Efficacy of Lysozyme as an Alternative to Antibiotics for Broiler Chickens

by

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TABLE OF CONTENTS

LIST OF TABLES v

LIST OF FIGURES x

ABSTRACT xi

LIST OF ABBREVIATIONS USED xii

ACKNOWLEDGEMENTS xiii

CHAPTER 1 INTRODUCTION 1

CHAPTER 2 LITERATURE REVIEW 3

2.1 THE GIT OF BROILER CHICKENS 3

2.2 INTESTINAL MICROBIOTA OF BROILER CHICKENS 6

2.2.1 THE DEVELOPMENT OF INTESTINAL MICROBIOTA 7

2.2.2 THE EFFECT OF DIET ON INTESTINAL MICROBIOTA 8

2.2.3 THE EFFECT OF ENVIRONMENT ON INTESTINAL MICROBIOTA 9

2.2.3.1 TEMPERATURE 9

2.2.3.2 LITTER 10

2.2.4 METHODS FOR ENUMERATION OF INTESTINAL MICROBIOTA 11

2.2.4.1 CONVENTIONAL CULTURE METHOD FOR ENUMERATION 11

2.2.4.2 SEQUENCING OF BACTERIAL GENETIC MATERIAL FOR BACTERIAL ENUMERATION 12

2.2.5 MICROBIOTA SPECIES OF INTEREST 13

2.2.5.1 *LACTOBACILLUS* 14

2.2.5.2 *BIFIDOBACTERIUM* 15

2.2.5.3 *SALMONELLA* 15

2.2.5.4 *CLOSTRIDIUM PERFRINGENS* 16

2.2.5.5 *E. COLI* 17

2.2.5.6 OTHER SPECIES OF INTEREST 18

2.3 ANTIBIOTICS 18

2.3.1 THE HISTORY OF ANTIBIOTIC USE IN ANIMAL AGRICULTURE 18

2.3.2 FUNCTION OF ANTIBIOTICS 19

2.3.3 ANTIBIOTIC RESISTANCE 20

2.3.4 ALTERNATIVES TO ANTIBIOTICS 20

2.3.4.1 ORGANIC ACIDS 21

2.3.4.2 PROBIOTICS 22

2.3.4.3 PREBIOTICS 23

2.3.4.4 OTHER ALTERNATIVES TO ANTIBIOTICS 24

2.4 LYSOZYME 24

2.4.1 FUNCTION OF LYSOZYME 25

2.4.2 APPLICATION OF LYSOZYME IN POULTRY AGRICULTURE 26

2.5 SUMMARY 28

CHAPTER 3 THE EFFECT OF LYSOZYME FOR BROILER CHICKENS GROWN UNDER TWO TEMPERATURE CONDITIONS 29

3.1 ABSTRACT 29

3.2 INTRODUCTION 29

3.3 OBJECTIVE **30**
3.4 HYPOTHESES **30**
3.5 MATERIALS AND METHODS **31**
3.5.1 ANIMAL HOUSING AND HUSBANDRY 31
3.5.2 DIETS 32
3.5.3 ANALYSIS OF GROWTH PERFORMANCE 35
3.5.4 DIGESTA SAMPLE COLLECTION 36
3.5.5 MICROBIOTA ANALYSIS 36
3.5.6 STATISTICAL ANALYSIS 39
3.6 RESULTS AND DISCUSSION **40**
3.6.1 GROWTH PERFORMANCE 40
3.6.2 INTESTINAL MICROBIOTA 48
3.7 CONCLUSIONS **52**

Chapter 4 THE EFFECT OF LYSOZYME IN EACH PERIOD OF THE GROWTH CYCLE OF BROILER CHICKENS GROWN IN OPTIMAL HOUSING CONDITION **54**
4.1 ABSTRACT **54**
4.2 INTRODUCTION **55**
4.3 OBJECTIVE **55**
4.4 HYPOTHESES **55**
4.5 MATERIALS AND METHODS **55**
4.5.1 ANIMAL HOUSING AND HUSBANDRY 56
4.5.2 DIETS 56
4.5.3 ANALYSIS OF GROWTH PERFORMANCE 59
4.5.4 DIGESTA SAMPLE COLLECTION 60
4.5.5 MICROBIOTA ANALYSIS 60
4.5.6 STATISTICAL ANALYSIS 62
4.6 RESULTS AND DISCUSSION **63**
4.6.1 GROWTH PERFORMANCE 63
4.6.2 INTESTINAL MICROBIOTA 69
4.7 CONCLUSIONS **73**

Chapter 5 THE EFFECT OF LYSOZYME IN EACH PERIOD OF GROWTH CYCLE ON BROILER CHICKENS GROWN ON USED LITTER **75**
5.1 ABSTRACT **75**
5.2 INTRODUCTION **76**
5.3 OBJECTIVE **76**
5.4 HYPOTHESES **76**
5.5 MATERIALS AND METHODS **77**
5.5.1 ANIMAL HOUSING AND HUSBANDRY 77
5.5.2 DIETS 77
5.5.3 ANALYSIS OF GROWTH PERFORMANCE 79
5.5.4 DIGESTA SAMPLE COLLECTION 79
5.5.5 MICROBIOTA ANALYSIS 79
5.5.6 STATISTICAL ANALYSIS 79

5.6 RESULTS AND DISCUSSION **80**
5.6.1 GROWTH PERFORMANCE 80
5.6.2 INTESTINAL MICROBIOTA 85
5.7 CONCLUSIONS **90**

Chapter 6 CONCLUSION AND RECOMMENDATIONS **91**
6.1 CONCLUSIONS **91**
6.2 RECOMMENDATION **91**

BIBLIOGRAPHY **93**

APPENDIX A Lighting schedules for cage trial and two floor trials **109**

APPENDIX B Temperature recorded by data logger for cage trial and two floor trials **110**

APPENDIX C Relative humidity recorded by data logger for cage trial and two floor trials **114**

LIST OF TABLES

Table 3.1 Composition of experimental starter diets containing lysozyme fed from 1-14 days of age……..…....…….…….…….33

Table 3.2 Composition of experimental starter diets containing lysozyme fed from 15-24 days of age……..…..……..…34

Table 3.3 Composition of experimental starter diets containing lysozyme fed from 25-35 days of age……...……...……...35

Table 3.4 Effect of inclusion level of lysozyme on body weight (g $bird^{-1}$) of broiler chickens on day 14 and 21……...……41

Table 3.5 Effect of inclusion level of lysozyme on body weight gain (g $bird^{-1}$ day^{-1}) of broiler chickens on day 14 and 21……...……41

Table 3.6 Effect of inclusion level of lysozyme and temperature conditions on body weight (g $bird^{-1}$) of broiler chickens on day 35……..…….42

Table 3.7 Effect of inclusion level of lysozyme and room temperature conditions on body weight gain (g $bird^{-1}$ day^{-1}) of broiler chickens from day 22-35…...42

Table 3.8 Effect of inclusion level of lysozyme on feed consumption (g $bird^{-1}$) per day per broiler chicken from day 0-14 and 15-21…….....45

Table 3.9 Effect of inclusion level of lysozyme and temperature conditions on feed consumption (g $bird^{-1}$ day^{-1}) of broiler chickens from day 22-35……..…45

Table 3.10 Effect of inclusion level of lysozyme on feed conversion ratio (feed $gain^{-1}$) of broiler chicken day 0-14 and 15-21……..……..…..46

Table 3.11 Effect of inclusion level of lysozyme and temperature conditions on feed conversion ratio (feed $gain^{-1}$) of broiler chicken from day 22-35……..…46

Table 3.12 ANOVA P-value for the percent of mortality in broilers fed different inclusion levels of lysozyme……..……..……...47

Table 3.13 Effect of inclusion level of lysozyme on total aerobes, coliforms, *E. coli* and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 14…….……..…48

Table 3.14 Effect of inclusion level of lysozyme and room temperature on total aerobes (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 35…….…49

Table 3.15 Effect of inclusion level of lysozyme and room temperature on total anaerobes (log_{10} cfu g^{-1}), in the ileum of broiler chickens on day 35…..…50

Table 3.16 Effect of inclusion level of lysozyme and room temperature on total coliforms (log_{10} cfu g^{-1}), in the ileum of broiler chickens on day 35……..51

Table 3.17 Effect of inclusion level of lysozyme and room temperature on *E. coli* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 35…….…...…51

Table 3.18 Effect of inclusion level of lysozyme and room temperature on *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 35……..…….……..52

Table 4.1 Composition of experimental diets containing lysozyme and antibiotic fed from 0-14, 15-24 and 25-35 days of age (Experiment 1)…….....57

Table 4.2 Composition of experimental diets containing lysozyme and antibiotic fed from 0-14, 15-24 and 25-35 days of age (Experiment 2)……..58

Table 4.3 Supplementation of lysozyme in different periods during the growth cycle of broiler chickens……..……..……..59

Table 4.4 The dilutions used for colony counts of total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens*…….…...61

Table 4.5 Effect of antibiotics and timing of introduction of lysozyme to the feed on body weight (g $bird^{-1}$) of broiler chickens……..……..….64

Table 4.6 Effect of antibiotics and timing of introduction of lysozyme to the feed on body weight gain (g $bird^{-1}$ day^{-1}) of broiler chickens…….....64

Table 4.7 Effect of antibiotics and timing of introduction of lysozyme to the feed on feed consumption (g $bird^{-1}$ day^{-1}) of broiler chickens……...…….65

Table 4.8 Effect of antibiotics and timing of introduction of lysozyme to the feed on feed conversion ratio (feed $gain^{-1}$) of broiler chickens…..……...……..65

Table 4.9 Effect of antibiotics and timing of introduction of lysozyme to the feed on mortality rate (%) of broiler chickens…….……...……68

Table 4.10 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 4……..…....……69

Table 4.11 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 14…….……...…70

Table 4.12 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 24……..……...…70

Table 4.13 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 35…….....……71

Table 4.14 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens throughout the trial…..71

Table 5.1 Composition of experimental diets containing lysozyme and antibiotic fed from 1-14, 15-24 and 25-35 days of age…….78

Table 5.2 Effect of antibiotics and timing of introduction of lysozyme to the feed on body weight (g $bird^{-1}$) of broiler chickens…….80

Table 5.3 Effect of antibiotics and timing of introduction of lysozyme to the feed on body weight gain (g $bird^{-1}$ day^{-1}) of broiler chickens……..81

Table 5.4 Effect of antibiotics and timing of introduction of lysozyme to the feed on feed consumption (g $bird^{-1}$ day^{-1}) of broiler chickens…….81

Table 5.5 Effect of antibiotics and timing of introduction of lysozyme to the feed on feed conversion ratio (feed $gain^{-1}$) of broiler chickens……...82

Table 5.6 Effect of antibiotics and timing of introduction of lysozyme to the feed on feed mortality rate (%) of broiler chickens……..84

Table 5.7 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anarobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 4……86

Table 5.8 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anarobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 14……..86

Table 5.9 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anarobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 24……..87

Table 5.10 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anarobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 35…..……..…..…..88

Table 5.11 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anarobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens throughout the trial……...…...89

LIST OF FIGURES

Figure 3.1 The daily 24 hours temperature schedule from day 22 to the end of the trial (day 35) for the room with sub optimal temperature…….....…….32

Figure 3.2 The culture *of Clostridium perfringens* on petri dish……..……...38

Figure 3.3 The culture of total aerobic bacteria on Petrifilm™ aerobic count plates.…38

Figure 3.4 The culture of total anaerobic bacteria on Petrifilm™ aerobic count plates……...38

Figure 3.5 The culture of *E. coli* and total coliform on Petrifilm™ E.coli/ coliform count plates…….....……38

Figure 4.1 The culture of lactic acid bacteria on Petrifilm™ aerobic count plates…….62

ABSTRACT

Antibiotics have been included in poultry feeds to improve growth performance. However, it is a concern that pathogens have become increasingly resistant to antibiotics. Lysozyme is a potential replacement for antibiotics. A trial with or without heat stress was conducted to investigate different inclusion levels (0, 50, 100 and 200ppm) of lysozyme on broiler chickens. Another two trials were conducted using clean or used litter to determine the effect of 100 ppm lysozyme on broiler chickens in each period of the growth cycle. Birds fed the 50 ppm treatment had heavier weight than birds fed the 200 ppm treatment on day 35 ($P<0.05$). When used litter was provided, feeding lysozyme to birds from days 5-14 and throughout the trial reduced the number of *E. coli* in the ileum compared with feeding antibiotic to birds ($P<0.05$). Dietary lysozyme positively influences bacterial numbers in the gastrointestinal tract of broiler chickens.

LIST OF ABBREVIATIONS USED

Association of Official Agricultural Chemists……...AOAC

Bacitracin methylene disalicylate...……..BMD

Buffered peptone water…….BPW

Canadian Council on Animal Care…….CCAC

Center for Disease Control and Prevention……...CDC

Colony forming unit……..cfu

Ethylenediaminetetraacetic acid……..EDTA

Escherichia coli……..….*E. coli*

Feed conversion ratio ……..……. FCR

Gastrointestinal tract.…….…… GIT

Necrotic enteritis.……..…...NE

Next generation sequencing……...……NGS

Polymerase chain reaction……..PCR

Relative humidity…….RH

Ribosomal RNA……...rRNA

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CHAPTER 1 INTRODUCTION

Antibiotics have been used for many years in animal production (Dibner and Richards 2005). Antibiotics are included in animal feeds to improve growth rate and feed efficiency for disease prophylaxis, and disease therapy (Solomons 1978). Even though antibiotics have been used by growers as effective supplements, the European Union banned the use of four growth promoting antibiotics (spiramycin, tylosin, bacitracin and virginiamycin). Bacitracin and virginiamycin are commonly used in poultry diets (Casewell et al. 2003). The reason for this ban is that potential human pathogens found on processed poultry carcasses have become increasingly resistant to certain antibiotics (Hofacre et al. 2003). Therefore, it is important to utilize improved management approaches. Improved management procedures coupled with increased biosecurity (Bojesen et al. 2010) or other alternative dietary supplements including probiotics, prebiotics and herbs have been suggested as ways to grow broiler chickens without antibiotics (Edens 2003; Patterson and Burkholder 2003; Guo et al. 2004).

Lysozyme is an example of a feed ingredient with potential to replace dietary antibiotics (Zhang et al. 2006). Lysozyme is defined as 1,4-β-N-acetylmuramidase. This enzyme cleaves the glycosidic bond between the N-acetylmuramic acid and N-acetylglucosamine in bacterial peptidoglycan of the cell wall (Phillips 1966). Lysozyme is a common enzyme that is commercially obtained from avian egg white, and is widespread in many tissues and secretions of animals (Grossowicz and Ariel 1983). Some studies have reported significant function of lysozyme in various organisms as a defender against bacteria (Biggar and Sturgess 1977; Ibrahim et al. 1996). In the body's defense

mechanisms, functions of lysozyme are associated with the monocyte macrophage system and immunoglobulins (Osserman 1976). Lysozymes play a role as an antibacterial agent which is mediated through its direct bacteriolytic action or stimulate macrophage phagocytic function (Biggar and Sturgess 1977; Thacore and Willett 1966).

Research regarding the use of lysozyme as an alternative to antibiotics for poultry is limited. An *in vitro* experiment conducted by Zhang et al. (2006) suggested that 200 μg/ml of lysozyme not only completely inhibited the growth of *Clostridium perfringens*, but also inhibited production of α-toxin which causes the lesions associated with necrotic enteritis (NE) in chickens. Some *in vivo* studies have shown lysozyme could be a potential candidate to eliminate *Clostridium perfringens* and improve growth performance in broiler chickens (Zhang et al. 2010; Liu et al. 2010). More *in vivo* studies are necessary to determine which levels of lysozyme are most effective, as well as determining which critical periods of the broiler growth cycle lysozyme may have the greatest impact on growth performance and microbiota populations of gastrointestinal tract (GIT) of broiler chickens.

CHAPTER 2 LITERATURE REVIEW

2.1 THE GIT OF BROILER CHICKENS

The GIT of chickens is the anatomically diverse organ system which provides an environment for mechanical and chemical reduction in the size and molecular complexity of food. The end products are absorbed, and the waste is excreted from the GIT. The GIT in chickens begins at the mouth and ends at the cloaca. The tract consists of a mouth, esophagus, crop, proventriculus, gizzard, small intestine, ceca, colon, rectum, and cloaca (Klasing 1999).

The beak or mouth is used to pick up food. Salivary glands in the mouth secrete saliva which wets the food, lubricating it to facilitate swallowing. Food is then pushed to the back of the mouth by the tongue. The esophagus carries food from the mouth to the crop and from the crop to the proventriculus via peristaltic action. Longitudinal folds in the mucosa allow the esophagus to expand to accommodate various feed sizes. The crop stores food until the gizzard can accommodate it. The crop can collapse or expand according to the amount of ingested food present. Nerves in the crop send hunger signals to the brain when the crop is empty stimulating chickens to eat more (Jacob et al. 2011 and Klasing 1999). Without the gastric antrum of mammalian species, the proventriculus in birds is the main site where gastric juice is produced. Hydrochloric acid and pepsin are secreted by oxynticopeptic cells in gastric glands in proventricular mucosa (Olowo-Okorun and Amure 1973). The function of the gizzard is to reduce the size of feed by

mechanically grinding it so that the surface areas of feed particles are increased (Klasing 1999).

The small intestine consists of the duodenum, jejunum and ileum. Food in the small intestine can be reduced to a relatively uniform and fluid chyme (Klasing 1999). The small intestine is the major site of nutrient absorption. The mixed secretions from the duodenum, liver and pancreas are used to digest nutrients (McDonald et al. 2002). Pancreatic juice secreted by the pancreas mainly takes part in protein digestion. The bile from the liver enters the GIT via the hepatic duct and plays a role in the digestion of lipids and the absorption of fat-soluble vitamins. The end of the jejunum and the start of ileum are marked by the Meckel's diverticulum, which is the remnant of the yolk sac (Jacob et al. 2011).

The ceca are blind pouches located at the connection of the small intestine and large intestine. The function of the ceca is to re-absorb some of the water in the fecal material and fermentation of remaining solid material. The large intestine is much shorter than the small intestine and mainly used to re-absorb water. In the cloaca, fecal material and urine are combined prior to excretion (Jacob et al. 2011; Sainsbury 2000).

Intestinal histology plays an important role in feed digestion. The use of feed additives may improve the growth performance of broiler chickens by changing the intestinal histology (Humphrey et al. 2002). The main role of villi is to absorb the nutrients coming into the intestine. Microvilli on the surface of the villi largely increase the surface area of

absorption by 15-fold. Food absorbed by villi will be transported to blood vessels via a capillary bed. In order to protect villi and microvilli from enzymes, mucous is secreted by goblet cells in the intestinal epithelium (Klasing 1999). The main function of intestinal crypts is cell generation. Some specialized cells such as absorptive cells, goblet cells and regenerative cells produce mucus and replace old epithelia cells. Generally, the deeper the crypts are the more goblet cells will be produced (De Los Santos et al. 2007). Turnover rate of a cell population is determined by rate of production or destruction. The cells to be produced by mitosis in the crypts travel to the tip of the villi in two to three days depending on the length of villi in the small intestine. The cells which are replaced are shed into the intestinal lumen (Creamer et al. 1961; Potten 1998).

The intestinal histology of broiler chickens is influenced by many factors including age, diet and intestinal microbiota. As chicks age, the villi become broader. This increases the inner surface area in the intestine available for food digestion. Nutrients such as pectin have been shown to improve villi surface area (Van Leeuwen et al. 2004). Additionally the presence of *Salmonella* typhimurium in the presence of pectin has been shown to increase villi surface area (Van Leeuwen et al. 2004). Pelicano et al. (2005) demonstrated that supplementation with probiotics, prebiotics, organic acids or the combination of each benefits the small intestine histological indexes. These measurements include villi height and crypt depth for 21-day-old broiler chickens. Other ingredients such as lysozyme, lactoferrin and antibiotics have increased the villi height in the small intestine, and subsequently improved the nutrient absorption by broiler chickens (Humphrey et al. 2002)

Along the GIT, bacteria attach to the mucosa of many sections including the crop, gizzard, duodenum, jejunum, ileum and ceca. The ceca have the most diverse bacteria population with clostridia being the dominant genus. Lactobacilli constitute the largest population in the upper GIT (Gong et al. 2007). Bacteria in the ceca can protect against an exogenous bacterial infection. In the small intestine, a healthy microbiota environment plays an important role in food digestion and food absorption which is directly related to growth performance of broiler chickens (Gong et al. 2002). The control of intestinal microbiota is important for the poultry industry. The phasing out of the use of antibiotics has led to the use of alternative feed supplements to maintain a healthy small intestine microbiota. Since the majority of bacteria in the ileum are Gram-positive bacteria (Gong et al. 2002), lysozyme could be an ideal alternative to antibiotics. Lysozyme directs its activity mostly Gram-positive bacteria including Clostridium (Zhang et al. 2006) and some of the Gram-negative bacteria (Pellegrini et al. 1992).

2.2 INTESTINAL MICROBIOTA OF BROILER CHICKENS

The intestinal microbiota in broiler chickens consists of bacteria, fungi and protozoa, among which bacteria are the predominant microorganisms (Yegani and Korver 2008). Facultative anaerobes mainly settle in the upper digestive tract, while obligate anaerobes dominate the ceca. The activities of microbiota are influenced by factors including animal age, environment and diet (Gabriel et al. 2006). The metabolism of bacteria can be useful or detrimental to the host animals. Bacteria can negatively affect the digestion of lipids, carbohydrates, proteins and vitamins, so that the requirement for energy and amino acids

increases. However, beneficial bacteria play an important role in the development of the intestinal immune system that protects birds against pathogens (Gabriel et al. 2006). The GIT commensal microorganisms can compete with the invading pathogens by occupying the attachment sites on the intestine mucosal surface. Newly hatched chicks which lack intestinal microbial communities are very vulnerable to invasion by pathogens (Lan et al. 2005).

2.2.1 THE DEVELOPMENT OF INTESTINAL MICROBIOTA

For newly hatched birds, evidence of intestinal microbiota varies due to the microbiological condition. In newly hatched chicks prior to receiving food and water, microbiota in the ceca are composed mainly of clostridia (Lev and Briggs 1956). Significant amounts of bacteria such as fecal streptococci and enterobacteria can be isolated from all sites of the GIT within a few hours of hatching. The number of bacteria increased first in the ceca and then throughout the GIT within the first 24 hours after hatching. (Barnes et al. 1972) Bacterial densities in the small intestine and the ceca will reach 10^8 and 10^{11} cfu/g of digesta respectively (Barnes 1972). During the period of 2 to 6 weeks after hatching, lactobacilli were the only group of microbes with numbers greater than 10^4 cfu/g of digesta from the small intestine (Barnes et al. 1972). Clostridia were found in the small intestine ranging from 10^2 to 10^4 cfu/g. *Clostridium perfringens* were isolated from only 15.7% of broiler chickens (Barnes et al. 1972). In the ceca, streptococci, lactobacilli, and coli-aerogenes bacteria ranged between 10^5 and 10^8 cfu/g, but the level of each type decreased by 6.5 weeks of age. (Barnes 1972). Using DNA technology, Amit-Romach et al. (2004) found that lactobacilli was dominant in the ceca of young chicks, with bifidobacteria becoming more dominant when chickens get older.

