DEVELOPMENT OF AN *IN VITRO* ASSAY TO EVALUATE ANTIMICROBIALS APPLIED *IN VIVO* FOR PREPARING MARKET-AGED BROILERS FOR SHIPPING

by

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Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia

in co-operation with

Nova Scotia Agricultural College
Truro, Nova Scotia

July 2012

DALHOUSIE UNIVERSITY

NOVA SCOTIA AGRICULTURAL COLLEGE

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "DEVELOPMENT OF AN *IN VITRO* ASSAY TO EVALUATE ANTIMICROBIALS APPLIED *IN VIVO* FOR PREPARING MARKET-AGED BROILERS FOR SHIPPING" by Regina Ofori in partial fulfilment of the requirements for the degree of Master of Science.

Dated: July 6, 2012

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DALHOUSIE UNIVERSITY
NOVA SCOTIA AGRICULTURAL COLLEGE

DATE: July 6th, 2012

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TITLE: DEVELOPMENT OF AN *IN VITRO* ASSAY TO EVALUATE ANTIMICROBIALS APPLIED *IN VIVO* FOR PREPARING MARKET-AGED BROILERS FOR SHIPPING

DEPARTMENT OR SCHOOL: Department of Plant and Animal Science

DEGREE: MSc CONVOCATION: October YEAR: 2012

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ABSTRACT

In vitro assays were developed using small intestinal contents from broilers fed maltodextrin in preparation for shipping. This was done to establish an effective bactericidal dose of allicin or lysozyme as ingredients in maltodextrin-based feed. The antimicrobials were added to overnight cultures of gut material bacteria from maltodextrin fed broilers and a pure culture of *Salmonella*. Following this, lysozyme was incorporated into maltodextrin feed at 0, 10 and 20 g.kg-1 of feed and offered for 9 h to 4 pens of 20 birds per treatment. Bacterial numbers were analyzed using Proc Mixed of SAS. Allicin and lysozyme inhibited Enterobacteriaceae and *Clostridium perfringens*, respectively, *in vitro*. Lysozyme showed the most promise; it reduced bacterial numbers in nutrient broth. Feeding lysozyme-enriched maltodextrin for 9 h inhibited bacilli growth ($P<0.05$) when evaluated using next generation sequencing. Lysozyme was effective in reducing specific bacterial numbers in the gut of market-aged broilers.

LIST OF ABBREVIATIONS USED

ANOVA	Analysis of variance
AOAC	Association of analytical chemists
AW	Water activity
BPW	Buffered peptone water
CFIA	Canadian food inspection agency
CFU	Colony forming units
DE	Dextrose equivalent
DGGE	Denaturing gradient gel electrophoresis
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
GIT	Gastrointestinal tract
HACCP	Hazard analysis critical control points
LB	Luria Bertani broth
LF	Lactoferrin
Log	Logarithm
LPOS	Lactoperoxidase system
LZ	Lysozyme
NGS	Next generation sequencing
NRC	National research council
OTU	Operational taxonomic units
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid

SAS	Statistical analytical software
TNTC	Too numerous to count
TSC	Tryptose sulfite cycloserine
TTGE	Temporal temperature gradient gel electrophoresis
WCR	Whole carcass rinse

ACKNOWLEDGEMENT

First, I will like to thank Chicken Farmers of Newfoundland and Labrador, and Agriculture and Agri-Food Canada for providing funding for this research.

In addition, I am grateful to my advisor, Dr. Bruce Rathgeber, and to my thesis committee members, Dr. Michelle Jendral and Dr. Derek Anderson for guiding and supporting in diverse ways during my graduate studies. They have been role models, wonderful advisors and good friends.

I would also like to thank Kristen Thompson and Krista Budgell for their help in the sampling and bacteria analysis of my experimental birds. They helped me even during weekends and nights. I am also grateful to the Rathgeber family for their hospitality and valuable help and to several graduate students for their friendship and motivation. I will never forget you because you are all true friends.

Finally, my family was of tremendous support and encouraged me to persevere.

Special thanks to Enoch Asante for his emotional support, trust in me, patience, love and understanding during my studies. Without your support in diverse ways, my road to finishing my thesis would have been very rough.

