.8	1418	381	317	4960	330	4.58	47.6	0.01
+	0.96	1249	412	350	4756	308	4.2	50.6	0.01
+	1.04	1324	375	332	4695	327	4.19	43.7	0.01
SEM		98	35	33	484	17	0.29	5.1	0.001
P-value		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[1] Each value represents the LS Means for 6 replicates for each treatment.

[2]Means with different superscripts within a column differ significantly ($P < 0.05$).

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Vita

Lindsay Good was born in Louisville, KY on June 5, 1990 to Amy and Bruce Good. She was active in 4-H and FFA growing up and graduated from Seneca High School in 2008. In the fall of 2008, Lindsay was awarded the Otis A. Singletary scholarship to attend the University of Kentucky. During her undergraduate career, Lindsay was active in undergraduate animal nutrition research and was a founding member of the Research in Equine and Agricultural Disciplines Club. She graduated from the University of Kentucky in 2011 with a Bachelor's in Equine Science.

After graduation, Lindsay participated in an internship program at Alltech in Nicholasville, which inspired her to pursue a Master's in poultry nutrition with Dr. Anthony Pescatore. Lindsay was awarded the Otis A. Singletary Graduate fellowship to assist in her graduate education.