Salmonella, *Campylobacter* and *E. coli* were also found in the ceca. *Clostridium* was found in small intestine (Amit-Romach et al. 2004).

2.2.2 THE EFFECT OF DIET ON INTESTINAL MICROBIOTA

The type and number of bacteria that participate in the composition of the microbiota within the GIT have an important relationship with animal health, well-being and productivity (Stevens and Hume 1998). Therefore, the maintenance of a healthy microbiological environment is important. Dietary manipulation has been shown to influence microbiota in the GIT (Barnes 1972).

Key components in the diet, such as proteins, fats, and carbohydrates and other growth-promoting supplements can have a significant effect on number and type of bacteria in the GIT of chickens (Barnes 1972). An example of the impact of dietary protein on broiler GIT microbiota was reported by Drew et al. (2004). Higher crude protein content, protein from animal products and higher methionine and glycine in broiler diets have been shown to stimulate the growth of *Clostridium perfringens* present in the ileum and ceca of broiler chickens compared to birds fed diets with lower crude protein, plant-based protein, and lower methionine and glycine content (Drew et al. 2004). Studies on the impact of manipulating the carbohydrates in broiler diets include work of Oyofo et al. (1989). They found that providing lactose and mannose in drinking water significantly reduced *Salmonella* typhimurium colonization in the intestine of broiler chickens. Corrier et al. (1990) also investigated the influence of carbohydrates reporting that dietary lactose reduced the cecal pH, increased the concentration of bacteriostatic acetic and propionic acids, and reduced the growth of *salmonella*. Others have investigated the impact of

supplementation of broiler diets with essential oils such as thymol, carvacrol, eugenol, curcumin, and piperin. Mitsch et al. (2004) found that inclusion of these essential oils in broiler diets inhibited the proliferation of *Clostridium perfringens* in the intestine and reduced the incidence of NE.

2.2.3 THE EFFECT OF ENVIRONMENT ON INTESTINAL MICROBIOTA

2.2.3.1 TEMPERATURE

The environment that birds are reared in can have a significant impact on the intestinal microbiota such as *E. coli* of broiler chickens (Maurer et al. 1998). Lymphoid organs including bursa, spleen and thymus are influenced by heat stress. Heat stress compromises function of certain organs and changes circulating levels of hormones, glucose, leukocytes and electrolytes (Abidin and Khatoon 2013). Al-Fataftah and Abu-Dieyeh (2007) evaluated the impact of elevated temperatures on broiler growth performance. For 4-8 weeks after hatch, broiler chickens reared at 25 C had better growth performance than those reared at 30 C and 35 C. Ain Baziz et al. (1996) conducted a similar study and found that the body weight of broilers reared at 32 C was 47% lower than birds reared at 25 C. Low temperature has a negative impact on growth performance of broiler chickens. Blahova et al. (2007) cold stressed 22-day-old birds with a temperature ranging from 4 to 13 C and found an increase in haematocrit and haemoglobin content and an increased heart and liver weight. The workload on the circulatory system was increased due to cold temperature conditions. The metabolism, feed intake and growth of broilers are influenced by environmental temperature outside of the optimum range. The efficiency of these fast growing birds is best for only a narrow

range of temperature (18-21 C over 4 weeks of age). Environmental temperatures that are too high or too low significantly impact the economy of broiler production as well as animal welfare (Blahova et al. 2007).

2.2.3.2 LITTER

In addition to the temperature, the quality of poultry litter is important to bird health. The quality of litter material under the feet of broilers is influenced by the absorbency of material used and the management of the moisture levels in the environment. Wet litter can reduce the growth rate of broiler chickens and lead to ulcerative dermatitis of the foot pad (Martland 1985). Litter can be a vehicle for harboring and transmitting harmful bacteria, which can be a concern for chicken production (Martin and MacCann 1998). Lu et al. (2003) reported litter samples from broiler flocks (age range from a few days to 42-day-old) with aerobic bacteria at levels of 10^9 cfu/g, with the majority being Gram-positive. The same study found that bacteria associated with human and poultry diseases are found in poultry litter. These include clostridia, staphylococci, and *Bordetella* spp. To minimize the impact of bacterial infection from litter material on broilers thorough cleaning and disinfection of production facilities and the use of clean litter are often a part of good management practices (Cardona and Kuney 2002). *Campylobacter jejuni*, an infectious bacterium for broiler chickens, can be transmitted to pathogen-free chicks when exposed to chicken litter from previous flocks (Montrose et al. 1984). There are also examples of infection of broilers with *Salmonella* typhimurium when grown on litter from a previous flock (Weinack et al. 1985). These studies would indicate that starting broiler chicks on previously used litter may provide a challenge to these birds as some bacteria that reside in this material could gain an early foothold in the GIT of these young

birds. This is an example of a practical situation or test for dietary manipulation intended to manage bacteria since in some parts of the world litter is reused a number of times in broiler production.

2.2.4 METHODS FOR ENUMERATION OF INTESTINAL MICROBIOTA

2.2.4.1 CONVENTIONAL CULTURE METHOD FOR ENUMERATION

The use of agar for quantitative determination of bacteria was first introduced by Koch in 1880 (Gilchrist et al. 1973). In 1916, the proper range for the counting of bacteria colonies (30-400) on agar filled petri dishes was determined. Any petri dish with a count outside this range is considered unreliable because it is shown to differ frequently for the same sample (Breed and Dotterer 1916). For the pour plate method, an unknown sample is diluted and mixed with buffer and poured into a petri dish along with liquid agar and mixed well prior to solidification. After incubation for 18-24 hours, bacteria, such as *E.coli* will grow on the plate. Only those plates with 30-300 colonies (depends on bacteria species) are counted, those beyond that range are discarded. This procedure is simple and relatively precise for bacteria enumeration (Gilchrist et al. 1973). However, this method can be time-consuming and labor intensive. This method inadvertently selects for some organisms while excluding others which imposes *a priori* bias (Amit-Romach et al. 2004). Modern approaches have overcome these difficulties by determining the sequencing of bacterial genetic material extracted from the community samples (Lan et al. 2005).

2.2.4.2 SEQUENCING OF BACTERIAL GENETIC MATERIAL FOR BACTERIAL ENUMERATION

The development of polymerase chain reaction (PCR) techniques allows for specific and rapid detection of microbiota in samples from the GIT of birds. The use of the 16S ribosomal RNA (rRNA) gene is important to determine phylogenetic relationships among bacteria. The rRNA and DNA based techniques can be used to identify different bacterial populations in chicken intestines without culturing (Amit-Romach et al. 2004).

The techniques of DNA sequencing has now become widely used by biologists in many fields including molecular cloning, breeding, as well as finding pathogenic genes. In the past 30 years, many DNA sequencing techniques have been developed. The ideal techniques should be fast, simple to perform, accurate and low cost (Liu et al. 2012). Since the early 1990's, the Sanger method has been widely used and is considered as first generation sequencing. This method significantly contributed to the completion of the human genome project. As new strategies for DNA sequencing developed, the cost of conventional DNA sequencing became lower and a wider range of biological phenomena were assessed (Shendure and Ji 2008). The alternative, next generation sequencing (NGS) replaced the Sanger method. Based on the combination of template preparation, sequencing and imaging, and genome alignment and assembly methods, NGS has application to a variety of biological and clinical practices with a much lower price (Metzker 2010).

Roche/454 technology, one of NGS methods, is derived from the combination of pyrosequencing and emulsion PCR techniques (Voelkerding et al. 2009). In this method, genomic DNA is mechanically cut into fragments of several hundred base pairs. Each of these fragments attach to one microbead. Then they are captured in droplets of an emulsion. The template amplification is performed on those droplets. Then, the microbeads are moved to a fiber-optic slide, where four DNA nucleotides: adenine (A), thymine (T), cytosine (C) and guanine (G) are added into a light signal via the firefly enzyme luciferase (Wicker et al. 2006). The other side of the fiber-optic slide face a charge-coupled device camera. Light from the DNA strand is detected by the camera. The light signal provides the identities of nucleotides that were incorporated within the original DNA strand to determine the sequence (Ansorge 2009). However, due to the large read numbers in 454 sequencing, there is noise from true sequence diversity in the data. The development of AmpliconNoise (PyroNoise algorithm) effectively removes 454 sequencing errors and PCR single base errors (Quince et al. 2011).

Genetic sequencing is a fast developing field. There are some new NGS systems including Illumina, HiSeq and Miseq. These new techniques significantly decrease the expense and shorten the time consumed compared to the previous techniques (Caporaso et al. 2012).

2.2.5 MICROBIOTA SPECIES OF INTEREST

Lactobacillus and *Bifidobacterium* are the two major beneficial genus of bacteria in the GIT of birds (Fooks and Gibson 2002). The problematic bacteria of current concern for

the poultry industry are *Salmonella*, *Clostridium*, *Camplylobacter* and *Escherichia coli* (*E. coli*) (Flickinger et al. 2003).

2.2.5.1 *LACTOBACILLUS*

Lactobacillus is a genus of common Gram-positive rod bacteria which can be either anaerobic or facultative anaerobic (Land et al. 2005). They are beneficial for animals because the colonization of *Lactobacillus* on the GIT inhibits the growth and colonization of many toxin-producing bacteria (Jin et al. 1996). The mechanisms of inhibition include creating a low pH environment, competing for nutrients and the binding sites with pathogenic bacteria (Raja et al. 2009) *In vitro*, *Lactobacillus* inhibits many intestinal bacteria, such as *Salmonella* typhimurium, *Staphylococcus aureus*, *E. coli*, *Clostridium perfringens* and *Clostridium difficile* (Meurman et al. 1995; Silva et al. 1987). *In vivo*, *Lactobacillus* mainly found in the crop and small and large intestine. The small intestine has the largest. An *in vitro* experiment on fragments of crop, large and small intestines showed that *Lactobacillus* effectively inhibited the adhesion of *Salmonella* pullorum, *Salmonella* enteritidis and *Salmonella* gallinarum to specific binding site on epithelia (Gusils et al. 1999). Providing *Lactobacillus* in broiler diets improves body weights and feed conversion ratio (FCR), and reduces coliforms in the cecum of broilers (Jin et al. 1998a). Most *Lactobacillus* strains isolated from the chicken intestine are resistant to bile salts and acids. Therefore, most *Lactobacillus* in the diet will not be interfered by the digestive fluid, and reach the lower GIT of broiler chickens as viable bacteria (Jin et al. 1998b).

2.2.5.2 *BIFIDOBACTERIUM*

Bifidobacterium are obligately anaerobic Gram-positive rod-shaped bacteria (Tomotari 1990). *Bifidobacterium* are regarded as one of the most important organisms related to human and animal health (Russell et al. 2011). In young rats, *Bifidobacterium* promoted maturation of the immune system and enhanced it by stimulating development of T-cells and antibody synthesis by peripheral blood mononuclear cells (Dong et al. 2010). Estrada et al. (2001) administered *Bifidobacerium bifidum* in the drinking water of broiler chickens and found a decrease of total aerobic bacteria and coliforms. Furthermore, the incidence of cellulitis was significantly reduced at slaughter. Feeding chickens kestoses oligosaccharides altered the intestinal microbiota by increasing the population of *Bifidobacterium*, so the intestinal health and digestion performance was strengthened (Patterson et al. 1997).

2.2.5.3 *SALMONELLA*

Salmonella belong to the family *Enterobacteriaceae*. It is a Gram-negative non-spore forming bacilli (Dunkley et al. 2009) that is the major cause and concern of bacterial enteric problems in humans and animals (Brenner et al. 2000). Food poisoning by *Salmonella* in humans can be attributed to poultry. Eggs and their by-products contaminated by *Salmonella* in humans are the major source of infection (Rabsch et al. 2001). *Salmonella* from ovaries, oviducts and eggs can infect newborn chicks, because those organisms can be isolated from those tissues and egg contents (Suzuki 1994). The infection with *salmonella* among chicken flocks induces general malaise, inflammation and damage of intestinal villi, and diarrhea which lead to anorexia and dehydration, and

increases the mortality rate (Barrow et al. 1987). The agricultural use of antibiotics has resulted in the mutation of *Salmonella* to drug resistant forms (Rabsch et al. 2001).

2.2.5.4 *CLOSTRIDIUM PERFRINGENS*

Clostridium perfringens is a Gram-positive anaerobic spore-forming rod-shaped bacterium (McDonel 1980). It can be found in the intestine of animals and humans and in the environment. In humans, the pathogen can cause gangrene and gastrointestinal disease, such as NE. Diseases caused by *Clostridium perfringens* more frequently occur in animals. It does not invade healthy cells in the intestine but produces various types of toxins and enzymes which cause lesions and other symptoms such as diarrhea, impaired digestive function, reduced nutrient absorption and decreased feed intake. The type of toxin depends on the *Clostridium perfringens* strains which are involved. Each type of toxin leads to a specific syndrome (Petit et al. 1999). There are five toxinotypes (A, B, C, D and E) of *Clostridium perfringens* strains that can produce four major toxins including α, β, ε and ι. Among them, toxin type A is the most common toxinotype in the environment. The toxin produces a significant impact on human and animal health (Petit et al. 1999; Van Immerseel et al. 2004).

NE is a disease mainly caused by *Clostridium perfringens*, type A and C (Van Immerseel et al. 2004) in broiler chickens. *Eimeria acervulina* and *Eimeria necatrix* are associated with NE as well (Al-Sheikhly and Al-Saieg 1979). It is described as one of the world's most prominent and severe poultry diseases. Choct and Kocher (2008) reported that the cost to the worldwide poultry industry from NE is estimated over two billion US dollars per year. In the acute clinical form, NE can cause high mortality rates of up to 50%. In

the subclinical form, intestinal mucosa is damaged by *Clostridium perfringens*, leading to decreased digestion, absorption, weight gain and poorer feed conversion ratio (Van Immerseel et al. 2004; Geier et al. 2010). NE usually occurs 2-6 weeks after hatching. In healthy broilers, the number of *Clostridium perfringens* in the small intestine is about 10^4 cfu g^{-1} of digesta. When NE occurs, the number of this bacterium reaches up to 10^7-10^9 cfu g^{-1} of digesta (Dahiya et al. 2006).

2.2.5.5 *E. COLI*

E. coli is a Gram-negative facultative anaerobic bacterium commonly found in the intestine of humans and animals. Most strains are harmless, but some types such as extraintestinal pathogenic *E. coli*, are disease causing. These types of *E. coli* can be transferred from birds to humans through consumption of contaminated chicken (Ron 2006; Center for Disease Control and Prevention 2011). In broiler chickens, *E. coli* can cause diseases including airsacculitis polyserositis, septicemia and intestinal diseases when birds are reared in a sub-optimal environment (Dho-Moulin and Fairbrother 1990). Oral administration of pathogenic *E. coli* and stress resulted in bacteremia and death of chickens (Leitner and Heller 1991). However, there was no impact on young chicks. When they were challenged later in life (after 12 days old), those birds became more resistant to *E.coli*. The early life infection might stimulate the immune system to produce antibodies (Leitner and Heller 1991). A lytic bacteriophage has the potential to prevent *E. coli* infection and the associated diseases in chickens (Barrow et al. 1998).

2.2.5.6 OTHER SPECIES OF INTEREST

The type and number of bacteria in the GIT of chickens is variable and large. In addition to those widely known bacteria already distributed, there are others that significantly impact the health and well-being of birds or humans (Allen and Fetterer 2002; Blaser et al. 1983). When *Campylobacter* infects chicken intestines, there are no symptoms of illness. However, these bacteria easily contaminate the broiler carcasses at slaughter. Humans who consume these products can become ill (Blaser et al. 1983). The supplementation of probiotics have been shown to help reduce *Campylobacter* contamination (Ghareeb et al. 2013).

Other than bacteria, some parasites also impact poultry health. Coccidiosis is a common disease in poultry that causes significant economic loss in production annually. *Eimeria* spp. are protozoans that lead to lesions of poultry GIT, and reduce feed efficiency (Allen and Fetterer 2002). The presence of coccidia increases susceptibility of the birds to NE when sufficient *Clostridium perfringens*, type A are in the intestine (Al-Sheikhly and Al-Saieg 1979). Vaccines for coccida based on live strains of this protozoan and recombinant-DNA are used for effective control of coccidiosis (Vermeulen et al. 2001).

2.3 ANTIBIOTICS

2.3.1 THE HISTORY OF ANTIBIOTIC USE IN ANIMAL AGRICULTURE

Antibiotics have been used as growth promoters in animal agriculture for more than 50 years. The benefit of inclusion of antibiotics in broiler chicken diets was first reported by Moore et al. (1946). Since then, antibiotics have been widely used in animal agriculture,

and have significantly increased animal growth performance (Gordon and Taylor 1954; Dibner and Richards 2005). Coates et al. (1955) showed that procaine penicillin accelerated the growth of broilers, while the weight and length of the small intestine were reduced. Even though antibiotics have been used by growers as effective supplements, regulatory agencies such as the World Health Organization and the Centers for Disease Control and Prevention (CDC) argue that the sub-therapeutic use of antibiotics should be restricted (Roe and Pillai 2003). In June of 1999, the European Union banned the use of four growth promoting antibiotics in poultry diets, of which, zinc bacitracin and virginiamycin were two (Hofacre et al. 2003). The reason for this ban is that potential human pathogens found on processed poultry carcasses have become increasingly resistant to certain antibiotics (Hofacre et al. 2003). Therefore, studies investigating alternatives to antibiotics which are abundant have become necessary.

2.3.2 FUNCTION OF ANTIBIOTICS

Antibiotics can be used as growth promoters for broiler chickens because they inhibit the growth or contribute to cell death of both Gram-positive and Gram-negative bacteria in the GIT (Kohanski et al. 2007). Antibiotics are classified into one of two categories: bactericidal drugs and bacteriostatic drugs. Bactericidal drugs kill bacteria with high efficiency while bacteriostatic drugs only inhibit the growth of bacteria (Pankey and Sabath 2004). The major functions of antibacterial drugs on bacteria are inhibition of DNA replication and repair, inhibition of protein synthesis and reduced cell wall turnover (Walsh 2000). For example, bacitracins can indirectly interfere with the biosynthesis of bacterial cell walls (Pollock et al. 1994). Virginiamycin target the ribosomes of bacteria,

and inhibits cell division. Gram-negative bacteria were not as sensitive as Gram-negative to virginiamycin in a study by Cocito (1979).

2.3.3 ANTIBIOTIC RESISTANCE

Antibiotic resistance has developed in some bacteria as a result of chromosomal changes and exchange of genetic material between bacteria (Neu 1992). Many disease-causing bacteria, including *Streptococcus pneumonia*, *Streptococcus pyogenes* and staphylococci have become resistant to antibiotics used in animal agriculture (Neu 1992). Those antibiotic-resistant bacteria arising within the agricultural industry can impact humans. During animal processing and slaughter, bacteria such as *Salmonella*, *Campylobacter* and *E. coli* are pathogens harbored in animal carcasses that can be a potential concern to human health (Wegener 2003). Antibiotic-resistant bacteria have been isolated from animal products such as poultry products and ham (Robredo et al. 2000; Gambarotto et al. 2001). The use of growth promoters in animal feed lead to a reservoir of antibiotic-resistant bacteria, with the problem of spreading to humans by food and animal contact (Wegener 2003). Animals excrete antibiotic-resistant bacteria via feces, which can be used on vegetable crops as fertilizer or for irrigation. If people become ill from these resistant bacteria, options for treatment are reduced (Khachatourians 1998).

2.3.4 ALTERNATIVES TO ANTIBIOTICS

Due to pressure from both consumer groups and government policy of some countries, it is important to find alternatives to antibiotics (United States Department of Agriculture 2012). Many studies have been performed to test possible alternative supplements that have similar beneficial effects on poultry health and growth performance (Edens 2003;

Patterson and Burkholder 2003; Guo et al. 2004). Other studies include management approaches to reduce bacterial infection instead of using antibiotics in the feed. Studies listed in the following sections are important to the sustainability of the poultry industry as well as human health.

2.3.4.1 ORGANIC ACIDS

Dietary organic acids have been the focus of numerous studies to provide the broiler chicken industry an option to replace the use of antibiotics in poultry feed. Organic acids are not classified as antibiotics, however, the supplementation of organic acids in broiler diets has been reported to function in a similar manner to antibiotics when fed to broilers (Abdel-Fattah et al. 2008). Organic acids maintain a healthy GIT environment and control pathogenic bacteria and intestinal diseases (Abdel-Fattah et al. 2008). Many studies found the use of organic acids in the diet or drinking water of broilers reduced problematic bacteria and improved growth performance, including body weight gain and feed conversion (Paul et al. 2007; Patten and Waldroup. 1988; Chaveerach et al. 2004; Veeramani et al. 2003). The undissociated form of organic acids can penetrate bacterial cell membranes, and reduce the pH of bacterial cytoplasm by production of H^+ ions (Nursey 1997). Organic acids can trigger the reduction of GIT pH, and increase the activities of pepsin in the bird GIT. The acid environment also increases the solubility of phytase in feed, which breaks down phytate. Less phytate will enter the small intestine and the formation of protein-mineral-phytate complexes will be reduced (Afsharmanesh and Pourreza 2005). The formation of protein-phytate complexes reduces the available endogenous amino acids (Selle et al. 2000). The combination of organic acids and

probiotics is more effective than individual supplements alone for reducing bacteria (Patten and Waldroup 1988; Gunal et al. 2006).

2.3.4.2 PROBIOTICS

The word "probiotics" was first introduced by Lilly and Stillwell (1965). This term was meant to describe the fact that some organisms produce substances that benefit other species. It was later described as organisms or substances that when supplemented into animal diets provide benefits to the host by influencing intestinal microbiota (Lilly and Stillwell 1965). However, it was not precise because this definition can include antibiotics. Since probiotics are not antibiotics, Fuller (1989) defined probiotics as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". A more recent definition was 'live microorganisms, which when consumed in adequate amounts, confer a health effect on the host' (Guarner and Schaafsma, 1998). In the diets of livestock, the most prevalent organisms used as probiotics are *Enterococcus*, *Saccharomyces* yeast and spore-forming *Bacillus* (Simon et al. 2001). For human consumption, *Lactobacillus* is a very common genus used as probiotics (Simon et al. 2001). The main functions of probiotics include suppressing pathogenic bacteria, altering microbial metabolism (enzyme activities) and improving the response of the immune system (Fuller 1989). Some studies found probiotics used as an alternative to antibiotics improved the growth of broiler chickens in a similar fashion to antibiotics (Owings et al. 1989; Fritts et al. 2000).