CHAPTER 1: INTRODUCTION

Once broiler chickens reach market weight, preparation for slaughter begins by withdrawing feed, crating and transporting the birds to a slaughter facility. Feed withdrawal is an important part of this preparation process, whereby the gastrointestinal tract (GIT) of poultry is given time to empty prior to catching and transport. The period that birds are denied access to feed normally lasts for 8-12h (Papa, 1991, Willis et al., 1996). Feed withdrawal is an important management practice because it helps to minimize carcass contamination (USDA, 1996) from exposure to GIT contents during automated evisceration at the processing plant (Bilgili, 1988, May and Lott, 1990). In the absence of feed withdrawal, birds GITs are full of digesta during processing at the slaughter facility. This material increases the GIT tension and a large volume of the digesta can escape in the event of GIT tear (Berrang et al., 2004). Northcutt and Buhr (1997) reported that without feed withdrawal, line speed typically slows down at the processing plant, resulting in increased cost of meat processing.

Although, there are benefits associated with withdrawing feed, there can also be negative impacts if not managed properly. Feed withdrawal can lead to stress in poultry, thereby raising animal welfare concerns (Delezie et al., 2007). Physiological responses to feed withdrawal are determined by the efforts that the birds need to exert in order to deal with the associated stress (Nijdam et al., 2005). Physiological responses such as enzymatic activities (creatine kinase) in the blood increase as feed withdrawal period increases, and may induce fear and fatigue in the birds (Rostagno, 2009, Nijdam et al., 2005). Excessive feed withdrawal periods can result in substantial weight loss, resulting in reduced processed carcass yield (Veerkamp, 1986, Lyon et al., 1991). Long feed

withdrawal periods can increase bacterial load in the gastrointestinal tract as birds may feed on manure-containing litter material (Corrier et al., 1999). Ramirez et al. (1997) reported that broilers denied feed for 8h had an increased incidence of *Salmonella* in the crop compared with birds from the same study that were fully fed. Similarly, withholding feed from broiler chickens prior to slaughter has been shown to result in increased recovery of *Campylobacter* from crops and ceca of commercially reared broilers (Byrd et al., 1998, Willis et al., 1996).

Some of the most common bacteria associated with contaminated poultry carcasses are *Salmonella* (Antunes et al., 2003), *Campylobacter*, *Escherichia coli* and *Clostridium perfringens* (Akhtar et al., 2009, Smith and Berrang, 2006). These are pathogens that are commonly found in the GIT of chickens (Smith and Berrang, 2006) and have been found to be a cause of foodborne illnesses in humans in the United States of America (Altekruse et al., 1997). Minimizing contamination of processed poultry with fecal material has been identified as an important part of reducing foodborne illness in humans (Smith et al., 2007, Kassaify, 2004).

An experimental alternative to feed withdrawal in market-age broilers is to provide birds with a highly digestible feed material such as maltodextrin resulting in birds with similar volumes of GIT content to birds that have been withdrawn from feed for a pre-slaughter period of 12h (Farhat et al., 2002). Providing nutrients at this time with maltodextrin feed can also help poultry retain body weight (Rathgeber et al., 2007, Farhat et al., 2002). Maltodextrin is a food ingredient used in human food (Grotz and Munro, 2009). However, including allicin or lysozyme in maltodextrin feed may result in additional benefits, and encourage acceptance of the practice by the poultry industry.

Attempts to control bacterial populations in the GIT of broiler chickens include adding antibiotics to poultry feed (Miranda et al., 2008, Smirnov et al., 2005). However, the use of antibiotics in poultry feed during the production period can result in development of antibiotic resistant bacteria (Schjorring and Krogfelt, 2011). Some bacterial isolates have been found to be resistant to antibiotics used in poultry, such as bambermycin, salinomycin, and bacitracin (Diarra et al., 2007). Such antibiotic resistant bacteria may be transferred to humans through the food chain (Perry et al., 2004). Use of alternatives to antibiotics and improved management practices may be options for reducing subtherapeutic antibiotic use in poultry feed (McEwen and Fedorka-Cray, 2002).

Some alternatives to using antibiotics include the use of prebiotics, probiotics and extracts from plant and animal origin (Hume, 2011). These are becoming common in poultry research due to their antimicrobial properties (Callaway et al., 2008, Dickens et al., 2000). Allicin from garlic has been found to strongly inhibit Gram-negative and Gram-positive bacteria (Ankri and Mirelman, 1999). Zhang et al. (2006) reported that lysozyme from hen egg white has antimicrobial properties against *Clostridium perfringens* isolates when used in a micro-broth dilution. In this study, allicin and lysozyme were evaluated *in vitro,* by utilizing digesta from market-aged broilers fed a maltodextrin-based diet. The objective of the current research is to develop an *in vitro* assay for the selection of effective levels of allicin or lysozyme prior to incorporation into a maltodextrin feed in preparation for shipping to slaughter.

CHAPTER 2: LITERATURE REVIEW

2.1 EFFECTS OF FEED WITHDRAWAL IN BROILER CHICKENS

2.1.1 Intestinal Integrity

The incidence of carcass contamination depends not only on the volume of digesta, but also on integrity of the digestive tract (Bilgili and Hess, 1997, Northcutt et al., 1997). Feed withdrawal is normally initiated to empty the GIT of broilers prior to slaughter (Zuidhof et al., 2004). However, Northcutt et al. (1997) indicated that feed withdrawal duration of 12 to 24 h resulted in heavy sloughing of the GIT. The authors suggested that the rapid deterioration of the GIT may cause contamination during poultry carcass processing.

A weak GIT has a higher tendency to tear during automated evisceration (Bilgili and Hess, 1997). Buhr et al. (1998) investigated the effects of feed withdrawal period on the shear strength of intestines of market-aged broilers. Withdrawing feed from birds for 0, 6, 8, 12 and 18 h had no influence on GIT shear strength of market-aged broilers. Bilgili and Hess (1997) investigated the effect of feed withdrawal periods (0, 6, 10, 14 and 18 h) and different ages (21, 42 and 49 days) on tensile strength of the GIT (Meckle's diverticulum to the ileocecal junction) of male and female broilers. The strength of the digestive tract decreased as feed withdrawal period increased beyond 14 h. The tensile strength of the broilers increased with age, male broilers showed greater hindgut strength than females (Bilgili and Hess, 1997).

2.1.2 Gastrointestinal Tract Bacteria

The level of pathogens in the GIT is influenced by the time birds are taken off feed and the amount of digesta in the GIT (Duke et al., 1997, Warriss et al., 2004).

Ramirez et al. (1997) and Corrier et al. (1999) reported that prolonged feed withdrawal periods have the potential to increase the incidence of *Salmonella* colonization in the crop of market-age broilers. Byrd et al. (1998) observed an increase in the incidence of *Campylobacter*-positive cases from 25% in the crop of market-aged broilers before feed withdrawal to 62.4% in the crops of birds after feed withdrawal period of 8 h. In contrast, Sengor et al. (2006) observed no differences in the *Enterococcus faecalis*, *Enterococcus faecium*, and sulfite reducing anaerobe bacterial count. However, the authors observed a reduction in coliform numbers in the ceca of market-aged broilers after withdrawing feed for 0, 6, 12 and 18 h. These findings indicate that long feed withdrawal periods up to 24 h may not always effectively reduce bacteria colonization. Controlling bacteria in the GIT is important because it is the main source of contamination during poultry carcass processing (Hinton et al., 2000, Rathgeber et al., 2007).

2.1.3 Meat Yield and Quality

There is growing interest in poultry meat quality (Baracho et al., 2006; Bolder, 2007) and carcass yield is important to poultry meat producers (Pollock, 1997). Improper conditioning of broilers before processing can lead to excessive digesta in the GIT and increased carcass contamination (Northcutt and Buhr, 1997). When contamination occurs, carcasses can be trimmed to help improve meat safety, but this action can result in increased processing costs (Northcutt and Buhr, 1997) and loss of saleable product. Buhr et al. (1998) found that a long feed withdrawal period (6-24 h) resulted in decreased body weight and reduced carcass yield. Orlic et al. (2007) reported that a feed withdrawal period of 6 h had no effect on body weight of broilers or meat yield, but helped to evacuate approximately 80% of excreta from the gastrointestinal tract. In contrast, the

live shrink per hour of feed withdrawal can range from 0.18 to 0.43% for broiler chickens (Buhr et al., 1998, Northcutt et al., 2003a). Although, the weight loss for a 2 kg broiler may not appear substantial when considered on a percentage basis for a single bird, when evaluated for a flock of 20,000 broilers, this translates to 72 to 172 kg of weight loss per hour (Northcutt et al., 2003a). Thus, effectively managing feed withdrawal periods in broilers is important.