There are potential problems associated with feeding probiotics. Probiotics are live organisms, so they have to attach to the intestinal epithelium to be effective growth

promoters. If animals are stressed, the conditions in the intestine could alter the opportunity for probiotics to thrive (Gibson and Roberfroid 1995). Furthermore, successful competition with other organisms is important for successful growth promotion, which becomes difficult when the competing organism is occupying attachment sites on the intestinal epithelium (Gibson and Roberfroid 1995). When birds are under stress, probiotics can effectively eliminate pathogenic bacteria in the GIT and improve growth performance. However, if birds are in an environment that provides optimal growing conditions, the beneficial effects of probiotics could be minimized and be difficult to detect (Patterson and Burkholder 2003).

2.3.4.3 PREBIOTICS

The concept of prebiotics was defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria species in the colon, and thus improves host health" (Gibson and Roberfroid 1995). Food ingredients that are classified as prebiotics have to be resistant to absorption by the GIT, be fermented by intestinal organisms, and selectively stimulate beneficial bacteria associated with animal health (Roberfroid 2007). Only inulin and trans-galactooligosaccharides are currently recognized as meeting all these criteria (Roberfroid 2007). However, many other ingredients such as kestoses, lactulose, fructooligosaccharides and galactooligosaccharides have been found to have prebiotic effects on GIT performance in broiler chickens (Collins and Gibson 1999; Xu et al. 2003). Prebiotics can increase digestion of nutrients in the feed by increasing the length and width of intestinal villi (Sinovec and Markovic 2005). Tasco® which is a product made from sun-dried brown seaweed has been reported to have prebiotic effects

when added to chicken diets. Wiseman (2012) demonstrated that feeding this product improves intestinal microstructure in broilers.

2.3.4.4 OTHER ALTERNATIVES TO ANTIBIOTICS

The extracts from some Chinese herbs including Tin Men Chu, Sey Lau Pai have been reported to inhibit the growth of food-borne bacteria such as *E. coli* (Chung et al. 1990), and improve the growth of broiler chickens (Guo et al. 2004). Other phyto-additives including garlic, oregano and essential oil (thymol and carvacrol) have been shown to have the potential to replace antibiotics in poultry diets (Windisch et al 2008; Mitsch et al. 2004).

There are management approaches for controlling intestinal microbiota that do not involve feed supplements. For example, the growth performance of broiler chickens has been reported to be related to the frequency of sanitizing water lines providing drinking water for the birds (Tablante et al. 2002). Improved biosecurity leads to reduce bacterial infections in broilers (Bojesen et al. 2010). Acidification of wood shavings with formic acid and propionic acid used as bedding has been reported to decreases the numbers of *Clostridium perfringens* and *Enterococcus* spp. in the GIT of broiler chickens without a negative impact on performance (Garrido et al. 2004).

2.4 LYSOZYME

In 1922, lysozyme was discovered by Alexander Fleming (Fleming 1922). Lysozymes are common antimicrobial enzymes which are widespread in many animal tissues and secretions but are mainly obtained commercially from avian egg white (Grossowicz and

Ariel 1983). The primary structure of egg white lysozyme is a single polypeptide chain with 129 amino acids. It is not a straight chain due to four pairs of cysteine (Position 6 and 127, 30 and 115, 64 and 80, and 76 and 94) that form disulfide bridges with each other (Proctor et al. 1988).

2.4.1 FUNCTION OF LYSOZYME

Lysozyme is defined as 1,4-β-N-acetylmuramidase. It cleaves the glycosidic bond between the N-acetylmuramic acid and N-acetylglucosamine in bacterial peptidoglycan which is an important component of cell walls (Phillips 1966), thus it provides protection against bacterial infection.

As part of the body defense mechanisms of animals, lysozymes are associated with the monocyte-macrophage system and immunoglobulins (Osserman 1976). Lysozymes play a role as antibacterial agents which are mediated through direct bacteriolytic action or stimulate macrophage phagocytic function (Biggar and Sturgess 1977; Thacore and Willett 1966). Until about 7 days before hatching, the egg and developing chicken embryo do not produce immunoglobulins. Therefore, the egg white contains a high level of lysozyme that provides immune function during this period (Jolles and Jolles 1984).

Lysozyme in the gut of many insects is related to the digestion of microorganisms (Imoto 2009). For invertebrates which do not produce immunoglobulins, lysozymes serve as a rudimentary protective system to destroy the bacterial cell wall (Jolles and Jolles 1984).

Common uses of lysozyme include incorporation in cheese, wine and pharmaceutical products (Neova Technologies Ltd. 2012). In cheese production, lysozyme prevents the growth of *Clostridium tyrobutyricum* which causes cracks and slits in the cheese (Wasserfall and Teuber 1979). Lysozyme lyses the vegetative form of this bacterium through enzymatic cleavage of the cell wall (Wasserfall and Teuber 1979). In the wine industry, lysozyme is used to prevent a wide range of Gram-positive bacteria. Lysozyme reduces the number of lactic acid bacteria and inhibits malolactic fermentation. It also increases amine levels including histamine, tyramine and putrescine in wine (Gerbaux et al. 1997). For pharmaceutical applications, lysozyme has been used as a drug or feed additive to cure periodontitis, prevent tooth decay, and fight against infective bacteria to improve immune response of the host (Proctor et al. 1988; Imoto 2009). Furthermore, lysozyme has been used in human infant formula to simulate human milk (Proctor et al. 1988). Lysozyme can prolong the shelf-life of food including fruit, vegetables and meat by reducing the harmful organisms which cause decomposition (Proctor et al. 1988). Some types of lysozyme have the ability to dissolve blood clots (Imoto 2009). Additional applications for lysozyme have yet to be determined (Imoto 2009).

2.4.2 APPLICATION OF LYSOZYME IN POULTRY AGRICULTURE

Studies regarding the use of lysozyme as an alternative to antibiotics for poultry are relatively limited. There are several studies that have been conducted to evaluate the benefit of inclusion of lysozyme in broiler diets to reduce the incidence of intestinal disease. Initially Zhang et al. (2006) conducted an *in vitro* experiment to find an effective dose of lysozyme for controlling *Clostridium perfringens.* They found that 200 μg/ml not

only completely inhibited the growth of *Clostridium perfringens*, but also inhibited production of α-toxin which caused the lesions associated with NE. A follow up study by Zhang et al. (2010) evaluated a radiant energy-treated lysozyme antimicrobial blend to control NE in broiler chickens. This blend at a level of 200 ppm was as effective as commonly used antibiotics (bacitracin methylene disalicylate (BMD)) for treatment of NE in the broiler chickens. Liu et al. (2010) found 40 ppm exogenous lysozyme effectively reduced the number of *Clostridium perfringens* in the ileum of broilers and prevented intestinal lesions when they were gavaged with *Clostridium perfringens*. Humphrey et al. (2002) used 10% of transgenic rice that expresses lysozyme and 5% of transgenic rice that expresses lactoferrin as a source of lysozyme and lactoferrin as a substitute for antibiotics in broiler diets. They reported that feed efficiency was improved and the birds had increased villi height in the small intestine compared to birds fed conventional rice. Sotirov and Koinarski (2003) showed that lysozyme can effectively help broiler chickens cope with *Eimeria* infection so that coccidiosis is prevented.

MacIsaac and Anderson (2008) conducted an experiment that showed a potential response to dietary lysozyme with increased turkey body weights. Turkeys fed diets with lysozyme or the combination of lysozyme and antibiotic had heavier body weights at 55 days of age compared to those fed diets without lysozyme or with antibiotic alone. Additionally, results showed that the feeding lysozyme in combination with BMD decreased the mortality rate (from 14.1% to 4.7%) of poults with lower initial body weights during the first two weeks of growth. Gillcrist (2012) found that the inclusion of

lysozyme and Oasis® (a hydrated hatchling feed supplement) in the transport containers during 24 hours of transport of female turkey poults increased day 55 and 69 body weight.

There are limited results published from studies focused on the effect of different levels of lysozyme on broiler chickens. Typically these studies look at providing lysozyme from one day of age to market age. There are no publications addressing the possibility that there may be specific critical periods of the growth cycle for which lysozyme may have the greatest impact on intestinal microbial populations and broiler growth performance.

2.5 SUMMARY

Alternatives to growth promoting antibiotics have been one of the priority areas for research identified by the Canadian Poultry Research Council and Chicken Farmers of Canada. Assessment of production performance of broiler chickens fed antibiotic alternatives requires controlled scientific studies. There is potential to use lysozyme extracted from egg white in poultry diets to effectively reduce the use of antibiotics and improve the intestinal microbiota and broiler growth performance. The supplementation of broiler diets with lysozyme will provide the poultry industry with poultry products that could be marketed as grown without antibiotics or raised “antibiotic free”. Knowledge regarding the most appropriate time period during the broiler growth cycle to provide lysozyme for the greatest effect may reduce the use of lysozyme and subsequently minimize the expense in broiler production.

CHAPTER 3 THE EFFECT OF LYSOZYME FOR BROILER CHICKENS GROWN UNDER TWO TEMPERATURE CONDITIONS

3.1 ABSTRACT

Antibiotics have been used in poultry feed for many years. However, pathogens have become increasingly resistant to antibiotics. Lysozyme could be a potential alternative to antibiotics. This trial was conducted to investigate inclusion of lysozyme levels (0, 50, 100 and 200 ppm) on growth performance and intestinal microbiota of broiler chickens subjected to heat stress. Birds fed 50 ppm lysozyme had heavier body weight (1709 g) ($P<0.05$) than the birds (1574 g) fed 200 ppm lysozyme. Birds fed 200 ppm lysozyme had the poorest FCR among all the treatments (Control, 50 ppm, 100 ppm and 200ppm are 2.22, 2.25, 2.25 and 2.54, respectively) ($P<0.05$). Heat stress reduced body weight, body weight gain, feed consumption, and reduced the population of total anaerobic bacteria and *Clostridium perfringens* in ileal samples from the birds. The use the lysozyme at the levels evaluated did not improve growth performance or changes in intestinal levels of *E. coli* or *Clostridium perfringens*.

Key words: antibiotics, lysozyme, heat stress, growth performance, intestinal microbiota.

3.2 INTRODUCTION

The use of antibiotics as a feed ingredient has become a concern to poultry industry and human health due to the development of bacterial resistance (Hofacre et al. 2003). Lysozyme, an enzyme with antimicrobial properties, could serve as an alternative to antibiotics. Lysozyme cleaves the glycosidic bond between the N-acetylmuramic acid

and N-acetylglucosamine in bacterial peptidoglycan (Phillips 1966). *In vitro,* 200 ppm of lysozyme was found to inhibit *Clostridium perfringens* (Zhang et al. 2006). Some *in vivo* studies with lysozyme were reported to positively improve the intestinal microbiota of broilers (Zhang et al. 2010; Liu et al. 2010). There is no published research on the effect of different levels of lysozyme on the combination of growth performance and intestinal microbiota of broilers. Often in studies evaluating potential alternatives to antibiotics it is important to provide environmental stress to the birds otherwise there is risk that bird performance will not be compromised in the absence of antibiotics. Heat stress compromises immune function of broilers (Abidin and Khatoon 2013) resulting in birds that are vulnerable to bacterial infections.

3.3 OBJECTIVE

To determine the effects of dietary inclusion levels of lysozyme on the growth performance and intestinal microbiota of broiler chickens grown in optimal or sub-optimal temperature conditions.

3.4 HYPOTHESES

Growth performance of the broiler chickens fed diets with lysozyme will improve compared to birds fed a control diet. Lysozyme can effectively modify the microbial population in the small intestine resulting in a decrease in *Clostridium perfringens,* detrimental *E. coli* and total coliforms at optimal or sub-optimal temperature conditions.

3.5 MATERIALS AND METHODS

3.5.1 ANIMAL HOUSING AND HUSBANDRY

Three hundred and eighty four male Ross 308 broiler chickens from Clark's Hatchery (Burtts Corner, NB) were used. Birds arrived at the Atlantic Poultry Research Centre in Truro, NS on the day of hatch, and were immediately randomly assigned to forty eight, 60 cm x 48 cm, cages within two of the controlled environmental rooms (CER). There are eight birds in one cage with the stocking density of 1.4 kg/m^2 on day 0 and 34.2 kg/m^2 on day 35. Upon arrival, the chicks were immediately provided with feed in troughs and water from nipple drinkers. Birds were introduced to water by dipping beaks in the nipple drinkers. Feed and water were supplied *ad libitum* throughout the trial. From Day 1 to 14, ambient temperature in the two rooms was decreased from 32 C to 26 C. From Day 14 to 21, ambient temperature in the room with optimal conditions continued to decrease to reach 23 C. The room with suboptimal conditions remained at 26 C for this period. From Day 22 to slaughter, the temperature in the room with optimal conditions decreased to 21 C. Fluctuating daily temperature was applied to the room with suboptimal conditions. Ambient temperature was set at 26 C for 12 hr then increased to 33 C for 12 hr. The temperature change was programed to take 1 hr to complete the 7 C change (Figure 3.1). Lighting was set according to standard operating procedure (Appendix A) Ambient temperature and relative humidity (RH) were recorded by data logger (Appendix B-1 and C-1). All procedures were carried out under the guidance of Canadian Council on Animal Care (CCAC 2009).

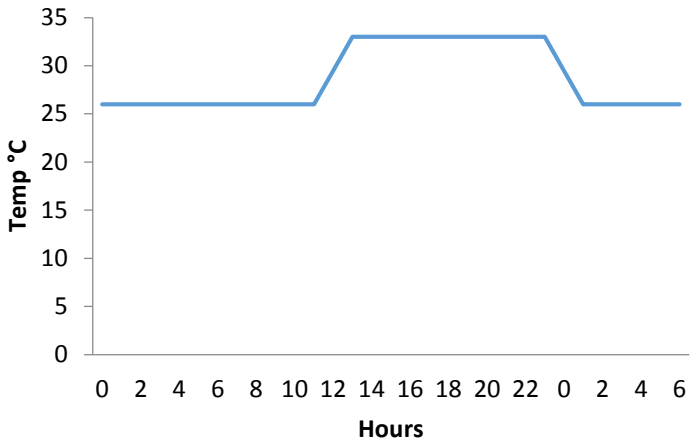

Figure 3.1 The daily 24 hours temperature schedule from day 22 to the end of the trial (day 35) for the room with sub optimal temperature.

3.5.2 DIETS

Diets were isocaloric and isonitrogenous within the starter (days 0-14) (Table 3.1), grower (days 15-22) (Table 3.2) and finisher (days 23-35) phases (Table 3.3). Diets were formulated to meet or exceed the National Research Council (1994) nutrient requirements for each stage. The diets were fed in mash form throughout the trial. Four test diets included the control diet and three diets containing lysozyme at levels of 50 ppm, 100 ppm or 200 ppm. Lysozyme, extracted from hen egg white, was provided by Neova Technologies Inc. Abbotsford, British Columbia. It was white powder with an enzymatic activity of 24,000 units/mg (Shugar 1952), and mixed with ethylenediaminetetraacetic acid (EDTA) at a ratio of 20:80. In each room, each diet was randomly assigned to six cages of broilers. Feed and water were provided *ad libitum*. Feed was weighed into the feeders as needed.

Table 3.1 Composition of experimental starter diets containing lysozyme fed from 1-14 days of age.

	Level of lysozyme (%)			
	0	0.005	0.01	0.02
Ingredients (%)				
Corn	44.51	44.50	44.48	44.46
Soybean meal	38.73	38.73	38.74	38.74
Wheat	10.00	10.00	10.00	10.00
Tallow blend	3.20	3.20	3.21	3.22
Limestone	1.65	1.65	1.65	1.65
Dicalcium phosphate	0.59	0.59	0.59	0.59
Iodized salt	0.43	0.43	0.43	0.43
Methionine premix[z]	0.39	0.39	0.39	0.39
Lysozyme[y]	-	0.005	0.01	0.02
Vitamin/mineral premix[x]	0.50	0.50	0.50	0.50
Calculated analysis				
AME_n (kcal/kg)[w]	3050	3050	3050	3050
Protein (%)	23.00	23.00	23.00	23.00
Calcium (%)	1.00	1.00	1.00	1.00
Nonphosphate phosphorus (%)	0.45	0.45	0.45	0.45
Lysine (%)	1.38	1.38	1.38	1.38
Methionine (%)	0.58	0.58	0.58	0.58
Methionine+cystine (%)	0.95	0.95	0.95	0.95
Sodium (%)	0.19	0.19	0.19	0.19
Determined analysis				
Crude protein (%)	23.80	23.16	24.36	22.80
Total calcium (%)	0.7	0.74	0.75	0.77
Total phosphorus (%)	0.48	0.51	0.50	0.49

[z] Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middlings, 0.5 kg.

[y] Inovapure 213 (active ingredient lysozyme at 20%, 24,000 Shugar units mg^{-1}) Neova Technologies, Inc., Abbotsford, BC., Canada.

[x] Supplied per kg diet: vitamin A, 9750 IU; vitamin D_3, 2000 IU; vitamin E, 25 IU; vitamin K, 2.97 mg; riboflavin, 7.6 mg; Dl Ca-pantothenate, 13.5 mg; vitamin B_{12}, 0.023 mg; niacin, 29.7; folic acid, 4.0 mg, choline, 801 mg; biotin, 0.3 mg; pyridoxine, 4.95 mg; thiamine, 2.91 mg; manganese, 70.2 mg; zinc, 80.0 mg; copper, 25 mg; selenium, 0.15 mg; ethoxyquin, 50 mg; wheat middlings, 1432 mg; ground limestone, 500 mg.

[w] Nitrogen-corrected apparent metabolizable energy.

Table 3.2 Composition of experimental grower diets containing lysozyme fed from 15-24 days of age.

	Level of lysozyme (%)			
	0.00	0.005	0.01	0.02
Ingredients (%)				
Corn	52.12	52.11	52.11	52.10
Soybean meal	30.97	30.97	30.97	30.97
Wheat	10.00	10.00	10.00	10.00
Tallow blend	3.76	3.76	3.76	3.76
Limestone	1.59	1.59	1.59	1.59
Dicalcium phosphate	0.50	0.50	0.50	0.50
Iodized salt	0.46	0.46	0.46	0.46
Methionine premix[z]	0.40	0.40	0.40	0.40
Lysozyme[y]	-	0.005	0.01	0.02
Vitamin/mineral premix[x]	0.20	0.20	0.20	0.20
Calculated analysis				
AME_n (kcal/kg)[w]	3150	3150	3150	3150
Protein (%)	20.00	20.00	20.00	20.00
Calcium (%)	0.92	0.92	0.92	0.92
Nonphosphate phosphorus (%)	0.40	0.40	0.40	0.40
Lysine (%)	1.15	1.15	1.15	1.15
Methionine (%)	0.44	0.44	0.44	0.44
Methionine +cystine (%)	0.76	0.76	0.76	0.76
Sodium (%)	0.18	0.18	0.18	0.18
Determined analysis				
Crude protein (%)	19.24	19.22	19.25	19.77
Total calcium (%)	0.89	0.96	0.88	0.90
Total phosphorus (%)	0.48	0.49	0.48	0.48

[z] Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middlings, 0.5 kg.

[y] Inovapure 213 (active ingredient lysozyme at 20%, 24,000 Shugar units mg^{-1}) Neova Technologies, Inc., Abbotsford, BC., Canada.

[x] Supplied per kg diet: vitamin A, 9750 IU; vitamin D_3, 2000 IU; vitamin E, 25 IU; vitamin K, 2.97 mg; riboflavin, 7.6 mg; Dl Ca-pantothenate, 13.5 mg; vitamin B_{12}, 0.023 mg; niacin, 29.7; folic acid, 4.0 mg, choline, 801 mg; biotin, 0.3 mg; pyridoxine, 4.95 mg; thiamine, 2.91 mg; manganese, 70. 2 mg; zinc, 80.0 mg; copper, 25 mg; selenium, 0.15 mg; ethoxyquin, 50 mg; wheat middlings, 1432 mg; ground limestone, 500 mg.

[w] Nitrogen-corrected apparent metabolizable energy.

Table 3.3 Composition of experimental finisher diets containing lysozyme fed from 25-35 days of age.

	Level of lysozyme (%)			
	0.00	0.005	0.01	0.02
Ingredients (%)				
Corn	57.95	57.94	57.93	57.91
Soybean meal	25.66	25.66	25.66	25.66
Wheat	10.00	10.00	10.00	10.00
Tallow blend	3.33	3.33	3.33	3.34
Limestone	1.62	1.62	1.62	1.62
Dicalcium phosphate	0.50	0.50	0.50	0.50
Iodized salt	0.44	0.44	0.44	0.44
Methionine premix[z]	0.40	0.40	0.40	0.40
Lysozyme[y]	-	0.005	0.01	0.02
Vitamin/mineral premix[x]	0.11	0.11	0.11	0.11
Calculated analysis				
AME_n (kcal/kg)[w]	3200	3200	3200	3200
Protein (%)	18.00	18.00	18.00	18.00
Calcium (%)	0.90	0.90	0.90	0.90
Nonphosphate phosphorus (%)	0.38	0.38	0.38	0.38
Lysine (%)	0.99	0.99	0.99	0.99
Methionine (%)	0.37	0.37	0.37	0.37
Methionine +cystine (%)	0.65	0.65	0.65	0.65
Sodium (%)	0.18	0.18	0.18	0.18
Determined analysis				
Crude protein (%)	17.97	17.35	16.89	17.26
Total calcium (%)	0.93	0.90	0.99	1.03
Total phosphorus (%)	0.44	0.45	0.45	0.44

[z] Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middlings, 0.5 kg.
[y] Inovapure 213(active ingredient lysozyme at 20%, 24,000 Shugar units mg^{-1}) Neova Technologies, Inc.,Abbotsford, BC., Canada.
[x] Supplied per kg diet: vitamin A, 9750 IU; vitamin D_3, 2000 IU; vitamin E, 25 IU; vitamin K, 2.97 mg; riboflavin, 7.6 mg; Dl Ca-pantothenate, 13.5 mg; vitamin B_{12}, 0.023 mg; niacin, 29.7; folic acid, 4.0 mg, choline, 801 mg; biotin, 0.3 mg; pyridoxine, 4.95 mg; thiamine, 2.91 mg; manganese, 70. 2 mg; zinc, 80.0 mg; copper, 25 mg; selenium, 0.15 mg; ethoxyquin, 50 mg; wheat middlings, 1432 mg; ground limestone, 500 mg.
[w] Nitrogen-corrected apparent metabolizable energy.