2.2 MICROORGANISMS AND THEIR IMPORTANCE IN THE POULTRY INDUSTRY

Microorganisms have both beneficial and detrimental effects on humans and animals. Known microorganisms include bacteria, protozoa, fungi (yeast and molds), microscopic algae, and viruses (Gibson and Williams, 2000). This research will focus on control of broilers digestive tract bacteria. Some of the more heavily studied bacteria associated with the digestive tract of poultry include *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes, Pseudomonas aeruginosa*, *Salmonella typhimurium, Staphylococcus aureus* and *Clostridium perfringens* (Buhr et al., 2005, Bohaychuk et al., 2006). Amit-Romach et al. (2004) reported that *Clostridium perfringens* and lactobacilli are major bacteria found in the ceca and small intestines of young birds, while *Bifidobacterium*, *Salmonella*, *Campylobacter*, and *E. coli* are more prevalent in the ceca of older chickens.

Research has shown that some bacteria can inhibit pathogenic bacteria (Hinton et al., 2002). Some beneficial bacteria produce lactic acid from carbohydrate sources such as glucose and sucrose (Hinton et al., 2002). The presence of lactic acid can increase the ability of lactic acid bacteria to compete with enteropathogens. Enteropathogens are

bacteria that disrupt normal function of the gastrointestinal tract and can become pathogenic bacteria if their populations become overgrown (Lillehoj et al., 2007). According to Taheri et al. (2009), lactic acid lowers the GIT pH and provides an environment conducive to the growth of beneficial bacterial while inhibiting enteropathogenic growth.

Although, some pathogenic bacteria may not be harmful to the host chicken, they may cause foodborne illness when contaminated chicken carcasses are consumed by humans (Jay, 1992). Human pathogens that are commonly found in the GIT of market-aged broilers and broilers meat products include *Clostridium perfringens*, *Escherichia coli*, coliforms, *Salmonella*, *Campylobacter*, *Bacilli, Staphylococcus aureus* and *Listeria monocytogenes* (Barbosa et al., 2005, Miranda et al., 2008, Ramirez et al., 1997).

2.3 COMMON BACTERIA ASSOCIATED WITH FOODBORNE ILLNESS

2.3.1 Clostridia Species

There are more than 100 clostridia species, approximately 20 of which are of zoonotic importance (Quinn et al., 2011). These bacteria are anaerobic, endospore-producing motile rods (containing spores with hard outer covers) with multiple flagella enabling them to move. Spores are difficult to eradicate (Whitaker et al., 2007). A temperature range of 43 to 45ºC and a pH of 5.5 have been shown to be favorable conditions for clostridial growth (Blackburn and McClure, 2002). According to Quinn et al. (2011), although, clostridia species have been found in soil and fresh water, most of the pathogenic clostridia are commonly found in the intestinal tracts of mammals. Clostridial species of clinical interest include *Clostridium haemolyticum, Clostridium*

argentinense, *Clostridium novyi, Clostridium botulinum* and *Clostridium perfringens* (Quinn et al., 2011).

Clostridium perfringens is one of the most virulent strains that can cause human foodborne illness (Udompijitkul et al., 2012). The spores can survive stressful environmental conditions such as radiation, desiccation and heat (Novak and Juneja, 2002). *Clostridium perfringens* is pathogenic in its vegetative state in which cells and spores are regularly multiplying (Byrne et al., 2006, Paredes-Sabja et al., 2007). This pathogen is mostly transmitted to humans through the consumption of cooked but heavily contaminated poultry and pork meat (Sinosh et al., 2010). The pathogen has been shown to have the ability to produce enterotoxins. Enterotoxins are toxins that disrupt the selective permeability of the plasma membrane to target cells (Kimura et al., 2010), and cause gastrointestinal problems in humans (McClane, 1996). Food poisoning in humans caused by *Clostridium perfringens* is characterized by abdominal cramping and diarrhea (Lederman et al., 2004). These symptoms occur between 8 to 24 h after ingestion of *Clostridium perfringens* from contaminated food (Lederman et al., 2004, Rood, 1998).

2.3.2 *Salmonella*

Salmonella belongs to the family Enterobacteriaceae (Chang et al., 1997). *Salmonella* is a facultative anaerobic, capable of growing both under aerobic and anaerobic conditions and a Gram-negative bacteria (Alakomi et al., 2000). *Salmonella* species have the potential to colonize and cause disease in humans due to their ability to multiply in an acidic gastric environment (Riesenberg-Wilmes et al., 1996). It has been reported that the optimum temperature for *Salmonella* species growth ranges from 35 to 37°C (Bronikowski et al., 2001), but can grow at a wider range, from 4 to 48°C (Dickson

et al., 1992, Velugoti et al., 2011). According to Schoeni et al. (1995), growth below 10°C is generally slow, but some *Salmonella* species can grow at temperatures as low as 4°C. In some cases, when *Salmonella* is exposed to sub-lethal heat shock, the bacteria build resistance to heat (Dodd et al., 2007). The optimal growth pH range for *Salmonella* species is found to be 4.5 to 7.5, depending on serovar (subspecies of *Salmonella*) and when pH and temperature are favorable (Curtis et al., 2003).

Salmonella causes illnesses such as enteric fever gastroenteritis, septicemia or bacteraemia and sequelae (Bell and Kyriakides, 2002). Foodborne illness occurs when 100-10,000 *Salmonella* bacterial cells are ingested (Bell and Kyriakides, 2002).

2.4 BACTERIA MULTIPLICATION

Huys et al. (2008) demonstrated that bacteria multiply and alter their genetic material through asexual and sexual processes. Asexual bacteria reproduce by binary fission, giving rise to two daughter cells with the same genetic (chromosomal) information as the mother cell (Smith et al., 1993). The genetic information is transferred to the descendants of the cell in which they are made, and mutations occurring over successive generations give rise to new lineages. New lineages are genetic diversity of bacteria (Achtman, 2012, Vellai and Vida, 1999). Sexual reproduction of bacteria is different from that of eukaryotes in that it does not involve the fusion of gametes. Rather, it involves the transfer of deoxyribonucleic acid (DNA) to generate bacterial diversity (Narra and Ochman, 2006). The transfer of genetic material occurs by one of three mechanisms: transformation, conjugation and transduction depending on the source of the DNA and the bacterial partners involved. Bacterial transformation, the simplest form of information exchange, involves the uptake of DNA from a dead bacterial cell

(Redfield, 1993). Bacterial conjugation is a sexual process whereby DNA is tranferred between and across cells of bacteria. Llosa et al. (2002) described bacteria conjugation as a "promiscuous DNA transport mechanism" due to the transfer of DNA between and among most bacterial cells. This is one of the major ways in which bacteria resistance is spread among pathogenic bacteria**.** Karimova et al. (1998) indicated that bacteria transduction occurs when there is an exchange between two bacteria cells.

As the exchange in genetic material accumulates, clonality decreases. Clonality refers to the degree to which bacterial species are distinguished with regards to genotype (Spratt, 2004). In this case, two extremes are formed which include highly clonal or non-clonal bacteria (Smith et al., 1993). For this reason, there is a need to determine the appropriate type of bacterial isolation analysis to use that will accurately represent the natural population and structure of bacteria (Spratt and Maiden, 1999), as some bacteria in complex matrices, examples include fermented meat products or fecal samples, may require complex methods for bacteria isolation (Furet et al., 2009).