3.5.3 Analysis of growth performance

Birds were mass weighed per cage on day 0, 14, 21 and 35. The feed remaining in the feeders was weighed on each weigh day and as mortality occurred. Mortality was recorded and the dead birds were sent to the veterinary pathologist for necropsy (Animal

Health Laboratory, Truro, Canada). Performance was determined by measuring body weight, body weight gain, feed consumption and FCR.

3.5.4 DIGESTA SAMPLE COLLECTION

On day 14 and 35, two birds per cage were euthanized by cervical dislocation for digesta sample collection. Approximately 15 cm of the ileum one cm anterior to the ileal cecal junction was aseptically harvested, placed in sterile plastic bags and taken to the level II microbiology laboratory. From each intestinal sample two cm from the proximal and distal ends of each ileal sample will be used for future NGS analysis (Results are not reported in this thesis). The remaining ileum was used for conventional microbial analysis. All samples were stored in a freezer at -80 C until analysis of microbiota.

3.5.5 MICROBIOTA ANALYSIS

Intestinal ileum samples were transferred to filtered stomacher bags and buffered peptone water (BPW) (Oxoid CM 0509, Basingstoke, Hampshire, England) was added in a 1:10 ratio. The samples were blended in a stomacher (Mix 2, AES Laboratories, Bruz, France) for 100 seconds to ensure homogeneity. A total of 6 ten-fold dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) were performed with BPW. For those samples collected on day 14, the dilutions of 10^{-1} and 10^{-2} were plated on *Clostridium perfringens* pour plates. The dilutions of 10^{-2}, 10^{-3} and 10^{-4} were plated on Petrifilm™ aerobic count plates (3M, St. Paul, MN) and the dilutions of 10^{-1}, 10^{-2} and 10^{-3} were plated on Petrifilm™ *E.coli*/coliform count plates (3M, St. Paul, MN). All samples were plated in duplicate for each dilution. For those samples collected on day 35, the same dilutions were used for *Clostridium perfringens*. The dilutions of 10^{-3}, 10^{-4} and 10^{-5} were used for aerobic count

plates in quadruplicate (half for incubated in a standard incubator (Geneq inc., Montreal, Quebec) and the other half incubated in a Bactron Anaerobic Chamber (model IV; Sheldon Manufacturing Inc., Cornelius, Oregon) under anaerobic conditions). The dilutions of 10^{-1}, 10^{-2} and 10^{-3} were plated on *E.coli*/coliform count plates. One ml of each dilution was plated on the plates.

Following the methodology described by Bolder et al. (1999), the *Clostridium perfringens* agar (Oxoid CM 0587, Basingstoke, Hampshire, England) was mixed with selective supplement (400 mg D-cycloserine per Liter) (Oxoid SR 0088E, Basingstoke, Hampshire, England). After the agar was solidified, the plates were incubated anaerobically in the Bactron Anaerobic Chamber at 37 C for 48 hours. The plates with black colonies within the range of 25-250 were selected for counting (Figure 3.1). Half of the aerobic count plates were incubated in the standard incubator at 37 C for 24 hours, while the other half were incubated in the Bactron Anaerobic Chamber at 37 C for 48 hours. The plates with red colonies within the same range were selected for counting. All red colonies were enumerated as aerobes (Figure 3.2) or anaerobes (Figure 3.3) depend on the incubators (AOAC 2005). For *E. coli* and total coliforms, the plates were incubated aerobically in the standard incubator at 37 C. After 24 hours, all the red and blue colonies with air bubbles attached or around were enumerated as coliforms. After 48 hours, the blue colonies with air bubbles were enumerated as *E, coli*. The plates with colonies within the range of 15-150 were selected for counting (Figure 3.4) (AOAC International 2005).

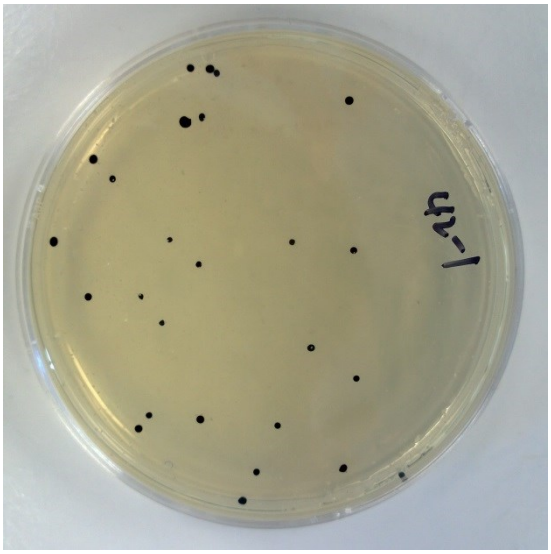

Figure 3.2 The culture of *Clostridium perfringens* on petri dish.

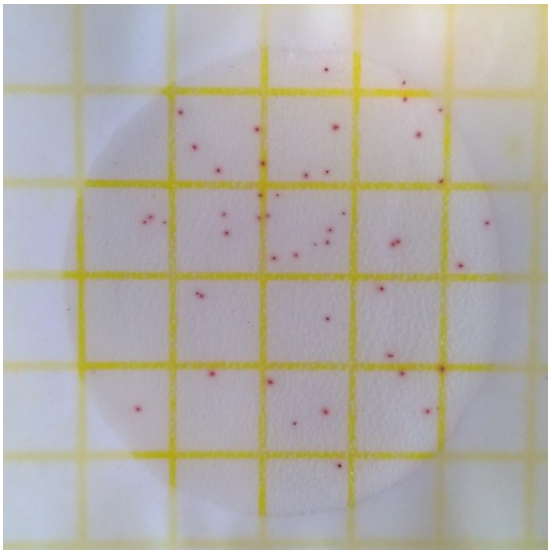

Figure 3.3 The culture of total aerobic bacteria on Petrifilm™ aerobic count plates.

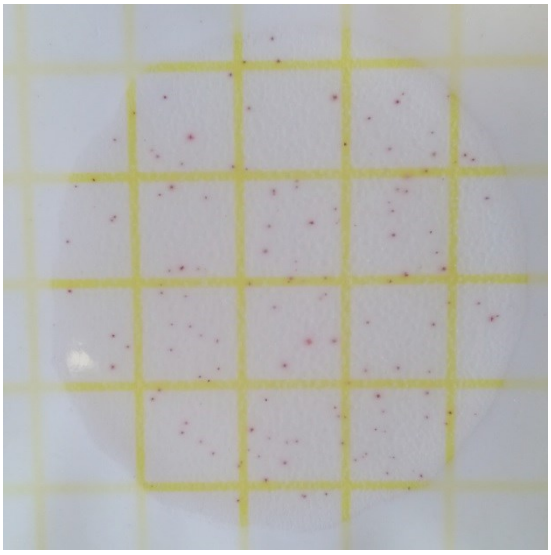

Figure 3.4 The culture of total anaerobic bacteria on Petrifilm™ count plates.

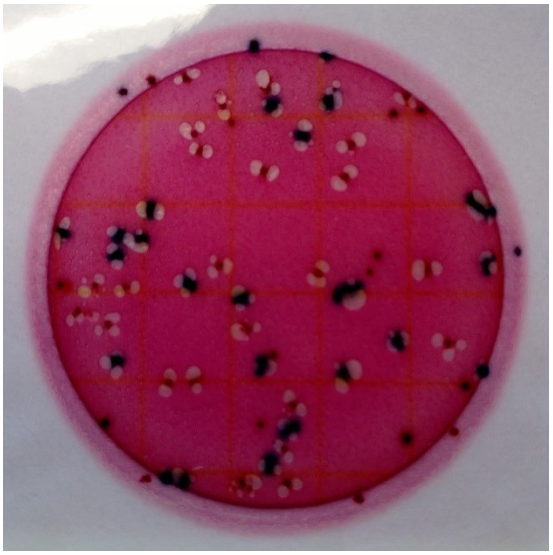

Figure 3.5 The culture of *E. coli* and total coliform on Petrifilm™ *E.coli*/ coliform count plates.

3.5.6 STATISTICAL ANALYSIS

From the start of this trial until day 22, this trial was treated as a completely randomized design with inclusion level of lysozyme as the main factor. Individual cages were the experimental units. Data were analyzed by ANOVA in SAS 9.3 (SAS Institute Inc., Cary, NC) (Littell et al. 1996). Growth data were analyzed as repeated measures with day as a factor. Where interactions with day were significant (α=0.05), data were sliced by day and analyzed separately. If significant main effects or interactions were found, the Tukey-Kramer method was used to compare differences among the least-square means (Gbur et al. 2012). The α-level for significance was $P<0.05$. The statistical model was: $Y_{ij} = \mu + Level_i + Day_k + Level * Day_{ik} + \varepsilon_{ik}$, where Y was the response of the variable (body weight, body weight gain, feed consumption and FCR), μ was the overall mean response of that parameter, $Level_i$ was the effect of inclusion level of lysozyme (i = 1-4), Day_k was the effect of level of day (k = 1-3), and ε_{ik} was the effect of the uncontrollable factors. The microbiology data were transformed to $\log_{10}$ before analysis. Y was the response variable for total aerobes, coliforms, *E. coli* and *Clostridium perfringens*.

For data collected from the days 22-35 period, the treatments were in a 2 x 4 factorial arrangement with level of inclusion and environmental condition as the main factors. Cages were considered the experimental units. The statistical model was: $Y_{ij} = \mu + Level_i + Condition_j + Level * Condition_{ij} + \varepsilon_{ij}$, where Y was the response variable (body weight, body weight gain, feed consumption, FCR, total aerobes, coliforms, *E. coli* and *Clostridium perfringens*), μ was the overall mean response of that parameter, $Level_i$ was

the effect of inclusion level of lysozyme (i = 1-4), $Condition_j$ was the temperature condition (j = 1-2), and ε_{ijk} was the effect of the uncontrollable factors.

3.6 Results and discussion

3.6.1 Growth performance

On day 14 and 21, the inclusion of lysozyme had no effect on body weight and body weight gain ($P>0.05$) (Table 3.4 and 3.5). Liu et al. (2010) found that the supplementation of 40 ppm lysozyme to broiler chickens did not increase body weight or body weight gain from day 14 to 28. In the current study when 50 ppm of lysozyme was added to the diet, the day 35 body weight of broiler chickens increased compared to birds fed 200 ppm of lysozyme ($P<0.05$) (Table 3.6). Lysozyme had no effect on body weight gain from day 22-35 ($P>0.05$) (Table 3.7). The supplementation of 200 ppm lysozyme in the diet might reduce some beneficial Gram-positive lactic acid bacteria such as *Lactobacillus* (Brown et al. 1962). *Lactobacillus* can improve the growth performance of broiler chickens (Jin et al. 1998a). Therefore, the growth performance might be compromised by the supplement of 200 ppm lysozyme in the diets due to inhibition of *Lactobacilllus*.

Table 3.4 Effect of inclusion level of lysozyme on body weight (g $bird^{-1}$) of broiler chickens on day 14 and 21.

Lysozyme level (ppm)	Age (day)		Level mean
	14	21	
0	385±13	748±13	566±11
50	394±13	758±13	576±11
100	389±13	739±13	564±11
200	374±13	732±13	553±11
Age mean	385±7[b]	744±7[a]	
ANOVA	P value		
Level	0.5284		
Age	<0.0001		
Level x Age	0.8826		

a-b: Lsmeans for age with different letters differ significantly (P<0.05)

Table 3.5 Effect of inclusion level of lysozyme on body weight gain (g $bird^{-1}$ day^{-1}) of broiler chickens on day 14 and 21.

Lysozyme level (ppm)	Age (day)		Level mean
	14	21	
0	24.0±1.5	51.9±1.5	38.0±1.13
50	24.7±1.5	52.0±1.5	38.3±1.13
100	24.3±1.5	49.9±1.5	37.2±1.13
200	23.2±1.5	51.2±1.5	37.1±1.13
Age mean	24.1±0.7[b]	51.2±0.7[a]	
ANOVA	P value		
Level	0.8450		
Age	<0.0001		
Level x Age	0.7902		

a-b: Lsmeans for age with different letters differ significantly (P<0.05)

Table 3.6 Effect of inclusion level of lysozyme and temperature conditions on body weight (g bird^{-1}) of broiler chickens on day 35.

Lysozyme level (ppm)	Temperature conditions		Level mean
	Control	Heat stress	
0	1735±46	1580±46	1657±32ab
50	1801±46	1618±46	1709±32a
100	1729±46	1528±46	1628±32ab
200	1648±46	1501±46	1574±32b
Temp. mean	1728±23[a]	1556±23[b]	
ANOVA	P value		
Level	0.0418		
Temp.	<0.0001		
Level x Temp.	0.9303		

a-b: Lsmeans with different letters within main effects differ significantly (P<0.05)

Table 3.7 Effect of inclusion level of lysozyme and room temperature conditions on body weight gain (g bird^{-1} day^{-1}) of broiler chickens from day 22-35.

Lysozyme level (ppm)	Temperature conditions		Level mean
	Control	Heat stress	
0	72.30±3.19	57.84±3.19	64.94±2.26
50	74.28±3.19	61.66±3.19	67.97±2.26
100	71.01±3.19	56.05±3.19	63.53±2.26
200	66.35±3.19	54.04±3.19	60.19±2.26
Temp. mean	70.92±1.60[a]	57.40±1.60[b]	
ANOVA	P value		
Level	0.1233		
Temp.	<0.0001		
Level x Temp.	0.9713		

a-b: Lsmeans with different letters for temperature differ significantly (P<0.05)

Broiler chickens under heat stress had reduced body weight and body weight gain (P<0.05) compared to those grown at the optimal temperatures (Table 3.6 and 3.7 respectively). Feeding lysozyme did not reduce the impact of heat stress on the reduction in body weight and body weight gain. On day 35, body weight of broiler chickens was reduced by 10% when cyclic diurnal heat stress was applied (Table 3.7). Dale and Fuller

(1980) found that broiler chickens reared in cool diurnally cycling temperature of 14 to 22 C. These birds gained more weight than those reared in hot diurnally cycling temperature of 22 to 33 C. Yahav et al. (1996) found a 33% increase in body weight for chickens reared at 25 C compared to 35 C, while Ain Baziz et al. (1996) found a 47% decrease of body weight at 32 C compared to 22 C environmental temperatures from hatch to day 49. Compared to other domestic animals, birds are less tolerant to temperature change. Heat stress induces physiological problems for broiler chickens (Abidin and Khatoon 2013). High temperature upsets the mineral balance in poultry blood (El Husseiny and Creger 1981). Ferket and Qureshi (1992) found less absorption of micronutrients in broiler chickens reared in a heat stressed environment. The optimum requirements of those micronutrients are barely met. The dilation of blood vessels caused by heat stress increased blood supply and energy consumption, so nutrients absorption is impacted (Bottie and Harrison 1987). Many panting activities of broiler chickens were observed in our experiment. Kadim et al. 2008 found heat stress led to panting and wing lifting.

The inclusion of lysozyme had no effect on feed consumption of broiler chickens at any age and FCR from day 0-14 and 15-24 ($P>0.05$) (Table 3.8, 3.9 and 3.10 respectively). There was an interaction with temperature and lysozyme on day 35 ($P<0.05$) (Table 3.11). Using data collected on day 35, it was clear that 200 ppm lysozyme reduced the feed conversion efficiency compared to 50 ppm and control treatment due to the lower body weight gain of the birds fed other treatments. Elevation of temperature eliminated the dietary treatment effect on feed efficiency (Table 3.11). Liu et al. (2010) added lysozyme

at 40 ppm to the diet and found no differences in feed intake or FCR for the period of day 14-28. However, when Humphrey et al. (2002) mixed 10% of transgenic rice that express lysozyme and 5% of transgenic rice that expressed lactoferrin into broiler diets, they found improved feed efficiency from days 3-20 for broiler chickens reared in a 25 C controlled environment room with 24 hours light. Research describing the effect of lysozyme inclusion level on poultry is limited. Supplementation of high level of lysozyme (200 ppm) might cause some problems that affect FCR. Tribst et al. (2008) conducted an *in vitro* study and reported that lysozyme reduced the population of *Lactobacillus*. The reduction of *lactobacillus* in the small intestine of broiler chicken helps with the development and colonization of harmful organisms, which compete for nutrient with the host (Jin et al. 1996). Future research of NGS will identify the effect of lysozyme on bacteria making up the population in the small intestine of broiler chickens.

Heat stress significantly reduced feed consumption from day 22-35 (Table 3.9), which is similar to that reported by Dale and Fuller (1980) and Meltzer (1983). In this experiment, FCR was not affected by heat stress (Table 3.10). Cooper and Washburn (1998) demonstrated that the body temperature of broiler chickens increased in response to heat stress. When body temperature was affected, there were negative correlations with body weight and feed intake. Heat stress had no effect on FCR from day 25-35 (Table 3.11). However, the correlation between temperature and FCR in the study of Cooper and Washburn (1998) was positive, so the effect of heat stress on FCR was negative.

Table 3.8 Effect of inclusion level of lysozyme on feed consumption (g bird^{-1}) per day per broiler chicken from day 0-14 and 15-21.

Lysozyme level (ppm)	Age (day)		Level mean
	0-14	15-21	
0	31.2±2.4	97.1±2.4	64.1±1.8
50	32.7±2.4	102.3±2.4	67.5±1.8
100	32.5±2.4	99.4±2.4	65.9±1.8
200	32.8±2.4	102.8±2.4	69.8±1.8
Age mean	32.3±1.2^{b}	100.4±1.2^{a}	
ANOVA	P value		
Level	0.4626		
Age	<0.0001		
Level x Day	0.7620		

a-b: Lsmeans for age with different letters differ significantly (P<0.05)

Table 3.9 Effect of inclusion level of lysozyme and temperature conditions on feed consumption (g bird^{-1} day^{-1}) of broiler chickens from day 22-35.

Lysozyme level (ppm)	Temperature conditions		Level mean
	Control	Heat stress	
0	150.6±6.6	133.4±6.6	142.0±4.7
50	159.2±6.6	143.6±6.6	151.4±4.7
100	160.1±6.6	125.4±6.6	142.7±4.7
200	176.2±6.6	128.1±6.6	152.1±4.7
Temp. mean	161.5±3.3^{a}	132.6±3.3^{b}	
ANOVA	P value		
Level	0.2689		
Temp.	<0.0001		
Level x Temp.	0.0554		

a-b: Lsmeans for temperature with different letters differ significantly (P<0.05)

Table 3.10 Effect of inclusion level of lysozyme on feed conversion ratio (feed gain^{-1}) of broiler chicken day 0-14 and 15-21.

Lysozyme level (ppm)	Age (day)		Level mean
	0-14	15-21	
0	1.31±0.07	1.90±0.07	1.60±0.05
50	1.32±0.07	1.98±0.07	1.65±0.05
100	1.34±0.07	2.00±0.07	1.67±0.05
200	1.41±0.07	2.05±0.07	1.73±0.05
Age mean	1.34±0.03^{b}	1.98±0.03^{a}	
ANOVA	P value		
Level	0.4247		
Age	<0.0001		
Level x Age	0.9285		

a-b: Lsmeans for age with different letters differ significantly (P<0.05)

Table 3.11 Effect of inclusion level of lysozyme and temperature conditions on feed conversion ratio (feed gain^{-1}) of broiler chicken from day 22-35.

Lysozyme level (ppm)	Temperature conditions		Level mean
	Control	Heat stress	
0	2.10±0.11^{b}	2.34±0.11	2.22±0.07^{b}
50	2.15±0.11^{b}	2.35±0.11	2.25±0.07^{b}
100	2.26±0.11^{ab}	2.24±0.11	2.25±0.07^{b}
200	2.70±0.11^{a}	2.38±0.11	2.54±0.07^{a}
Temp. mean	2.30±0.05	2.33±0.05	
ANOVA	P value		
Level	0.0129		
Temp.	0.7714		
Level x Temp.	0.0448		

a-b: Lsmeans for level with different letters differ significantly (P<0.05)

The total mortality was 10.1%, with most of these occurring in the period of days 0-14. The majority mortalities were caused by omphalitis and leg deformities. The diseases were not related to the treatments. Lysozyme had no effect on mortality rate of broiler chickens during days 0-21 or 22-35 or throughout the trial (P>0.05). Lysozyme did not

reduce the rate of mortality which decreased with heat stress (P>0.05) (Table 3.12). Zhang et al. (2010) found that lysozyme can reduce mortality when birds were given a bacterial challenge. Chickens gavaged with *Clostridium perfringens* had reduced NE mortality (from 32.06% to 9.04%) when supplement with 200 ppm radiant energy treated lysozyme (Zhang et al. 2010). In our experiment, lysozyme was directly extracted from egg white. This form of lysozyme might not be as effective as the treated lysozyme or the bacterial challenge may be a more appropriate stress to measure the effect of treatment with lysozyme. Heat stress did not directly cause mortality and in fact birds in the elevated temperature treatment had lower mortality rate compared to birds in the control environment during days 22-35 (Table 3.12). In the control environment, 80% of the mortalities were birds that were culled due to leg problems. However, research has shown high temperature increased the mortality rate of broiler chickens (Al-Fataftah 1987). High ambient temperature increased body temperature leading to many conditions such as cardiac arrest, adrenal cortical inefficiency or ionic imbalance in the blood of broiler chickens which increased the mortality rates (Deaton et al. 1984).

Table 3.12 ANOVA P-value for the percent of mortality in broilers fed different inclusion levels of lysozyme.

	Time period (days)			
	0-14	15-21	22-35	0-35
Level	0.324	0.578	0.425	0.678
Temp	-	-	0.022	-
Temp x Level	-	-	0.424	-

3.6.2 INTESTINAL MICROBIOTA

On day 14, aerobes, coliforms, *E. coli* and *Clostridium perfringens* in the ileum were not influenced by inclusion level of lysozyme on broiler chickens (P>0.05) (Table 3.13). Liu et al. (2010) found 40 ppm of lysozyme did not change the number of *E. coli* and *Clostridium perfringens* from ileal samples collected from 22 day old broilers. Compared to lysozyme treated birds, those fed the control diet had similar number of bacteria (P>0.05). If more samples were included in this study, there were possibilities of significant differences for bacteria numbers.

Table 3.13 Effect of inclusion level of lysozyme on total aerobes, coliforms, *E. coli* and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 14.

Lysozyme Level (ppm)	Aerobes	Coliforms	*E. coli*	*Clostridium perfringens*
0	6.15±0.20	3.77±0.19	3.43±0.22	1.95±0.19
50	5.56±0.20	3.36±0.19	2.96±0.22	1.63±0.19
100	5.72±0.20	3.15±0.19	2.82±0.22	1.55±0.19
200	5.85±0.20	3.45±0.19	3.11±0.22	1.83±0.19
mean	5.82	3.43	3.08	1.74
ANOVA				
Number of Observations	96	96	96	96
P value	0.2079	0.1597	0.2611	0.4375

Ileal samples collected from 35 day old broilers had similar numbers of aerobic bacteria regardless of lysozyme levels, environment temperature or any combination of the two main effects (Table 3.14). The total aerobic bacteria measured in this experiment included obligate aerobes and facultative anaerobes. Amit-Romach et al. (2004) detected six groups of bacteria including *Lactobacillus*, *Bifidobacterium*, *Salmonella*, *Campylobacter*, *E. coli*, and *Clostridium* in chicken digesta by using 16S rDNA primers.

Lactobacillus, *Salmonella* and *E. coli* are facultative anaerobes while most *Campylobacter* prefer an oxygen concentration of 3-15% to grow (Chynoweth et al. 1998).

Table 3.14 Effect of inclusion level of lysozyme and room temperature on total aerobes (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 35.

Lysozyme level (ppm)	Temperature conditions		Level mean
	Control	Heat stress	
0	6.02±0.20	5.69±0.20	5.86±0.14
50	6.25±0.20	5.94±0.20	6.10±0.14
100	5.65±0.20	5.85±0.20	5.75±0.14
200	5.47±0.20	5.91±0.20	5.69±0.14
Temp. mean	5.85±0.10	5.85±0.10	
ANOVA	P value		
Level	0.2029		
Temp.	0.9954		
Level x Temp.	0.1561		

There were no changes to the number of total anaerobes in the ileum samples when lysozyme was added to the diet. However, heat stress reduced the number of total anaerobes per gram of sample for all 35-day-old birds (Table 3.15). Perhaps birds under heat stress drink more water leading to the dilution of ileal digesta. There is evidence of wet droppings. However, the interaction of heat stress and lysozyme had no effect on the number of anaerobes cultured (Table 3.15). In the ileum, the majority of total anaerobes are *Lactobacillus*, and clostridia are also present (Barnes et al. 1972).

When birds are under heat stress, lymphocyte numbers are reduced (Borges et al. 1999). Heat stress decreased immune response as well. Zulkifli et al. (2000) reported a reduction

in antibodies of birds exposed to high temperature. However, an increase in bacterial numbers with the use of heat stress was not observed in this case.

Table 3.15 Effect of inclusion level of lysozyme and room temperature on total anaerobes (log_{10} cfu g^{-1}), in the ileum of broiler chickens on day 35.

Lysozyme level (ppm)	Temperature conditions		Level mean
	Control	Heat stress	
0	7.60±0.17	7.07±0.17	7.33±0.12
50	7.76±0.17	7.43±0.17	7.59±0.12
100	7.69±0.17	7.16±0.17	7.42±0.12
200	7.82±0.17	6.93±0.17	7.37±0.12
Temp. mean	7.72±0.09[a]	7.15±0.09[b]	
ANOVA	P value		
Level	0.4432		
Temp.	<0.0001		
Level x Temp.	0.4291		

a-b: Lsmeans for temperature with different letters differ significantly (P<0.05)

The inclusion of lysozyme did not affect the total coliforms and *E. coli* numbers in the ileal samples (Table 3.16 and 3.17 respectively). Liu et al. (2010) found *E. coli* numbers in the ileum were not changed by dietary lysozyme at 40 ppm. Others have found lysozyme *in vitro* is effective against *E. coli* growth (Deckers et al. 2008). Temperature and the interaction with lysozyme treatment had no effect on total coliforms and *E. coli* in the ileum. In our study, birds were housed in the wire cages, which may have negated the effort to stress the birds with the cycling high temperatures. Willis et al. (2002) reported that bacteria isolation from chickens was lower in birds reared in wire cages than those in floor pens.

Table 3.16 Effect of inclusion level of lysozyme and room temperature on total coliforms (log_{10} cfu g^{-1}), in the ileum of broiler chickens on day 35.

Lysozyme level (ppm)	Temperature conditions		Level mean
	Control	Heat stress	
0	3.49±0.32	3.78±0.32	3.63±0.23
50	4.68±0.32	4.03±0.32	4.35±0.23
100	3.99±0.32	4.22±0.32	4.10±0.23
200	3.95±0.32	3.56±0.32	3.75±0.23
Temp. mean	4.03±0.16	3.89±0.16	
ANOVA	P value		
Level	0.1001		
Temp.	0.5584		
Level x Temp.	0.3743		

Table 3.17 Effect of inclusion level of lysozyme and room temperature on *E. coli* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 35.

Lysozyme level (ppm)	Temperature conditions		Level mean
	Control	Heat stress	
0	2.94±0.32	3.14±0.32	3.04±0.23
50	4.17±0.32	3.28±0.32	3.73±0.23
100	3.55±0.32	3.79±0.32	3.67±0.23
200	3.46±0.32	3.00±0.32	3.23±0.23
Temp. mean	3.53±0.16	3.30±0.16	
ANOVA	P value		
Level	0.1010		
Temp.	0.3198		
Level x Temp.	0.2387		

Lysozyme had no effect on *Clostridium perfringens* in the ileum (Table 3.18). Liu et al. (2010) reported that lysozyme did not reduce *Clostridium perfringens* in an optimal environmental temperature situation, but when challenge was provided (oral supplementation of *Clostridium perfringens*), lysozyme reduced those bacteria. In this study, *Clostridium perfringens* in the ileum of birds were fewer when exposed to heat stress compared to those in the normal environment. We would expect that when birds

are under heat stress, bacterial growth should increase since the immune response of the birds can be reduced. Chicken intestinal microbiota is a complicated environment. Many bacteria compete with each other for nutrients and substrates (Yegani and Korver 2008). *Clostridium perfringens* might be less competitive than the others, so the population decreased while other bacteria grew faster. When testing the effect of antimicrobials on *Clostridium perfringens*, heat stress may not be an idea challenge.

Table 3.18 Effect of inclusion level of lysozyme and room temperature on *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 35.

Lysozyme level (ppm)	Temperature conditions		Level mean
	Control	Heat stress	
0	1.73±0.22	1.30±0.22	1.51±0.15
50	2.10±0.22	1.17±0.22	1.63±0.15
100	2.18±0.22	1.45±0.22	1.82±0.15
200	2.34±0.22	1.39±0.22	1.86±0.15
Temp. mean	2.09±0.11[a]	1.39±0.11[b]	
ANOVA	P value		
Level	0.3438		
Temp.	<0.0001		
Level x Temp.	0.6052		

a-b: Lsmeans for temperature with different letters differ significantly (P<0.05)

3.7 CONCLUSIONS

Weight gain by birds fed the 50 ppm lysozyme were greater (P<0.05) than when the birds were fed 200 ppm lysozyme. Birds fed 200 ppm lysozyme had the poorest FCR. Heat stress reduced body weight, body weight gain, feed consumption, and reduced the population of total anaerobic bacteria and *Clostridium perfringens*. The use lysozyme in feed provided no improvement for growth performance and no changes in intestinal numbers of *E. coli* or *Clostridium perfringens*.

Birds in cages have limited access to bacteria compared to those in floor pens, which reduces the possibility of finding effect of lysozyme on intestinal microbiota. Performance of broilers in cages is usually poorer than that achieved in floor pens. Floor pens are the usual housing method employed with broilers. Antibiotics were not included in the present study which was a cage study. Both antibiotics and lysozyme should be testing in broilers to determine whether antibiotics can be replaced by lysozyme in broiler chickens. Lysozyme may have differential effects if added at different periods during the specific period of the growth cycle. Finding the most effective time to include lysozyme over the 35-day growth period may identify a time to focus its use. Adding lysozyme to diets only the days of the growth cycle that considered critical periods may be effective. The use of 100 ppm lysozyme did not cause any negative effect on growth performance of intestinal microbiota of broiler chickens from the cage trial. Subsequently, two growth trials were conducted to determine the critical periods during the growth cycle of broiler chickens for which dietary lysozyme supplementation at 100 ppm may have the greatest impact on growth and feed efficiency.

CHAPTER 4 THE EFFECT OF LYSOZYME IN EACH PERIOD OF THE GROWTH CYCLE OF BROILER CHICKENS GROWN IN OPTIMAL HOUSING CONDITION

4.1 ABSTRACT

Lysozyme could be used as a potential alternative to antibiotics in poultry feed. Adding lysozyme in only the critical periods of the growth cycle of broiler chickens would be the most effective way to use this ingredient due to its high cost ($40 kg^{-1}). This study was conducted to determine the effect of dietary lysozyme on the growth performance and intestinal microbiota in each period of the growth cycle of broiler chickens grown on new pine shavings. Two replicate experiments were designed as a one-way analysis of variance with length of time in which 100 ppm lysozyme was fed to the birds as the main factor (-ve lysozyme days 0-35; +ve lysozyme days 0-5; +ve lysozyme days 6-14; +ve lysozyme days 15-24; +ve lysozyme days 25-35; +ve lysozyme days 0-35; +ve virginiamycin (250 ppm) days 0-35). The inclusion of lysozyme and virginiamycin had no effect on the growth performance and intestinal microbiota of broiler chickens ($P>0.05$). The average day 35 body of broiler chickens fed control diet and diet with virginiamycin and lysozyme throughout the trial were 1884, 1897 and 1863 g, respectively. The growing conditions provided in this study which included strict biosecurity may have minimized the chances for birds to be exposed to harmful organisms. Potential effects of lysozyme were not detected under this situation.

Key words: antibiotics, lysozyme, new pine shavings, growth cycle, growth performance, intestinal microbiota, biosecurity

4.2 INTRODUCTION

Floor pens are the usual housing method used with broilers in commercial farms. More bacteria can be isolated from the birds reared on floor pens than those in wire cages (Willis et al. 2002). Using floor pens to determine the effect of lysozyme on growth performance and intestinal microbiota is more practical compared to caged birds. Compared to the price of virginiamycin at 250 ppm ($3/tonne feed), lysozyme at 100 ppm cost more ($4/tonne feed). Adding lysozyme in the critical periods of the growth cycle of broiler chickens may be an effective way to reduce the cost of using it as an alternative to antibiotics.

4.3 OBJECTIVE

To determine the effect of dietary lysozyme on the growth performance and intestinal microbiota in each period of the growth cycle of broiler chickens grown under optimal housing conditions when new litter is provided.

4.4 HYPOTHESES

Feeding broiler chickens lysozyme at key time periods will improve growth performance and reduce *Clostridium perfringens,* detrimental *E. coli* and total coliforms in the small intestine.

4.5 MATERIALS AND METHODS

4.5.1 ANIMAL HOUSING AND HUSBANDRY

This experiment had two replicate trials. In each trial, 2240 male Ross 308 broiler chickens from Clark's Hatchery (Burtts Corner, NB) were used. Birds arrived in the Atlantic Poultry Research Centre in Truro, NS on the day of hatch, and were immediately randomly assigned to fifty-six, 150 cm x 218 cm, floor pens equally distributed within four rooms. The stocking density of the first and second experiment was 0.6 kg/m^2 and 0.6 kg/m^2 on day 0 and 18.3 kg/m^2 and 24.60 kg/m^2 on day 35, respectively. Upon arrival, the chicks were immediately provided with feed and water. Feed was provided *ad libitum* in cardboard box lids for the first week and in tube feeders for the entire experiment. Birds were introduced to water by dipping beaks in the nipple drinker. Lighting was set according to standard operating procedure (Appendix A). Ambient temperature and RH were recorded by data logger (Appendix B-2 B-3 and C-2). The RH of Experiment 2 was not reported due to malfunction of data logger. All procedures were carried out under the guidance of the Canadian Council on Animal Care (CCAC 2009).

4.5.2 DIETS

Diets were isocaloric and isonitrogenous within the starter (days 0-14), grower (days 15-24) and finisher phases (days 25-35) (Experiment 1, Table 4.1 and Experiment 2, Table 4.2). Diets were formulated to meet or exceed the National Research Council (1994) nutrient requirements for each phase. The diets were fed in mash form throughout the trial. There were seven treatments in this experiment with eight replications. Three of these treatments, control (no growth promotants), lysozyme (100 ppm) and antibiotic (virginiamycin 250 ppm) diets were fed throughout the trial. Lysozyme, extracted from hen egg white, was provided by Neova Technologies Inc. Abbotsford, British Columbia.

Table 4.1 Composition of experimental diets containing lysozyme and antibiotic fed from 0-14, 15-24 and 25-35 days of age (Experiment 1).

Growth periods	Starter			Grower			Finisher		
Days of age	0-14			15-24			25-35		
Ingredients (%)	C	A	L†	C	A	L	C	A	L
Corn	44.40	44.35	44.38	52.03	51.98	52.01	56.90	56.86	56.89
Soybean meal	38.75	38.76	38.75	30.98	30.98	30.98	25.85	25.85	25.85
Wheat	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Tallow blend	3.24	3.26	3.24	3.78	3.80	3.79	3.66	3.68	3.67
Limestone	1.65	1.65	1.65	1.59	1.59	1.59	1.40	1.40	1.40
Dicalcium phosphate	0.59	0.59	0.59	0.50	0.50	0.50	0.64	0.64	0.64
Vitamin/mineral premix[z]	0.50	0.50	0.50	0.46	0.46	0.46	0.50	0.50	0.50
Iodized salt	0.43	0.43	0.43	0.40	0.40	0.40	0.40	0.40	0.40
Methionine premix[y]	0.39	0.39	0.39	0.20	0.20	0.20	0.53	0.53	0.53
Coban[x]	0.05	0.05	0.05	0.05	0.05	0.05	-	-	-
Lysine 98%		-	-	-	-	-	0.128	0.128	0.128
Stafac 44[w]	-	0.025	-	-	0.025	-	-	0.025	-
Lysozyme[v]	-	-	0.01	-	-	0.01	-	-	0.01
Calculated analysis									
AME_n (kcal/kg)[u]	3050	3050	3050	3150	3150	3150	3200.20	3200.00	3200.00
Crude protein (%)	23.00	23.00	23.00	20.00	20.00	20.00	18.00	18.00	18.00
Calcium (%)	1.00	1.00	1.00	0.92	0.92	0.92	0.85	0.85	0.85
Available Phosphorus (%)	0.45	0.45	0.45	0.40	0.40	0.40	0.42	0.42	0.42
Lysine (%)	1.38	1.38	1.38	1.15	1.15	1.15	1.09	1.09	1.09
Methionine (%)	0.58	0.58	0.58	0.44	0.44	0.44	0.58	0.58	0.58
Meth+cyst (%)[t]	0.95	0.95	0.95	0.76	0.76	0.76	0.86	0.86	0.86
Sodium (%)	0.19	0.19	0.19	0.18	0.18	0.18	0.18	0.18	0.18
Determined analysis									
Crude protein (%)	23.95	23.99	23.62	21.24	20.27	20.25	18.08	17.97	18.58
Total calcium (%)	1.20	0.92	0.91	0.79	0.90	0.86	0.80	0.80	0.79
Total phosphorus (%)	0.48	0.51	0.52	0.44	0.45	0.45	0.40	0.41	0.41

†C: Control; A: Antibiotics; L: Lysozyme.

[z] Supplied per kg diet: vitamin A, 9750 IU; vitamin D_3, 2000 IU; vitamin E, 25 IU; vitamin K, 2.97 mg; riboflavin, 7.6 mg; Dl Ca-pantothenate, 13.5 mg; vitamin B_{12}, 0.023 mg; niacin, 29.7; folic acid, 4.0 mg, choline, 801 mg; biotin, 0.3 mg; pyridoxine, 4.95 mg; thiamine, 2.91 mg; manganese, 70.2 mg; zinc, 80.0 mg; copper, 25 mg; selenium, 0.15 mg; ethoxyquin, 50 mg; wheat middlings, 1432 mg; ground limestone, 500 mg.

[y] Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middlings, 0.5 kg.

[x] Coccidiostat - Coban (active ingredient monensin sodium, 200 g kg^{-1}) Elanco Animal Health, Division Eli Lilly Canada Inc., Guelph, ON, Canada.

[w] Virginiamycin 44 g kg^{-1}, Zoetis Animal Health, London, ON, Canada.

[v] Inovapure 213 (active ingredient lysozyme at 20%, 24,000 Shugar units mg^{-1}) Neova Technologies, Inc.,Abbotsford, BC., Canada.

[u] Nitrogen-corrected apparent metabolizable energy.

[t] Methionine and cystine

Table 4.2 Composition of experimental diets containing lysozyme and antibiotic fed from 0-14, 15-24 and 25-35 days of age (Experiment 2).

Growth periods	Starter			Grower			Finisher		
Days of age	0-14			15-24			25-35		
Ingredients (%)	C	A	L†	C	A	L	C	A	L
Corn	43.16	43.13	43.16	50.76	50.69	50.74	56.90	56.86	56.89
Soybean meal	38.98	38.98	38.98	31.21	31.23	31.22	25.85	25.85	25.85
Wheat	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Tallow blend	3.68	3.68	3.68	4.23	4.27	4.24	3.66	3.68	3.67
limestone	1.67	1.67	1.67	1.43	1.43	1.43	1.40	1.40	1.40
Dicalcium phosphate	0.83	0.83	0.83	0.71	0.71	0.71	0.64	0.64	0.64
Vitamin/mineral premix[z]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Iodized salt	0.43	0.43	0.43	0.40	0.40	0.40	0.40	0.40	0.40
Methionine premix[y]	0.64	0.64	0.64	0.58	0.59	0.58	0.53	0.53	0.53
Coban[x]	0.05	0.05	0.05	0.05	0.05	0.05	-	-	-
Lysine 98%	0.06	0.06	0.06	0.12	0.12	-	0.128	0.128	0.128
Stafac 44[w]	-	0.025	-	-	0.025	-	-	0.025	-
Lysozyme[v]	-	-	0.01	-	-	0.01	-	-	0.01
Calculated analysis									
AME_n (kcal/kg)[u]	3050.61	3050	3050	3150	3150	3150	3200.20	3200.00	3200.00
Protein (%)	23.00	23.00	23.00	20.00	20.00	20.00	18.00	18.00	18.00
Calcium (%)	1.05	1.05	1.05	0.90	0.90	0.90	0.85	0.85	0.85
Available Phosphorus (%)	0.50	0.50	0.50	0.45	0.45	0.45	0.42	0.42	0.42
Lysine (%)	1.43	1.43	1.43	1.24	1.24	1.24	1.09	1.09	1.09
Methionine (%)	0.69	0.69	0.69	0.63	0.63	0.63	0.58	0.58	0.58
Meth+cyst (%)[t]	1.07	1.07	1.07	0.95	0.95	0.95	0.86	0.86	0.86
Sodium (%)	0.19	0.19	0.19	0.18	0.18	0.18	0.18	0.18	0.18
Determined analysis									
Crude protein (%)	23.62	23.58	23.74	21.29	20.07	21.23	18.28	18.89	17.83
Total calcium (%)	1.01	1.12	1.09	0.80	0.99	0.89	0.68	0.68	0.78
Total phosphorus (%)	0.54	0.55	0.59	0.47	0.50	0.50	0.43	0.45	0.44

†C: Control; A: Antibiotics; L: Lysozyme.

[z] Supplied per kg diet: vitamin A, 9750 IU; vitamin D_3, 2000 IU; vitamin E, 25 IU; vitamin K, 2.97 mg; riboflavin, 7.6 mg; Dl Ca-pantothenate, 13.5 mg; vitamin B_{12}, 0.023 mg; niacin, 29.7; folic acid, 4.0 mg, choline, 801 mg; biotin, 0.3 mg; pyridoxine, 4.95 mg; thiamine, 2.91 mg; manganese, 70.2 mg; zinc, 80.0 mg; copper, 25 mg; selenium, 0.15 mg; ethoxyquin, 50 mg; wheat middlings, 1432 mg; ground limestone, 500 mg.

[y] Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middlings, 0.5 kg.

[x] Coccidiostat - Coban (active ingredient monensin sodium, 200 g kg^{-1}) Elanco Animal Health, Division Eli Lilly Canada Inc., Guelph, ON, Canada.

[w] Virginiamycin 44 g kg^{-1}, Zoetis Animal Health, London, ON, Canada.

[v] Inovapure 213(active ingredient lysozyme at 20%, 24,000 Shugar units mg^{-1}) Neova Technologies, Inc.,Abbotsford, BC., Canada.

[u] Nitrogen-corrected apparent metabolizable energy.

[t] Methionine and cystine

It was a white powder with an enzymatic activity of 24,000 units mg^{-1} (Shugar 1952) and mixed with EDTA at a ratio of 20:80. For the other four treatments, the diet with 100 ppm lysozyme was fed during specific periods of the growth cycle of broiler chickens (days 0-4 5-14, 15-24 or 25-35). Control diets were fed to the birds for the remaining period of the trial for each of these treatments (Table 4.3).

Table 4.3 Supplementation of lysozyme in different periods during the growth cycle of broiler chickens.

Treatment	Time period (days)			
	0-4	5-14	15-24	25-35
1	Control	Control	Control	Control
2	Antibiotic	Antibiotic	Antibiotic	Antibiotic
3	Lysozyme	Lysozyme	Lysozyme	Lysozyme
4	Lysozyme	Control	Control	Control
5	Control	Lysozyme	Control	Control
6	Control	Control	Lysozyme	Control
7	Control	Control	Control	Lysozyme

4.5.3 Analysis of growth performance

In both experiments, birds were mass weighed per pen on days 0, 4, 14, 24 and 35. The feed remaining in the feeders was weighed on each weigh day and as mortality occurred. Mortality was recorded and the dead birds were sent to the veterinary pathologist for necropsy (Animal Health Laboratory, Truro, Canada). Performance was determined by measuring body weight, body weight gain, feed consumption and FCR.

4.5.4 DIGESTA SAMPLE COLLECTION

For the first experiment of this trial, two birds per pen were euthanized by cervical dislocation on days 4, 14, 24 and 35. Approximately 15 cm of the ileum one cm anterior to the ileal cecal junction was aseptically harvested, placed in sterile plastic bags on ice and taken to the level II microbiology laboratory. From each intestinal sample, two cm from the proximal and distal ends of each ileum sample will be used for future NGS analysis (Results are not reported in this thesis). The remaining ileum was used for conventional microbial analysis. All samples were stored in a freezer at -80 C until analysis of microbiota. Digesta samples were not collected from Experiment 2.

4.5.5 MICROBIOTA ANALYSIS

For the first experiment, intestinal ileal samples were transferred to filtered stomacher bags and BPW was added in a 1:10 ratio. The samples were blended in the stomacher for 100 seconds to ensure homogeneity. The initial dilution was 10^{-1}, and then a total of six dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) were performed with BPW for culturing total aerobes, anaerobes, coliforms, *E. coli* and *Clostridium perfringens*. One ml of 10^{-2}, 10^{-3} or 10^{-4} BPW with sample, depend on the age of broilers when samples were collected, was added to 9 ml of MRS buffer (Oxoid CM 0361, Basingstoke, Hampshire, England) to create five dilutions (10^{-3} 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) for lactic acid bacteria counts. The dilutions used for colony counts of total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* were listed (Table 4.4).

Table 4.4 The dilutions used for colony counts of total aerobes, anaerobes, coliforms, *E.coli*, lactic acid bacteria and *Clostridium perfringens*.