2.5 METHODS OF CULTURING BACTERIA

Isolation of bacteria is often carried out using the traditional culturing procedures which include non-selective pre-enrichment media, used to culture a wide range of bacteria or selective enrichment media, for isolation of bacteria that require specific environmental conditions for growth. This is followed by plating on selective agar (prevents growth of other bacteria) or differential agar (colonies of bacteria are identified by color or morphological characteristics promoted by agar ingredients) (Rauen et al., 2005, Lastovica and le Roux, 2000). Traditional methods of plating include pour plating and spread plating. Pour plating involves the plating of a dilution of bacterial suspension

in a petri dish with a liquid agar medium at a temperature of 45-55°C which is then poured into the petri dish. The inoculum is rotated for uniform distribution. Following that, the plates are inverted after agar solidification and incubated at the appropriate temperature. Spread plating comprises of pre-pouring of the cooled liquid agar medium. The inoculum is then spread on the solidified medium with a sterile glass spreader. The plates are inverted and incubated at appropriate temperature. These methods are followed with the enumeration of presumptive colonies after incubation (Whitman and MacNair, 2004). Presumptive colonies of bacteria grown using one of the above described plating methods are isolated, and the bacteria are confirmed biochemically and serologically (Fratamico et al., 2005). Most pathogens are easily isolated on laboratory media. However, others may need more time as resuscitation of sub-lethal injured cells may require specialized media and controlled incubation conditions (Sharan et al., 2011, Hoefel et al., 2003). These traditional culturing methods require 4 to 7 days for bacterial colony detection and confirmation. However, the media may not be sensitive enough to detect some bacteria (Whyte et al., 2002).

Due to the difficulty in culturing injured bacteria, several alternative electrophoretic techniques of identifying bacteria have been developed (Lehner et al., 2005). The polymerase chain reaction (PCR) of bacterial deoxyribonucleic acid (DNA) amplification is a prerequisite for the electrophoretic techniques (Amann et al., 1992). These techniques include temporal temperature gradient gel electrophoresis (TTGE), which is an electrophoretic technique based on the separation of 16S ribosomal DNA (rDNA) fragments by using a high temperature gradient (Ogier et al., 2002) and is good for identifying population dynamics of complex bacteria population ecosystems (Roth et

al., 2010). 16S rRNA is a gene marker that is used to study bacterial phylogeny and taxonomy as 16S rRNA gene can only be found in bacteria and it is large enough (about 1,500bp) for sequencing information purposes (Janda and Abbott, 2007). Real-time PCR is fast and efficient at detecting clinical microorganisms within an hour or less, as it combines amplification and detection into one step, and collects data throughout the PCR process (Patel et al., 2006, Espy et al., 2006). Denaturing gradient gel electrophoresis (DGGE) is another sensitive technique for bacterial identification. DGGE is commonly used for determining *Salmonella* and *Helicobacter* species bacterial populations in complex ecosystems in the GIT of monogastrics (Ricke et al., 2004, Al-Soud et al., 2003). It has been demonstrated that these techniques are more effective at detecting complex microbial population, compared with traditional culturing methods (Ward et al., 1990, Muyzer et al., 1993, Zhu et al., 2002, Hume et al., 2003). Currently there is the introduction of next generation sequencing (NGS) technique. This technique is used to sequence complex genomic DNA (Harismendy et al., 2009).

Although, the traditional methods of culturing bacteria may prove challenging, they can be helpful when combined with an electrophoretic technique (Al-Soud et al., 2003, Cocolin et al., 2002). The electrophoretic technique, together with the traditional culturing method, is effective in providing an accurate profile of the cells that cannot be detected by either method alone (Whyte et al., 2002). The combination of traditional culturing and electrophoretic technique has made it possible to isolate culturable and non-culturable forms of bacteria (Fera et al., 2004, Maugeri et al., 2005).

2.5.1 Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is an electrophoretic technique (Mohlenhoff et al., 2001) that has been used to characterize bacterial communities (Zhou et al., 2007). A common form of DDGE analysis begins with the extraction of total DNA from a complex microbial mixture, followed by PCR-based amplification of a specific DNA fragment using universal primers. DGGE analysis involves partial separation of double-stranded DNA molecules of the same length that differ in base pair sequence as they move through a polyacrylamide gel containing a linearly increasing gradient of denaturants (Kassem et al., 2011, Huber and Peduzzi, 2004). DGGE is a widely used technology for digesta analysis in pigs and poultry (Torok et al., 2008, La-ongkhum et al., 2011). Hume et al. (2003) demonstrated that DGGE allows for identification of microbes in the digestive tract of chicken that are fastidious (difficult to culture bacteria that have complex nutritional requirements), and suggested that DGGE could be useful in monitoring changes in the bacterial community of the digestive tract.