Day	Aerobes	Anaerobes	Coliforms and *E. coli*	Lactic acid bacteria	*Clostridium perfringens*
4	10^{-4} 10^{-5} 10^{-6}	10^{-4} 10^{-5} 10^{-6}	10^{-2} 10^{-3} 10^{-4}	10^{-4} 10^{-5} 10^{-6}	10^{-1} 10^{-2}
14	10^{-3} 10^{-4} 10^{-5}	10^{-4} 10^{-5} 10^{-6}	10^{-2} 10^{-3} 10^{-4}	10^{-3} 10^{-4} 10^{-5}	10^{-1} 10^{-2}
24	10^{-4} 10^{-5} 10^{-6}	10^{-4} 10^{-5} 10^{-6}	10^{-2} 10^{-3} 10^{-4}	10^{-4} 10^{-5} 10^{-6}	10^{-1} 10^{-2}
35	10^{-4} 10^{-5} 10^{-6}	10^{-5} 10^{-6} 10^{-7}	10^{-2} 10^{-3} 10^{-4}	10^{-5} 10^{-6} 10^{-7}	10^{-1} 10^{-2}

Samples were pour plated in Petri dishes for the counting of *Clostridium perfringens*. Petrifilm™ aerobic count plates were used for the counting of aerobes, anaerobes, lactic acid bacteria and Petrifilm™ *E.coli*/coliform count plates for the counting of *E.coli*/coliform. Each dilution was plated in duplicate.

Following the methodology described by Bolder et al. (1999), the samples were plated with *Clostridium perfringens* agar mixed with selective supplement. After the agar was solidified, the plates were incubated anaerobically in a Bactron Anaerobic Chamber (model IV) at 37 C for 48 hours. The plates with black colonies numbering within 25-250 were selected for counting *Clostridium perfringens*. The plates for the counting of anaerobes and lactic acid bacteria were incubated in the Bactron Anaerobic Chamber for 24 hours. Red colonies on the aerobic count Petrifilm™ with BPW were enumerated as anaerobes and plates with samples diluted in MRS were enumerated as lactic acid bacteria (Figure 4.1). The aerobic count plates were incubated in a standard incubator at 37 C for 24 hours. The plates with the number of red colonies within the range of 25-250 were selected for counting (AOAC International 2005). For *E. coli* and total coliforms, the plates were incubated aerobically at 37 C. After 24 hours, all red and blue colonies with air bubbles were counted as coliforms. After 48 hours, the blue colonies associated

with air bubbles were enumerated as *E. coli* (AOAC 2005). The plates with 15-150 cfu were selected for counting. All of the bacterial count data were transformed using $\log_{10}$ prior to statistical analyses. Microbiota analysis was not performed for Experiment 2.

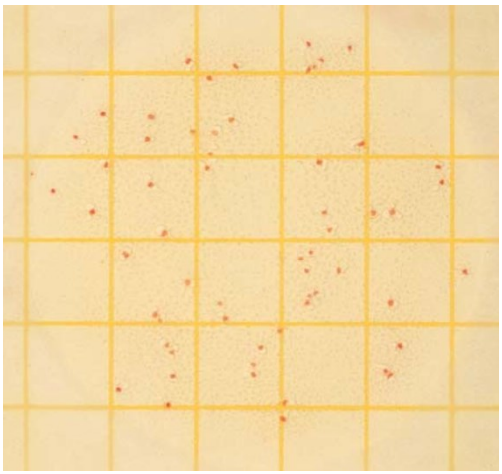

Figure 4.1 The culture of lactic acid bacteria on Petrifilm™ aerobic count plates.

4.5.6 Statistical analysis

This trial consists of two experiments. For growth performance parameters, this trial was a completely randomized block design with period of time in which the lysozyme or antibiotic were fed to the birds as the main factor and rooms of two experiments as the block. Pen was used as the experimental unit. Data were analyzed by ANOVA in SAS 9.2 (SAS Institute Inc., Cary, NC) (Littell et al. 1996). Growth data were analyzed as repeated measures with day as a factor. Where interactions with day were significant (α=0.05), data were sliced by day and analyzed separately. If significant main effects or interactions were found, the Tukey-Kramer method was used to compare differences among the least-square means (Gbur et al. 2012). The α-level for significance was $P<0.05$. The statistical model was: $Y_{ij} = \mu + Treatment_i + Day_j + Treatment*Day_{ij} + Room_k + \varepsilon_{ijk}$, where Y is the response of the variable (body weight, body weight gain,

feed consumption, FCR, μ is the overall mean response of that parameter), $Treatment_i$ is the effect of antibiotic or lysozyme included in the diet during different time periods (i = 1-6), Day_j is the effect of level of day (j = 1-3), $Room_k$ is the effect of the k^{th} level of the block (room) (k = 1-4), and ε_{ijk} is the effect of the uncontrollable factors.

For the bacterial population data of Experiment 1, tha same design was used except the data were not analyzed as repeated measures with day as a factor. The statistical model was: $Y_{ij} = \mu + Treatment_i + Room_k + \varepsilon_{ik}$, where Y is the response of the variable (Total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* on log_{10} basis). $Treatment_i$ is the effect of antibiotic or lysozyme included in the diet during different time periods (i = 1-6), Day_j is the effect of level of day (j = 1-3), $Room_k$ is the effect of the k^{th} level of the block (room) (k = 1-4), and ε_{ijk} is the effect of the uncontrollable factors. For *Clostridium perfringen*s, the data were not distributed normally due to the small number of positive observations and transformations that were tried did not change this.

4.6 RESULTS AND DISCUSSION

4.6.1 GROWTH PERFORMANCE

When the growth performance data for Experiments 1 and 2 were analyzed initially, there was a significant ($P<0.05$) effect of trial on the growth parameters. Therefore, the growth performance data were analyzed together with rooms in the two trials as blocks. The inclusion of antibiotic or lysozyme in the broiler diets at different periods during the production cycle had no effects ($P>0.05$) on body weight (Table 4.5), daily weight gain

(Table 4.6), feed consumption (Table 4.7) or FCR (Table 4.8). There was a blocking effect on all the growth parameter ($P<0.05$).

Table 4.5 Effect of antibiotics and timing of introduction of lysozyme to the feed on body weight (g bird^{-1}) of broiler chickens.

Treatment	Age (day)				
	4	14	24	35	Treatment mean
Control	89±17	345±17	927±17	1884±17	811±8
Antibiotic	90±17	360±17	939±17	1897±17	821±8
Lysozyme	89±17	354±17	942±17	1863±17	812±8
Lysozyme Days 0-4	90±17	352±17	935±17	1891±17	817±8
Lysozyme Days 5-14	87±17	351±17	924±17	1868±17	808±8
Lysozyme Days 15-24	91±17	360±17	947±17	1893±17	823±8
Lysozyme Days 25-35	88±17	359±17	946±17	1902±17	824±8
Day mean	89±17^{d}	354±17^{c}	937±17^{b}	1885±17^{a}	
ANOVA	P value				
Room	<0.0001				
Treatment	0.6741				
Age	<0.0001				
Treatment x Age	0.9999				

a-d: Lsmeans for age with different letters differ significantly ($P<0.05$)

Table 4.6 Effect of antibiotics and timing of introduction of lysozyme to the feed on body weight gain (g bird^{-1}day^{-1}) of broiler chickens.

Treatment	Age (days)				
	0-4	5-14	15-24	25-35	Treatment mean
Control	10.2±1.2	28.5±1.2	58.2±1.2	86.9±1.2	46.0±0.5
Antibiotic	10.5±1.2	29.9±1.2	57.9±1.2	87.0±1.2	46.3±0.5
Lysozyme	10.3±1.1	29.4±1.1	58.6±1.1	82.6±1.1	45.5±0.5
Lysozyme Days 0-4	10.4±1.2	29.2±1.2	58.3±1.2	87.0±1.2	46.2±0.5
Lysozyme Days 5-14	10.2±1.2	29.3±1.2	57.3±1.2	85.8±1.2	45.7±0.5
Lysozyme Days 15-24	10.6±1.2	30.0±1.2	58.8±1.2	86.1±1.2	46.4±0.5
Lysozyme Days 25-35	10.2±1.2	30.1±1.2	58.7±1.2	86.8±1.2	46.5±0.5
Age mean	10.3±0.4^{d}	29.5±0.4^{c}	58.3±0.4^{b}	86.2±0.4^{a}	
ANOVA	P value				
Room	<0.0001				
Treatment	0.7489				
Age	<0.0001				
Treatment x Age	0.9917				

a-d: Lsmeans for age with different letters differ significantly ($P<0.05$)

Table 4.7 Effect of antibiotics and timing of introduction of lysozyme to the feed on feed consumption (g bird^{-1}day^{-1}) of broiler chickens.

Treatment	Age (days)				
	0-4	5-14	15-24	25-35	Treatment mean
Control	13.2±2.1	51.1±2.1	99.8±2.1	155.6±2.1	79.9±1.0
Antibiotic	13.5±2.1	54.9±2.1	101.3±2.1	155.6±2.1	81.3±1.0
Lysozyme	12.7±2.1	51.1±2.1	96.2±2.1	150.9±2.1	77.8±0.9
Lysozyme Days 0-4	13.2±2.1	52.6±2.1	98.7±2.1	158.1±2.1	80.7±1.0
Lysozyme Days 5-14	12.8±2.2	51.9±2.1	98.0±2.1	154.3±2.1	79.3±1.0
Lysozyme Days 15-24	13.2±2.1	50.8±2.1	98.8±2.1	157.7±2.1	80.1±1.0
Lysozyme Days 25-35	13.0±2.1	51.5±2.1	93.0±2.1	157.6±2.1	78.7±0.9
Age mean	13.1±0.8^{d}	52.0±0.8^{c}	98.0±0.8^{b}	155.7±0.8^{a}	
ANOVA	P value				
Room	<0.0001				
Treatment	0.2052				
Age	<0.0001				
Treatment x Age	0.8135				

a-d: Lsmeans for age with different letters differ significantly (P<0.05)

Table 4.8 Effect of antibiotics and timing of introduction of lysozyme to the feed on feed conversion ratio (feed gain^{-1}) of broiler chickens.

Treatment	Age (days)				
	0-4	5-14	15-24	25-35	Treatment mean
Control	1.27±0.05	1.80±0.05	1.70±0.05	1.79±0.05	1.64±0.02
Antibiotic	1.27±0.05	1.83±0.05	1.75±0.05	1.80±0.05	1.66±0.02
Lysozyme	1.27±0.04	1.76±0.04	1.65±0.04	1.82±0.04	1.62±0.02
Lysozyme Day 0-4	1.29±0.05	1.78±0.05	1.70±0.05	1.83±0.05	1.65±0.02
Lysozyme Days 5-14	1.26±0.05	1.78±0.05	1.71±0.05	1.80±0.05	1.64±0.02
Lysozyme Days 15-24	1.26±0.04	1.71±0.04	1.68±0.04	1.85±0.04	1.62±0.02
Lysozyme Days 25-35	1.27±0.04	1.72±0.04	1.59±0.04	1.83±0.04	1.60±0.02
Age mean	1.27±0.02^{c}	1.77±0.02^{a}	1.68±0.02^{b}	1.82±0.02^{a}	
ANOVA	P value				
Room	0.0116				
Treatment	0.5512				
Age	<0.0001				
Treatment x Age	0.9215				

a-c: Lsmeans for age with different letters differ significantly (P<0.05)

FCR in the period of days 5-14 was poorer than those in the period of days 0-4 and 15-24. The reason was birds from days 5-14 consumed more feed but had less weight gain caused be some environmental factors such as temperature and RH. Liu et al. (2010) found adding 40 ppm lysozyme to the diet did not increase broiler weight gain or feed intake from days 14-28 when broilers were reared on litter covered floor with optimal environmental temperatures. Humphrey et al. (2002) reported that a combination of 10% of transgenic rice expressing lysozyme and 5% of transgenic rice expressing lactoferrin in the diets improved FCR of broiler chickens from days 3-20. MacIsaac and Anderson (2008) conducted an experiment that showed turkeys fed diets with 200 ppm lysozyme or a combination of 200 ppm lysozyme and 4.4 ppm antibiotics (BMD) had heavier body weight on day 55 than those fed diets with antibiotics the entire production cycle or without any supplement. Gillcrist (2012) included that the inclusion of lysozyme and Oasis® (a hydrated hatchling feed supplement) in the transport containers during 24 hours of transport of female turkey poults and were able to detect an increase in body weight at 55 and 69 days of age. Studies about feeding lysozyme in different periods of the growth cycle of broiler chickens are limited.

There are a number of examples of studies that have demonstrated the benefit of including virginiamycin in broiler diets. Dumonceaux et al. (2006) conducted a study with broiler chickens found birds fed virginiamycin had increased body weight and better FCR compared to those fed without virginiamycin. Cavazzoni et al. (1998) found that virginiamycin increased the weight gain, but did not impact feed consumption or FCR of broilers. George et al. (1982) reported that virginiamycin protected chickens against

experimentally induced NE and significantly increased the growth performance compared to birds given no antibiotic at all. Donabedian et al. (2003) found that chickens fed virginiamycin had a 13% increased mean body weight. In the same study, however, some strains of *E. faecium* became resistant to virginiamycin.

However, the current study failed to demonstrate a benefit to growth performance for either virginiamycin or lysozyme. The clean litter bedding and effective biosecurity in this trial may have reduced the likelihood that the chickens would be challenged by bacteria in the environment (Bojesen et al. 2010). When broilers are not under an environmental stress, dietary antibiotics or alternative antimicrobials may not improve growth performance.

Lysozyme had no effect on mortality rate of broiler chickens ($P>0.05$) (Table 4.9). There was a blocking factor on mortality rate ($P<0.05$). The period of days 25-35 had a higher mortality rate than days 0-4 ($P<0.05$). The majority of mortalities were caused by ascites and leg deformities. Ascites is also known as pulmonary hypertension syndrome (Wideman et al. 1997). The factors that induce ascites include extremes in environmental temperature, elevated level of dust, carbon dioxide, ammonia and a lack of oxygen (McGovern et al. 1999). Low temperature is the main cause for ascites (Canadian Poultry Consultants Ltd. 2013).

Table 4.9 Effect of antibiotics and timing of introduction of lysozyme to the feed on mortality rate (%) of broiler chickens.

Treatment	Age (days)				Treatment mean	Age (days)
	0-4	5-14	15-24	25-35		0-35
Control	0.5±0.5	0.3±0.5	0.8±0.5	4.0±0.5	1.4±0.2	5.6±0.9
Antibiotic	0.0±0.5	0.5±0.5	1.0±0.5	4.6±0.5	1.5±0.2	6.1±0.9
Lysozyme	0.0±0.5	0.3±0.5	1.2±0.5	2.5±0.5	1.0±0.2	4.0±0.9
Lysozyme Days 0-4	0.3±0.5	0.2±0.5	1.4±0.5	2.5±0.5	1.1±0.2	4.4±0.9
Lysozyme Days 5-14	0.5±0.5	0.3±0.5	1.2±0.5	4.0±0.5	1.5±0.2	5.9±0.9
Lysozyme Days 15-24	0.2±0.5	0.3±0.5	0.5±0.5	3.4±0.5	1.1±0.2	4.3±0.9
Lysozyme Days 25-35	0.2±0.5	1.3±0.5	0.3±0.5	3.5±0.5	1.3±0.2	5.2±0.9
Age mean	0.2±0.2[b]	0.5±0.2[ab]	0.9±0.2[ab]	3.5±0.2[a]		
ANOVA	P value					
Room	0.0004					<0.0001
Treatment	0.6094					0.5227
Age	<0.0001					
Treatment x Age	0.4007					

a-b: Lsmeans for days with different letters differ significantly (P<0.05)

4.6.2 INTESTINAL MICROBIOTA

The number of aerobic bacteria, anaerobic bacteria, coliforms, *E. coli.*, lactic acid bacteria and *Clostridium perfringens* in the ileum were not influenced (P>0.05) by the inclusion of the antibiotic or lysozyme in the diets on day 4 (Table 4.10), day 14 (Table 4.11), day 24 (Table 4.12), day 35 (Table 4.13) or throughout the trial (Table 4.14). On day 4, there was a blocking effect (P<0.05) on the numbers of aerobes, coliforms and *E. coli*. On day 14, these blocking effects were significant (P<0.05) for the numbers of aerobes, anaerobes and lactic acid bacteria. There was no blocking effect (P>0.05) on bacterial numbers for samples collected from 24-day-old birds. On day 35, the blocking effect was only apparent (P<0.05) for the number of coliforms. Blocking effects were not significant (P>0.05) when data were combined throughout the trial.

Table 4.10 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 4.

Treatment	Aerobes	Anaerobes	Coliforms	*E. coli*	Lactic acid bacteria	*Clostridium perfringens*
Control	6.48±0.26	8.59±0.15	5.59±0.36	5.30±0.40	7.78±0.05	1.41±0.15
Antibiotic	6.77±0.26	8.56±0.15	5.11±0.36	5.05±0.40	7.92±0.05	1.00±0.15
Lysozyme	5.86±0.26	8.32±0.15	5.05±0.36	4.78±0.40	7.94±0.05	1.08±0.15
Lysozyme Days 0-4	6.93±0.26	8.21±0.15	5.16±0.36	4.82±0.40	7.70±0.05	1.08±0.15
Lysozyme Days 5-14	6.93±0.26	8.26±0.15	5.91±0.36	5.81±0.40	7.80±0.05	1.00±0.15
Lysozyme Days 15-24	6.64±0.26	8.38±0.15	5.14±0.36	5.00±0.40	7.86±0.05	1.26±0.15
Lysozyme Days 25-35	6.60±0.26	8.25±0.15	5.66±0.36	5.47±0.40	7.86±0.05	1.14±0.15
ANOVA	P-value					
Room	0.0018	0.9169	0.0357	0.0255	0.5400	0.2891
Treatment	0.0891	0.4061	0.4996	0.5114	0.2085	0.4661

Table 4.11 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfrigens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 14.

Treatment	Aerobes	Anaerobes	Coliforms	*E. coli*	Lactic acid bacteria	*Clostridium perfringens*
Control	6.39±0.26	6.68±0.22	3.48±0.30	3.41±0.29	6.46±0.22	1.10±0.15
Antibiotic	6.38±0.26	6.44±0.22	3.95±0.30	3.86±0.29	6.31±0.22	1.41±0.15
Lysozyme	6.40±0.26	6.43±0.22	3.95±0.30	3.77±0.29	6.57±0.22	1.12±0.15
Lysozyme Days 0-4	6.41±0.26	6.37±0.22	3.71±0.30	3.89±0.31	6.36±0.22	1.32±0.15
Lysozyme Days 5-14	6.28±0.26	6.44±0.21	3.88±0.30	3.41±0.29	6.48±0.22	1.17±0.15
Lysozyme Days 15-24	6.64±0.26	6.60±0.21	4.00±0.30	3.96±0.29	6.69±0.22	1.15±0.15
Lysozyme Days 25-35	6.29±0.26	6.42±0.21	3.85±0.30	3.76±0.29	6.59±0.22	1.23±0.15
ANOVA	P-value					
Room	0.0024	0.0024	0.1502	0.1874	0.0063	0.3903
Treatment	0.9724	0.9525	0.8915	0.7347	0.8901	0.7302

Table 4.12 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfrigens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 24.

Treatment	Aerobes	Anaerobes	Coliforms	*E. coli*	Lactic acid bacteria	*Clostridium perfringens*
Control	6.49±0.23	6.77±0.20	4.26±0.31	4.66±0.33	6.86±0.22	1.43±0.26
Antibiotic	6.29±0.23	6.69±0.20	4.92±0.31	4.26±0.33	6.73±0.22	1.25±0.26
Lysozyme	6.35±0.23	6.79±0.20	5.01±0.31	4.31±0.36	6.92±0.24	1.34±0.29
Lysozyme Days 0-4	6.47±0.23	6.63±0.20	4.77±0.31	4.47±0.33	7.12±0.21	1.37±0.26
Lysozyme Days 5-14	6.75±0.23	6.91±0.20	4.63±0.31	4.65±0.33	6.61±0.22	1.77±0.26
Lysozyme Days 15-24	6.16±0.23	6.48±0.20	4.68±0.31	4.72±0.33	6.83±0.22	1.40±0.27
Lysozyme Days 25-35	6.58±0.23	6.80±0.20	5.09±0.31	4.64±0.33	6.99±0.22	1.64±0.26
ANOVA	P-value					
Room	0.6016	0.7509	0.9361	0.9568	0.8030	0.1908
Treatment	0.6566	0.8168	0.5488	0.9341	0.7091	0.8186

Table 4.13 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfrigens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 35.

Treatment	Aerobes	Anaerobes	Coliforms	*E. coli*	Lactic acid bacteria	*Clostridium perfringens*
Control	7.01±0.18	7.40±0.18	4.88±0.27	4.03±0.42	7.44±0.19	1.13±0.17
Antibiotic	6.94±0.18	7.37±0.18	5.18±0.27	4.51±0.42	7.42±0.19	1.57±0.17
Lysozyme	7.28±0.18	7.53±0.18	5.33±0.27	5.02±0.42	7.69±0.19	1.62±0.17
Lysozyme Days 0-4	7.38±0.18	7.74±0.18	5.00±0.27	4.22±0.42	7.78±0.19	1.20±0.17
Lysozyme Days 5-14	7.08±0.18	7.32±0.18	4.86±0.27	4.29±0.42	7.40±0.19	1.25±0.17
Lysozyme Days 15-24	7.13±0.18	7.45±0.18	4.97±0.27	4.38±0.42	7.56±0.19	1.19±0.17
Lysozyme Days 25-35	7.05±0.18	7.64±0.18	4.78±0.27	4.23±0.42	7.68±0.19	1.29±0.17
ANOVA	P-value					
Room	0.3243	0.8613	0.0397	0.1694	0.9188	0.2035
Treatment	0.6452	0.6618	0.8133	0.7372	0.7026	0.2693

Table 4.14 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfrigens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens throughout the trial.

Treatment	Aerobes	Anaerobes	Coliforms	*E. coli*	Lactic acid bacteria	*Clostridium perfringens*
Control	6.59±0.14	7.40±0.16	4.55±0.19	4.35±0.20	7.13±0.13	1.26±0.10
Antibiotic	6.59±0.14	7.30±0.16	4.79±0.19	4.41±0.20	7.10±0.13	1.30±0.10
Lysozyme	6.47±0.14	7.29±0.16	4.83±0.19	4.51±0.20	7.27±0.13	1.35±0.10
Lysozyme Day 0-4	6.80±0.14	7.26±0.16	4.66±0.19	4.36±0.20	7.24±0.13	1.24±0.10
Lysozyme Day 5-14	6.76±0.14	7.23±0.16	4.82±0.19	4.55±0.20	7.07±0.13	1.31±0.10
Lysozyme Day 15-24	6.64±0.14	7.23±0.16	4.70±0.19	4.49±0.20	7.24±0.13	1.21±0.10
Lysozyme Day 25-35	6.63±0.14	7.30±0.16	4.84±0.19	4.50±0.20	7.29±0.13	1.31±0.10
ANOVA	P-value					
Room	0.3744	0.6570	0.1321	0.1081	0.4756	0.3739
Treatment	0.6985	0.9934	0.9083	0.9984	0.8474	0.9675

Zhang et al. (2006) conducted *in vitro* studies using the same lysozyme as our study and found that 50 ppm lysozyme in a micro-broth dilution assay reduced the α-toxin produced by *Clostridium perfringens*. They reported that 200 ppm lysozyme completely inhibited the growth of *Clostridium perfringens* and toxin. Another *in vitro* experiment using EDTA modified lysozyme from egg white reported that 500 ppm and 1250 ppm inhibited *Clostridium perfringens* in digesta collected from broilers fed maltodextrin (Ofori 2012). Liu et al. (2010) reported that lysozyme fed to broilers at 40 ppm did not reduce the presence of *Clostridium perfringens*, *E. coli* or *Lactobacillus* in ileum samples of broiler chickens. Ofori (2012) found that *in vivo* 20 g lysozyme in 1kg maltodextrin feed had no effect on the number of *Clostridium perfringens* in the ileum samples using traditional bacteria culturing methods, but reduced on Gram-positive bacteria (bacilli) when NGS methods were employed. The birds grown in our trial were raised under optimal environmental conditions, therefore, the bacteria in the intestinal tract may have been different in type and number compared to samples from birds grown under sub-optimal environmental conditions. Additionally, the lysozyme used in this experiment was EDTA modified. The activity of lysozyme against bacteria may not have been maximized for this reason. Zhang et al. (2010) and Liu et al. (2010) both challenged the birds in their studies by directly feeding *Clostridium perfringens*. The challenge significantly increased the population of *Clostridium perfringens* in the intestine and compromised the bird's growth performance. Both of these studies reported that supplementation of lysozyme in the diets prevented intestinal lesions typical of NE caused by *Clostridium perfringens.* The number of *Clostridium perfringens* was reduced on day 28 with 200 ppm radiant energy–treated lysozyme (Zhang et al. 2010) and day 21 with 40 ppm lysozyme (Liu et al.

2010). The authors did not mention what kind of lysozyme was used and whether it was modified in any way (Liu et al. 2010). Dumonceaux et al. (2006) found that dietary supplementation with virginiamycin increased the number of *Lactobacillus* in the GIT, and reduced the bacteria species in the distal location.

Processing and supplementation of other feed ingredients can strengthen the antimicrobial function. EDTA acts synergistically with lysozyme against bacteria, such as *Listeria monocytogene* (Branen and Davidson 2004). The combination of Lysozyme and EDTA suppressed the growth of *Listeria monocytogenes*, and inhibited microbiota to prevent meat spoilage (Wang and Shelef 1992). In this study, lysozyme was mixed with EDTA in a ratio of 20:80, but had no detectable effect on intestinal microbiota. The effect of lysozyme might be improved by other methods. The combination of lysozyme with other supplements such as nisin, has been shown to improve overall antibacterial activity (Chung et al. 2000). Other than supplementation with other substances, the activities of lysozyme against bacteria may be improved by thermal modification, which was conducted by heating the solutions to 80 C for 20 min, and then spray drying (Lesnierowski et al. 2001). Compared to common chicken egg white lysozyme, ultrafiltration-modified lysozyme had improved antimicrobial function (Lesnierowski et al. 2009).

4.7 Conclusions

The inclusion of 100 ppm of lysozyme and 250 ppm of virginiamycin had no effect on the growth performance and intestinal microbiota of broiler chickens. The egg white

lysozyme mixed with EDTA provided in this study might be less effective than other versions of lysozyme. If appropriate processing was applied to the lysozyme, or a combination of lysozyme with other substances, such as nisin and lactoferrin, the effect on growth performance and intestinal microbiota might be more evident. The growing conditions provided in this study which included strict biosecurity may have minimized the chances for birds to get harmful organisms infection. Potential effects of lysozyme were not detected under this condition. However, neither was there an improvement in performance when virginiamycin was administered. The likely lack of a challenge for the broiler in this study have led to a subsequent trial with used litter provided as a potential challenge to the birds. The trial will be reported in the next chapter.

CHAPTER 5 THE EFFECT OF LYSOZYME IN EACH PERIOD OF GROWTH CYCLE ON BROILER CHICKENS GROWN ON USED LITTER

5.1 ABSTRACT

Lysozyme could be used as alternatives to antibiotics in broiler production. Adding lysozyme in only the critical periods of the growth cycle of broiler chickens may be an effective to reduce the impact of the high cost of lysozyme ($40 kg^{-1}). This study was conducted to determine the effect of dietary lysozyme on the growth performance and intestinal microbiota in each period of the growth cycle of broiler chickens grown on previously used litter. This trial was designed as a one way analysis of variance with length of time in which the lysozyme at 100 ppm was fed to the birds as the main factor (-ve lysozyme days 0-35; +ve lysozyme days 0-5; +ve lysozyme days 6-14; +ve lysozyme days 15-24; +ve lysozyme days 25-35; +ve lysozyme days 0-35; +ve virginiamycin (250 ppm) days 0-35). The supplementation of 100 ppm lysozyme and 250 ppm of antibiotics had no effect on growth performance of broiler chickens ($P>0.05$). On day 35, inclusion of the antibiotics throughout the trial significantly lowered the number of total aerobic and anaerobic bacteria in the ileum compared to those fed the control diets and lysozyme throughout the trial and from days 15-24 ($P<0.05$). The *E. coli* numbers in the ileum of the birds fed lysozyme for the duration (4.28 log_{10} cfu g^{-1}) of the trial as well as during days 5 to 14 (4.23 log_{10} cfu g^{-1}) were lower than samples from birds fed the antibiotics (4.97 log_{10} cfu g^{-1}). Lysozyme has potential to maintain healthy intestinal microbiota.

Key words: antibiotics, lysozyme, used litter, growth cycle, growth performance, intestinal microbiota, *E. coli*.

5.2 INTRODUCTION

When birds are grown under optimal housing conditions with strict biosecurity, the possibility for development of bacterial infections is low (Bojesen et al. 2010). In this situation, the effect of lysozyme or antibiotics on intestinal microbiota might be reduced. Used litter reserves and transmits harmful bacteria such as clostridia and staphylococci from flock to flock, which can be a concern for chicken production (Martin and MacCann 1998; Lu et al. 2003). Zhang et al. (2010) and Liu et al. (2010) both challenged broilers with an oral *Clostridium perfringens* treatment when testing the effect of lysozyme on growth performance and intestinal microbiota of broilers and found positive effect on microbiota. There are no published studies focused on determing the effect of lysozyme on broilers using previously used litter as a method for evaluating a feed ingredient in a potentially stressful environment.

5.3 OBJECTIVE

To determine the effect of dietary lysozyme on the growth performance and intestinal microbiota in each period during the growth cycle of broiler chickens grown on used litter material.

5.4 HYPOTHESES

Feeding broiler chickens lysozyme at key time periods will improve growth performance and reduce *Clostridium perfringens*, detrimental *E. coli* and total coliforms in the small intestine when used litter is provided.

5.5 Materials and Methods

5.5.1 Animal housing and husbandry

In this trial, 2184 male Ross 308 broiler chickens from Clark's Hatchery (Burtts Corner, NB) were used. Birds arrived at the Atlantic Poultry Research Centre in Truro, NS on the day of hatch, and were immediately randomly assigned to eighty-four, 99 cm x 218 cm, floor pens equally distributed within four rooms. There were 26 birds in each pen with a stocking density of 0.6 kg/m^2 on day 0 and 22.1 kg/m^2 on day 35. Upon arrival, the chicks were immediately provided with feed and water. Feed was provided *ad libitum* in cardbord box lids for the first week and in tube feeders for the entire experiment. Birds were introduced to water by dipping beaks in the nipple drinker. Litter from a previous broiler flock was mixed with clean hard wood shavings at a ratio of 50:50 to provide an environmental challenge to the birds. Lighting was set according to standard operating procedure (Appendix A) Ambient temperature was recorded by data logger (Appendix B-3). The RH of this trial was not reported due to malfunction of data logger. All procedures were carried out under the guidance of Canadian Council on Animal Care (CCAC 2009).

5.5.2 Diets

Diets were isocaloric and isonitrogenous within starter (days 0-14), grower (days 15-24) and finisher (days 25-35) phases (Table. 5.1). Diets were formulated to meet or exceed the National Research Council (1994) nutrient requirements for each stage. Dietary treatments provided to the birds followed the same protocol as in Section 4.5.2.

Table 5.1 Composition of experimental diets containing lysozyme and antibiotic fed from 1-14, 15-24 and 25-35 days of age.

Growth periods	Starter			Grower			Finisher		
Days of age	1-14			15-24			25-35		
Ingredients (%)	C	A	L†	C	A	L	C	A	L
Corn	43.16	43.13	43.16	50.76	50.69	50.74	56.90	56.86	56.89
Soybean meal	38.98	38.98	38.98	31.21	31.23	31.22	25.85	25.85	25.85
Wheat	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Tallow blend	3.68	3.68	3.68	4.23	4.27	4.24	3.66	3.68	3.67
limestone	1.67	1.67	1.67	1.43	1.43	1.43	1.40	1.40	1.40
Dicalcium phosphate	0.83	0.83	0.83	0.71	0.71	0.71	0.64	0.64	0.64
Vitamin/mineral premix[z]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Iodized salt	0.43	0.43	0.43	0.40	0.40	0.40	0.40	0.40	0.40
Methionine premix[y]	0.64	0.64	0.64	0.58	0.59	0.58	0.53	0.53	0.53
Coban[x]	0.05	0.05	0.05	0.05	0.05	0.05	-	-	-
Lysine 98%	0.06	0.06	0.06	0.12	0.12	-	0.128	0.128	0.128
Stafac 44[w]	-	0.025	-	-	0.025	-	-	0.025	-
Lysozyme[v]	-	-	0.01	-	-	0.01	-	-	0.01
Calculated analysis									
AME_n (Kcal/kg) [u]	3050	3050	3050	3150	3150	3150	3200	3200.00	3200.00
Protein (%)	23.00	23.00	23.00	20.00	20.00	20.00	18.00	18.00	18.00
Calcium (%)	1.05	1.05	1.05	0.90	0.90	0.90	0.85	0.85	0.85
Phosphorus (%)	0.50	0.50	0.50	0.45	0.45	0.45	0.42	0.42	0.42
Lysine (%)	1.43	1.43	1.43	1.24	1.24	1.24	1.09	1.09	1.09
Methionine (%)[t]	0.69	0.69	0.69	0.63	0.63	0.63	0.58	0.58	0.58
Meth+cyst (%)	1.07	1.07	1.07	0.95	0.95	0.95	0.86	0.86	0.86
Sodium (%)	0.19	0.19	0.19	0.18	0.18	0.18	0.18	0.18	0.18
Determined analysis									
Crude protein (%)	23.94	22.87	23.11	20.44	19.94	20.06	19.19	19.24	19.13
Total calcium (%)	0.91	0.97	0.97	0.76	0.83	0.88	0.83	0.78	0.82
Total phosphorus (%)	0.61	0.57	0.60	0.53	0.53	0.54	0.49	0.51	0.53

†C: Control; A: Antibiotics; L: Lysozyme.

[z] Supplied per kg diet: vitamin A, 9750 IU; vitamin D_3, 2000 IU; vitamin E, 25 IU; vitamin K, 2.97 mg; riboflavin, 7.6 mg; Dl Ca-pantothenate, 13.5 mg; vitamin B_{12}, 0.023 mg; niacin, 29.7; folic acid, 4.0 mg, choline, 801 mg; biotin, 0.3 mg; pyridoxine, 4.95 mg; thiamine, 2.91 mg; manganese, 70.2 mg; zinc, 80.0 mg; copper, 25 mg; selenium, 0.15 mg; ethoxyquin, 50 mg; wheat middlings, 1432 mg; ground limestone, 500 mg.

[y] Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middlings, 0.5 kg.

[x] Coccidiostat - Coban (active ingredient monensin sodium, 200 g kg^{-1}) Elanco Animal Health, Division Eli Lilly Canada Inc., Guelph, ON, Canada.

[w] Virginiamycin 44 g kg^{-1}, Zoetis Animal Health, London, ON, Canada.

[v] Inovapure 213 (active ingredient lysozyme at 20%, 24,000 Shugar units mg^{-1}) Neova Technologies, Inc.,Abbotsford, BC., Canada.

[u] Nitrogen-corrected apparent metabolizable energy.

[t] Methionine and cystine

5.5.3 Analysis of growth performance

Growth performance data collected was similar and followed the same procedures as reported in Section 4.5.3.

5.5.4 Digesta sample collection

Two birds per pen on day 4 and one bird per pen on days 14, 24 and 35 were euthanized by cervical dislocation. Approximately 15 cm of ileum, one cm anterior to the ileal cecal junction, was aseptically harvested. From each intestinal sample, Two cm from the proximal and distal ends of each sample was cut and stored in -80 C freezers until NGS analysis will be performed in the future. Another two cm from the distal end was cut and submerged in a 2% formalin solution until the future analysis of intestinal histology (NGS and histology results are not reported in this thesis). The remaining samples were analyzed by conventional bacteria culture methods on the same day of collection.

5.5.5 Microbiota analysis

Microbiota data were collected at the same time points following the same procedures as described in Section 4.5.5.

5.5.6 Statistical analysis

Data of growth performance and intestinal microbiota in this trial were analyzed the same way as described in Section 4.5.6.

5.6 RESULTS AND DISCUSSION

5.6.1 GROWTH PERFORMANCE

The antibiotic and lysozyme treatments had no effects (P>0.05) on growth performance including body weight (Table 5.2), body weight gain (Table 5.3), feed consumption (Table 5.4) and FCR (Table 5.5). The blocking effects were significant for body weight gain and FCR.

Table 5.2 Effect of antibiotics and timing of introduction of lysozyme to the feed on body weight (g bird^{-1}) of broiler chickens.

Treatment	Age (day)				
	4	14	24	35	Treatment mean
Control	80±10	386±10	1102±10	2170±10	935±7
Antibiotic	80±11	375±11	1061±11	2175±11	923±7
Lysozyme	81±11	385±11	1095±11	2147±11	927±7
Lysozyme Days 0-4	80±10	377±10	1089±10	2195±10	935±7
Lysozyme Days 5-14	77±11	363±11	1065±11	2154±11	915±7
Lysozyme Days 15-24	80±10	386±10	1106±10	2187±10	940±7
Lysozyme Days 25-35	78±10	377±10	1083±10	2163±10	925±7
Age mean	79±4^{d}	378±4^{c}	1086±4^{b}	2170±4^{a}	
ANOVA	P value				
Room	0.0921				
Treatment	0.1623				
Age	<0.0001				
Treatment x Age	0.1330				

a-d: Lsmeans for age with different letters differ significantly (P<0.05)

Table 5.3 Effect of antibiotics and timing of introduction of lysozyme to the feed on body weight gain (g bird^{-1}day^{-1}) of broiler chickens.

Treatment	Age (days)				
	0-4	5-14	15-24	25-35	Treatment mean
Control	8.6±0.9	34.0±0.9	71.57±0.9	97.09±0.9	52.8±0.4
Antibiotic	8.1±0.9	32.9±0.9	68.8±0.9	101.3±0.9	52.8±0.4
Lysozyme	8.5±1.0	33.9±0.9	71.1±0.9	95.7±0.9	52.3±0.4
Lysozyme Days 0-4	8.3±1.0	33.0±0.9	71.2±0.9	100.5±0.9	53.2±0.4
Lysozyme Days 5-14	7.7±0.9	31.8±0.9	70.1±0.9	99.0±0.9	52.2±0.4
Lysozyme Days 15-24	8.5±1.0	34.0±0.9	72.0±0.9	98.2±0.9	53.2±0.4
Lysozyme Days 25-35	8.2±1.0	33.2±0.9	70.6±0.9	98.2±0.9	52.5±0.4
Age mean	8.3±0.4^{d}	33.3±0.3^{c}	70.8±0.3^{b}	98.6±0.3^{a}	
ANOVA	P value				
Room	0.0229				
Treatment	0.3731				
Age	<0.0001				
Treatment x Age	0.0339				

a-d: Lsmeans for age with different letters differ significantly (P<0.05)

Table 5.4 Effect of antibiotics and timing of introduction of lysozyme to the feed on feed consumption (g bird^{-1}day^{-1}) of broiler chickens.

Treatment	Age (days)				
	0-4	5-14	15-24	25-35	Treatment mean
Control	10.6±1.1	44.8±1.1	101.7±1.1	167.4±1.1	81.1±0.7
Antibiotic	10.4±1.2	42.8±1.2	97.2±1.2	164.8±1.2	78.8±0.7
Lysozyme	10.6±1.2	44.9±1.2	101.1±1.2	167.0±1.2	80.9±0.7
Lysozyme Day 0-4	10.8±1.2	42.9±1.1	101.5±1.1	167.4±1.1	80.6±0.7
Lysozyme Day 5-14	10.1±1.2	41.9±1.2	98.9±1.2	168.7±1.2	79.9±0.7
Lysozyme Days 15-24	10.7±1.3	45.1±1.1	102.1±1.1	165.2±1.1	80.8±0.7
Lysozyme Days 25-35	10.5±1.2	44.1±1.1	100.5±1.1	166.8±1.1	80.5±0.7
Age mean	10.5±0.5^{d}	43.8±0.4^{c}	100.4±0.4^{b}	166.8±0.4^{a}	
ANOVA	P value				
Room	0.2232				
Treatment	0.2191				
Age	<0.0001				
Treatment x Age	0.2987				

a-d: Lsmeans for age with different letters differ significantly (P<0.05)

Table 5.5 Effect of antibiotics and timing of introduction of lysozyme to the feed on feed conversion ratio (feed gain^{-1}) of broiler chickens.

Treatment	Age (day)				
	4	14	24	35	Treatment mean
Control	1.27±0.02	1.32±0.02	1.42±0.02	1.73±0.02	1.44±0.01
Antibiotic	1.30±0.02	1.30±0.02	1.42±0.02	1.63±0.02	1.41±0.01
Lysozyme	1.29±0.02	1.33±0.02	1.42±0.02	1.75±0.02	1.45±0.01
Lysozyme Days 0-4	1.30±0.02	1.30±0.02	1.43±0.02	1.67±0.02	1.42±0.01
Lysozyme Days 5-14	1.31±0.02	1.32±0.02	1.41±0.02	1.71±0.02	1.44±0.01
Lysozyme Days 15-24	1.29±0.02	1.33±0.02	1.42±0.02	1.68±0.02	1.43±0.01
Lysozyme Days 25-35	1.31±0.02	1.33±0.02	1.42±0.02	1.69±0.02	1.44±0.01
Age mean	1.30±0.01^{c}	1.32±0.01^{c}	1.42±0.01^{b}	1.69±0.01^{a}	
ANOVA	P value				
Room	0.0067				
Treatment	0.0913				
Age	<0.0001				
Treatment x Age	0.4463				

a-c: Lsmeans for age with different letters differ significantly (P<0.05)

The used litter in this experiment was from a previous broiler flock. This litter would contain bacteria from the previous flock of broilers, which would pose a potential challenge for the incoming chicks (Lu et al. 2002). The lack of a difference between the negative control birds (no antibiotic) and the birds given antibiotic indicated that use of reused litter was not enough of a challenge that restricted growth or affected the health of these birds (P>0.05). Other researchers have found improved performance with inclusion of dietary lysozyme. Humphrey et al. (2002) added 10% transgenic rice expressing lysozyme as a substitute for antibiotics in broiler diets. The reported results indicated an improvement of feed intake and FCR. Some have demonstrated the importance of a good challenge when evaluating alternatives to antibiotics. Zhang et al. (2010) reported that a lysozyme-based antimicrobial blend either treated with radiant energy or untreated when included at 200 ppm in broiler feed improved the body weight and FCR for birds gavaged

with *Clostridium perfringens*. With the same challenge, Liu et al. (2010) found 40 ppm lysozyme had no effect on body weight gain and FCR of broilers. Virginiamycin supplement in broiler diets as an antibiotic was found to improve growth performance (George et al. 1982; Cavazzoni et al. 1998; Dumonceaux et al. 2006). In our study, birds fed lysozyme did not have different growth compared to those fed no supplement or the commercial antibiotics. When providing challenge to the birds for growth performance analysis, the use of previously used litter mixed in new litter may not provide enough challenge when 50% dirty litter was used. The used litter may need to come from a flock that had poor performance to provide a real challenge to the next flock of birds.

The antibiotic and lysozyme had no effects on mortality rate ($P>0.05$) (Table 5.6). There was no day effect on the mortality rate ($P>0.05$).

The total mortality calculated through the study was 2.81%. Cause of mortality was mainly septicemia, leg deformities and ascites. No NE was diagnosed in birds necropsied. Diseases were not related to these treatments. In other challenge studies, researchers have successfully increased the incidence of NE in a flock of birds. Zhang et al. (2010) fed *Clostridium perfringens* to chicken orally, and this resulted in an increase of NE mortality from 0.09% to 32.06%. The supplementation of 200 ppm radiant energy treated lysozyme antimicrobial blend reduced the mortality of birds fed *Clostridium perfringens* to 9.04% ($P<0.05$).

Table 5.6 Effect of antibiotics and timing of introduction of lysozyme to the feed on mortality rate (%) of broiler chickens.

Treatment	Age (day)				Treatment mean	Age (day)
	0-4	5-14	15-24	25-35		0-35
Control	0.0±0.5	1.4±0.5	0.0±0.5	1.6±0.5	0.7±0.3	3.0±1.2
Antibiotic	1.4±0.5	2.0±0.5	0.2±0.5	1.4±0.5	1.2±0.3	4.7±1.2
Lysozyme	0.0±0.5	0.8±0.5	0.0±0.5	0.0±0.5	0.2±0.3	0.8±1.2
Lysozyme Day 0-4	1.6±0.5	0.3±0.5	1.5±0.5	0.8±0.5	1.1±0.3	4.2±1.2
Lysozyme Day 5-14	0.0±0.5	1.5±0.5	1.1±0.5	1.3±0.5	1.0±0.3	4.0±1.2
Lysozyme Day 15-24	0.3±0.5	0.4±0.5	0.4±0.5	0.0±0.5	0.3±0.3	1.0±1.2
Lysozyme Day 25-35	0.0±0.5	0.7±0.5	0.4±0.5	0.8±0.5	0.5±0.3	1.8±1.2
Day mean	0.5±0.2	1.0±0.2	0.5±0.2	0.8±0.2		
ANOVA	P value					
Room	0.5480					0.5708
Treatment	0.1217					0.1131
Day	0.1267					
Treatment x Day	0.1310					

There was no statistical analysis performed between data from the trials using new litter (Chapter 4) and the trial using used litter. In the new litter trials, the average day 35 body weight was 1885±17 g, compared to 2170±4 g in the used litter trial. The mortality during the used litter trial was 44.6% lower than previous clean litter trials. The expectation was that growth performance would be compromised in the used litter trial due to the presence of pathogenic bacteria from the used litter that could challenge bird health. The opposite of the expected results from those trials might be caused by a number of factors. The clean litter trials were conducted in the winter while the used litter trial was conducted in the summer. Even through the temperature should strictly follow the standard operation procedure, the weather outside still had an impact on the room temperature and RH. The used litter is from a trial (no lysozyme was provided) where the average day 35 body weight was over 2200 g. These fast growing healthy birds may have provided some beneficial bacteria such as lactobacilli in the used litter (Paco et al. 2003). These could serve as a probiotics that improve the bird growth performance. The birds used for each trial were from different parent stock. The health condition of the parent flocks might have been different.

5.6.2 INTESTINAL MICROBIOTA

The inclusion of the commercial antibiotic in the diets for the duration of the trial and lysozyme at different periods during the life cycle of the broilers had no impact on the number of aerobic bacteria, anaerobic bacteria, coliforms, lactic acid bacteria or *Clostridium perfringens* on day 4 (Table 5.7), day 14 (Table 5.8) and day 24 (Table 5.9). Blocking effects were significant for *Clostridium perfringens* on day 4 and 35 and aerobes, coliform and *E. coli* on day 24.

Table 5.7 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 4.

Treatment	Aerobes	Anaerobes	Coliforms	*E. coli*	Lactic acid bacteria	*Clostridium perfringens*
Control	7.65±0.12	7.73±0.13	4.71±0.25	4.23±0.26	7.78±0.23	3.35±0.24
Antibiotic	7.53±0.12	7.46±0.13	4.95±0.25	4.65±0.26	7.70±0.23	3.57±0.24
Lysozyme	7.41±0.12	7.41±0.13	4.33±0.25	3.82±0.26	7.85±0.23	3.13±0.24
Lysozyme Days 0-4	7.69±0.12	7.43±0.13	4.95±0.25	4.46±0.26	7.84±0.23	3.51±0.24
Lysozyme Days 5-14	7.56±0.12	7.51±0.13	4.43±0.25	4.03±0.26	7.48±0.23	3.10±0.24
Lysozyme Days 15-24	7.49±0.12	7.48±0.13	4.99±0.25	4.59±0.26	7.72±0.23	3.33±0.24
Lysozyme Days 25-35	7.49±0.12	7.48±0.13	4.19±0.25	3.79±0.26	7.48±0.23	3.33±0.24
ANOVA	P-value					
Room	0.3993	0.2785	0.7519	0.3820	0.9015	0.0178
Treatment	0.7107	0.6428	0.1238	0.0936	0.8393	0.7901

Table 5.8 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 14.

Treatment	Aerobes	Anaerobes	Coliforms	*E. coli*	Lactic acid bacteria	*Clostridium perfringens*
Control	5.86±0.26	6.25±0.24	4.78±0.27	4.23±0.26	6.31±0.20	2.25±0.33
Antibiotic	6.82±0.27	6.93±0.25	5.20±0.29	4.65±0.26	7.00±0.21	2.53±0.35
Lysozyme	5.76±0.27	5.99±0.25	4.29±0.29	3.82±0.26	6.34±0.21	2.67±0.35
Lysozyme Days 0-4	5.71±0.26	6.16±0.24	4.28±0.27	4.46±0.26	6.34±0.20	2.25±0.33
Lysozyme Days 5-14	5.68±0.26	6.33±0.24	4.45±0.27	4.03±0.26	6.44±0.20	2.39±0.33
Lysozyme Days 15-24	6.68±0.26	6.63±0.24	4.93±0.27	4.59±0.26	6.77±0.20	1.81±0.33
Lysozyme Days 25-35	6.35±0.27	6.54±0.25	5.16±0.29	3.79±0.26	6.64±0.21	2.70±0.35
ANOVA	P-value					
Room	0.8543	0.7143	0.4299	0.3820	0.1058	0.6043
Treatment	0.0078	0.1641	0.0866	0.0936	0.1678	0.5491

Table 5.9 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 24.

Treatment	Aerobes	Anaerobes	Coliforms	*E. coli*	Lactic acid bacteria	*Clostridium perfringens*
Control	6.31±0.23	7.49±0.18	5.27±0.28	5.10±0.28	7.08±0.19	1.74±0.30
Antibiotic	6.51±0.23	7.53±0.18	5.81±0.28	5.69±0.28	7.07±0.19	2.21±0.30
Lysozyme	6.28±0.23	7.35±0.18	5.19±0.28	5.09±0.28	6.85±0.19	1.99±0.30
Lysozyme Days 0-4	6.14±0.23	7.41±0.18	5.72±0.28	5.46±0.28	7.17±0.19	2.39±0.30
Lysozyme Days 5-14	5.75±0.23	7.21±0.18	4.90±0.28	4.64±0.28	6.80±0.19	2.26±0.30
Lysozyme Days 15-24	6.09±0.23	7.39±0.18	5.29±0.28	4.99±0.28	6.98±0.19	2.29±0.30
Lysozyme Days 25-35	6.08±0.23	7.20±0.18	5.10±0.28	4.52±0.28	6.68±0.19	1.94±0.30
ANOVA	P-value					
Room	0.0024	0.3716	0.0078	0.0118	0.2571	0.6486
Treatment	0.3953	0.8058	0.2294	0.0714	0.5585	0.7402

Others have reported similar findings. Liu et al. (2010) reported that lysozyme had no impact on *Lactobacillus* (the major lactic acid bacteria in the ileum) and *Bifidobacterium* on day 22 and day 28 under *Clostridium perfringens* challenge. However, the number of *Clostridium perfringens* was reduced by lysozyme supplementation at 40 ppm in the feed (Liu et al. 2010).

For samples collected on day 35, inclusion of the antibiotic significantly ($P<0.05$) lowered the number of total aerobic and anaerobic bacteria (Table 5.10) in the ileum compared to those fed the control diets and lysozyme throughout the trial and lysozyme from days 15-24.

Table 5.10 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* (log_{10} cfu g^{-1}) in the ileum of broiler chickens on day 35.

Treatment	Aerobes	Anaerobes	Coliforms	*E. coli*	Lactic acid bacteria	*Clostridium perfringens*
Control	6.31±0.19^{ab}	7.13±0.23^{a}	5.26±0.30	4.03±0.33	7.43±0.19	3.15±0.28
Antibiotic	5.86±0.19^{b}	6.16±0.23^{b}	4.76±0.30	4.60±0.33	6.89±0.19	2.32±0.28
Lysozyme	6.52±0.19^{ab}	7.53±0.23^{a}	4.84±0.30	4.35±0.33	7.46±0.19	2.30±0.28
Lysozyme Days 0-4	6.08±0.19^{ab}	6.68±0.23^{ab}	4.28±0.30	3.99±0.33	7.16±0.19	1.93±0.28
Lysozyme Days 5-14	6.29±0.20^{ab}	6.79±0.24^{ab}	4.51±0.32	4.02±0.36	7.12±0.21	2.44±0.30
Lysozyme Days 15-24	6.71±0.19^{a}	7.17±0.23^{a}	4.88±0.30	4.53±0.33	7.32±0.19	2.65±0.28
Lysozyme Days 25-35	6.05±0.19^{ab}	6.87±0.23^{ab}	4.87±0.30	4.50±0.33	7.21±0.19	2.67±0.28
ANOVA	P-value					
Room	0.6474	0.2799	0.3339	0.2579	0.3422	0.0443
Treatment	0.0459	0.0043	0.4076	0.3576	0.3836	0.1085

a-b: Lsmeans for aerobes and anaerobes numbers with different letters differ significantly ($P<0.05$)

The total aerobic bacteria measured in this experiment included obligate aerobes and facultative anaerobes. In the small intestine, most of the bacteria are facultative anaerobic, with *Streptococci*, *Lactobacillus* and *E. coli* making up the majority of the total bacteria. Other anaerobic bacteria including, *Eubacterium*, *Propionibacterium*, *Clostridium*, *Gemmiger* and *Fusobacterium* have been isolated from the small intestine of broilers (Salanitro et al. 1978). Streptococci are associated with some poultry diseases, such as peritonitis, salpingitis (Edwards and Hull 1937) and endocarditis (Jortner and Helmboldt 1971). Virginiamycin inhibited the growth of bacteria (Dumonceaux et al. 2006). However, this antibiotic led to resistant bacteria that can be transferred from chickens to humans (Cox and Popken 2004).

The *E. coli* numbers (Table 5.11) in the ileum samples of the birds fed lysozyme for the duration of the trial as well as during days 5 to 14 were lower ($P<0.05$) than the ileal number from those fed the antibiotic for the duration of the trial. Before transformation of the number of the data to $\log_{10}$ basis, the numbers of *E. coli* in the ileum when birds fed antibiotic, lysozyme, and lysozyme only on days 5-14 were 93325, 19054 and 16982 cfu g^{-1}, respectively. Lysozyme added in the diet reduced the numbers of *E. coli* by over 80% in the ileum compared to antibiotic.

Table 5.11 Effect of antibiotics and timing of introduction of lysozyme to the feed on total aerobes, anaerobes, coliforms, *E. coli*, lactic acid bacteria and *Clostridium perfringens* ($\log_{10}$ cfu g^{-1}) in the ileum of broiler chickens throughout the trial.

Treatment	Aerobes	Anaerobes	Coliforms	*E. coli*	Lactic acid bacteria	*Clostridium perfringens*
Control	6.53±0.15	7.15±0.13	5.00±0.15	4.76±0.16^{ab}	7.15±0.13	2.62±0.17
Antibiotic	6.67±0.16	7.02±0.13	5.17±0.15	4.97±0.16^{a}	7.17±0.13	2.67±0.17
Lysozyme	6.51±0.16	7.10±0.13	4.67±0.15	4.28±0.16^{b}	7.14±0.13	2.52±0.17
Lysozyme Days 0-4	6.41±0.15	6.92±0.13	4.81±0.15	4.43±0.16^{ab}	7.13±0.13	2.52±0.17
Lysozyme Days 5-14	6.32±0.16	6.97±0.13	4.58±0.15	4.23±0.16^{b}	6.95±0.13	2.55±0.17
Lysozyme Days 15-24	6.74±0.15	7.17±0.13	5.02±0.15	4.67±0.16^{ab}	7.20±0.13	2.52±0.17
Lysozyme Days 25-35	6.50±0.16	7.04±0.13	4.82±0.15	4.40±0.16^{ab}	7.01±0.13	2.66±0.17
ANOVA	P-value					
Room	0.4705	0.5080	0.4101	0.2111	0.8620	0.8863
Treatment	0.5251	0.7958	0.0764	0.0118	0.8085	0.9872

a-b: Lsmeans for *E. coli* numbers with different letters differ significantly ($P<0.05$)

This data indicates that the EDTA modified lysozyme can influence numbers of Gram-negative *E. coli*. Dean and Ward (1992) found that the use of an EDTA modified lysozyme lysed the cell wall of *E. coli* and released intracellular protein. Ellison and Giehl (1991) reported that lysozyme together with lactoferrin inhibited many Gram-

negative bacteria, such as *E. coli*. EDTA increases of permeability of *E. coli* outer member (Ellison and Giehl 1991). Deckers et al. (2008) found that *E. coli* was partially inactivated by egg white lysozyme. However, Liu et al. (2010) reported that lysozyme did not significantly reduce ileal *E. coli* numbers. *E. coli* infections in poultry are related to several diseases including omphalitis, salpingitis, cellulitis, enterocilitis and septicaemia (Dinev 2010).

Compared to the first floor trial with the average numbers of *E. coli*, coliforms and *Clostridium perfringens* of 4.45, 4.74 and 1.28 in the ileum of broiler chickens, those numbers increased to 4.53, 4.87 and 2.58, respectively in this trial where used litter was provided. Improved biosecurity reduced bacterial infections in broiler chickens (Bojesen et al. 2010).

5.7 CONCLUSIONS

The supplementation of 100 ppm lysozyme and 250 ppm of virginiamycin had no effect on growth performance of broiler chickens. On day 35, inclusion of virginiamycin throughout the trial significantly lowered the number of total anaerobic bacteria in the ileum compared to those fed the control diets and lysozyme throughout the trial and lysozyme from days 15-24. The used litter from a previous trial might not provide enough challenge to the birds, especially if it is derived from a flock of high performing birds with low mortality. The *E. coli* numbers in the ileum of the birds fed lysozyme for the duration of the trial as well as during days 5 to 14 were lower than the ileal number for those fed the antibiotic. Lysozyme has potential to create healthy intestinal microbiota.

CHAPTER 6 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSIONS

The supplementation of EDTA modified lysozyme in diets did not improve the growth performance of broiler chickens compared to control diets. Heat stress suppressed the growth performance, and reduced the numbers of anaerobic bacteria and *Clostridium perfringens*. Feeding lysozyme did not reduce the impact of heat stress on the reduction in the body weight and body weight gain. The inclusion of 200 ppm lysozyme had a negative effect on growth performance. For the trial with used litter was provided, feeding 100 ppm lysozyme to birds from day 5 to 14 during the starter period and throughout the trial reduced the population of *E. coli* in the ileum compared with feeding the antibiotic to the birds. These data indicate that lysozyme can influence bacterial numbers, in particular Gram-negative *E. coli*.

6.2 RECOMMENDATION

When testing the effect of antimicrobials on *Clostridium perfringens*, heat stress might not be an ideal challenge. The used litter, which provides challenge to birds, should come from a previous flock with relative poor growth performance and should be mixed in new litter in a higher portion than 50%. Lysozyme might be processed or mixed with another growth promoter to enhance its function instead of being modified by EDTA before feeding to birds.

Further study is necessary to determine the effect of lysozyme on other parameters including ileum and cecal microbiota analysis by NGS and histology. NGS has been initiated but due to technical difficulties at the Lethbridge Experimental Centre, Agriculture Agrifood Canada has not yet been completed. Further research will be conducted on the cecal samples collected and retained from the floor trials using traditional culturing methods as well as NGS to identify shifts in relative abundance of bacterial groups at the phylum, class order, family, and genus levels. Intestinal histology analysis has been proceeding on the used litter trial. The parameters including mucosal width, villus height, crypt depth, villus midwidth and villus surface area will be measured. The effect of lysozyme on histology will be determined. All those further studies will give a more complete picture of the effects of lysozyme on broiler chickens.

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APPENDIX A Lighting schedules for cage trial and two floor trials

Table A-1: Lighting schedules for broiler chickens housed in controlled environment room at the Atlantic Poultry Research Centre during cage trial.

Days post hatch	Light hours	Light intensity (lux)
0-2	24	20
3-4	23	20
5-6	16	15
7-9	16	10
10-11	16	5
12-13	16	5
14-16	16	5
17-18	16	5
19-20	16	5
21	16	5
22-23	16	5
24-27	16	5
28	17	5
29-32	18	5
34-35	19	5

APPENDIX B Temperature recorded by data logger for cage trial and two floor trials

Table B-1: Temperature recorded by data logger for broiler chickens housed in controlled environment room at the Atlantic Poultry Research Centre during cage trial.

Days post hatch	Control room				Heat stress room			
	Temp Setting	Ave. Temp	Min. Temp	Max. Temp	Temp Setting	Ave. Temp	Min. Temp	Max. Temp
0-2	32	33.3	24	38	32	32.7	29	37
3-4	31	30.4	25	38	31	31.3	27	36
5-6	30	31.1	26	36	30	28.9	26	33
7-9	29	29.6	25	33	29	28.2	24	32
10-11	28	28.2	25	31	28	26.6	22	31
12-13	27	26.0	20	32	27	24.4	19	31
14-16	26	25.4	22	30	26	23.6	19	29
17-18	25	24.1	19	27	26	23.6	19	28
19-20	24	22.6	19	26	26	23.6	20	26
21	23	21.1	18	25	26	23.3	21	27
22-23	23	20.6	18	25	26-33†	31.1	19	39
24-27	22	19.7	16	24	26-33	28.3	18	38
28-35	21	19.4	16	25	26-33	27.9	18	42

†26-33：Fluctuating daily temperature.

Table B-2 Temperature (C) recorded by computer for broiler chickens housed in at the Atlantic Poultry Research Institute during clean litter floor trials (Experiment 1).

Day post hatch	Temp setting	Room 151			Room 152			Room 153			Room 156		
		Ave. Temp	Min. Temp	Max. Temp	Ave. Temp	Min. Temp	Max. Temp	Ave. Temp	Min. Temp	Max. Temp	Ave. Temp	Min. Temp	Max. Temp
0-2	32	29.7	28	31	29.7	29	31	29.5	28	31	29.4	28	30
3-4	31	29.8	28	31	29.8	29	31	29.8	28	31	29.7	28	30
5-6	30	29.3	28	30	29.1	28	30	29.1	28	30	28.8	28	29
7-9	29	28.6	27	30	28.5	28	30	28.3	27	29	28.2	27	29
10-11	28	28.1	27	29	28.1	27	29	27.9	27	29	27.8	27	28
12-13	27	26.9	25	28	27.0	26	28	26.8	26	27	26.7	25	27
14-16	26	25.5	24	26	25.7	25	26	25.4	24	27	25.5	24	26
17-18	25	25.0	24	26	25.1	24	26	24.8	24	26	24.8	24	25
19-20	24	24.0	24	25	24.1	24	25	23.9	23	25	23.9	23	25
21-23	23	23.2	23	24	23.2	23	24	22.9	22	24	22.9	22	24
24-27	22	23.1	23	24	23.1	23	24	23.0	22	24	23.0	22	24
28-35	21	22.8	22	24	22.8	22	24	22.7	21	24	22.4	21	24

Table B-3 Temperature (C) recorded by computer for broiler chickens housed in at the Atlantic Poultry Research Institute during clean litter floor trials (Experiment 2).

Day post hatch	Temp setting	Room 151			Room 152			Room 153			Room 156		
		Ave. Temp	Min. Temp	Max. Temp	Ave. Temp	Min. Temp	Max. Temp	Ave. Temp	Min. Temp	Max. Temp	Ave. Temp	Min. Temp	Max. Temp
0-2	32	30.5	27	31	30.3	29	31	30.3	28	31	30.3	29	31
3-4	31	29.9	26	31	29.7	29	30	29.7	28	30	29.6	28	31
5-6	30	28.9	26	30	28.9	28	30	28.9	28	30	29.0	28	30
7-9	29	28.0	25	29	28.1	26	29	28.0	27	29	28.0	27	29
10-11	28	27.7	25	29	27.8	26	28	27.7	26	28	27.6	27	29
12-13	27	26.8	25	28	26.7	26	27	26.6	26	27	26.9	26	28
14-16	26	25.5	23	27	25.5	24	27	25.5	24	27	25.6	24	27
17-18	25	24.8	23	26	24.8	24	26	24.7	23	26	24.8	24	26
19-20	24	23.9	23	25	23.7	23	25	23.7	23	25	23.7	23	25
21-23	23	23.3	23	24	23.1	23	24	23.1	23	24	23.2	22	24
24-27	22	23.3	23	24	23.1	23	24	23.1	23	24	23.2	22	24
28-35	21	23.0	21	24	22.9	20	24	22.9	19	24	22.8	19	25

Table B-4 Temperature (C) recorded by computer for broiler chickens housed in at the Atlantic Poultry Research Institute during used litter floor trials.

Day post hatch	Temp setting	Room 151			Room 152			Room 153			Room 156		
		Ave. Temp	Min. Temp	Max. Temp	Ave. Temp	Min. Temp	Max. Temp	Ave. Temp	Min. Temp	Max. Temp	Ave. Temp	Min. Temp	Max. Temp
0-2	32	30.4	29	32	30.2	29	31	30.2	29	31	29.7	27	31
3-4	31	29.4	27	31	29.0	28	30	29.1	28	30	28.1	27	30
5-6	30	-†	-	-	-	-	-	-	-	-	-	-	-
7-9	29	-	-	-	-	-	-	-	-	-	-	-	-
10-11	28	27.3	26	28	27.2	27	28	27.0	26	28	27.0	26	28
12-13	27	26.6	26	29	26.4	25	29	26.4	25	29	26.1	25	29
14-16	26	25.8	25	27	25.7	24	27	25.7	24	27	25.5	24	27
17-18	25	26.6	25	27	26.4	24	27	25.9	24	27	25.7	24	26
19-20	24	24.5	23	28	24.5	24	27	24.5	24	27	24.1	23	27
21-23	23	23.4	22	25	23.3	23	25	23.4	23	24	23.1	23	24
24-27	22	23.9	22	28	23.7	23	28	23.6	23	27	23.5	23	27
28-35	21	23.7	23	26	23.5	22	25	23.4	22	25	23.2	22	25

† Data were not collected due to the malfunction of data logger.

APPENDIX C Relative humidity recorded by data logger for cage trial and two floor trials

Table C-1 Relative humidity (RH) (%) recorded by data logger for broiler chickens housed in controlled environment room at the Atlantic Poultry Research Centre during cage trial.

Day post hatch	Control room			Heat stress room		
	Ave. RH	Min. RH	Max. RH	Ave. RH	Min. RH	Max. RH
0-5	45.5	32	65	45.6	33	68
6-10	44.2	29	59	46.3	30	68
11-15	49.4	36	66	51.0	37	68
16-20	56.9	42	68	57.0	43	70
21-25	56.5	43	68	50.0	39	70
26-30	59.2	45	65	49.2	32	67
31-35	56.3	43	65	45.1	33	72

Table C-2 Relative humidity (RH) (%) recorded by data logger for broiler chickens housed in at the Atlantic Poultry Research Institute during clean litter floor trials (Experiment 1).

Day post hatch	Room 151			Room 152			Room 153			Room 156		
	Ave. RH	Min. RH	Max. RH	Ave. RH	Min. RH	Max. RH	Ave. RH	Min. RH	Max. RH	Ave. RH	Min. RH	Max. RH
0-5	57.1	3	73	31.3	26	36	21.7	14	30	24.7	20	42
6-10	51.2	24	74	37.4	28	53	31.1	15	53	36.1	21	58
11-15	42.9	22	66	21.2	14	41	24.6	13	33	27.9	20	35
16-20	53.3	30	75	35.3	21	48	33.0	23	42	38.3	26	47
21-25	56.0	3	76	51.3	30	66	43.2	25	58	51.4	33	64
26-30	56.8	3	76	54.6	38	79	45.3	24	46	51.9	33	84
31-35	61.6	3	78	54.6	39	77	45.5	30	68	52.1	36	75