DGGE is cheaper relative to next generation sequencing (NGS) and the data from microbial populations are ready to be viewed right after electrophoresis (Hanning and Ricke, 2011). Research has identified a limitation to this method. DGGE band extraction and sequencing may provide vague results when two 16S ribosomal ribonucleic acid (16S rRNA) fragments from different taxa migrate to the same position in the DGGE gel (Jackson et al., 2000, Gafan and Spratt, 2005). In general, DGGE suffers from limitations in both resolution and sensitivity.

2.5.2 Next Generation Sequencing

Sanger sequencing has been used over the past three decades to completely sequence hundreds of microbial genomes and a few higher eukaryote genomes including that of mice (Wicker et al., 2006, Voelkerding et al., 2009). Recently, there has been a shift from Sanger sequencing, also referred to as "first generation technology", to next generation sequencing (NGS) due to the benefits of this newly developed technique (Metzker, 2010). NGS permits parallel sequencing reactions to be carried out on a larger scale and for a lower cost, compared with Sanger sequencing (Harismendy et al., 2009, Metzker, 2010). Rothberg and Leamon (2008) reported that the next generation sequencing technique has led to a better understanding of the structure of the human genome. Barbazuk et al. (2007) noted that next generation sequencing is an excellent method for analyzing gene-associated single nucleotide polymorphisms. These are the single-based DNA variations among the same individuals. Roche/454 GS FLX, Illumina Genome Analyzer *II*x, Life Technologies SOLiD and Helicos HeliScope are the four next generation sequencing platforms on the market (Kircher and Kelso, 2010). Illumina GA has recently been replaced by Illumina HiSeq (Rothberg et al., 2011), and Life Tech has introduced the Ion Torrent and Proton platforms (Meldrum et al., 2011). Each of the four platforms has a different protocol, but the sequencing technology consists of four steps, including: template preparation, sequencing, imaging, and data analysis (Metzker, 2010).

Roche/454 GS FLX (454 sequencing) is a sequencing technology developed in 2005 (Voelkerding et al., 2009). It involves continuous addition of nucleotide primers: thymine (T), adenine (A), cytosine (C) and guanine (G) (Margulies et al., 2005, Aydin et al., 2006). The DNA isolates are PCR-amplified using primers to target specific regions

of the bacterial 16S rRNA gene (V1-2) (Wu et al., 2010). Genomic DNA fragments are linked to microbeads at a 1:1 ratio. The microbeads are captured in droplets of emulsion which serve as microreactors for the polymerase chain reaction (PCR) template amplification. The microbeads are then distributed in a fibre-optic slide (PicoTiterPlate™) where the four DNA nucleotides are then added. Integration of a nucleotide into a growing DNA strand in one of the wells is converted into a light signal by the firefly enzyme, luciferase (Wicker et al., 2006). The light signals are captured by a camera and recorded. The number recorded by a computer is directly proportional to the number of nucleotides in the light signal (Margulies et al., 2005). Unlike Sanger sequencing, 454 sequencing allows for increased numbers read from the polymerase chain reaction (PCR) amplicons (Wicker et al., 2006, Balzer et al., 2010). However, due to the large volume of numbers read, it is necessary to distinguish sequencing error and PCR chimeras. PCR chimeras are incomplete extensions of DNA during the PCR resulting in fragments that effectively acts as primers in the next round of PCR. These may lead to overestimation of the operational taxonomic units (OTUs) data present. AmpliconNoise and Chimera removal are used to manage the sequencing errors (Quince et al., 2011). AmpliconNoise is a PyroNoise algorithm that is capable of removing 454 sequencing errors.

2.6 METHODS OF CONTROLLING BACTERIAL GROWTH IN POULTRY

Antimicrobials are effective against the growth of a wide range of microorganisms, including fungi, viruses and bacteria (Li et al., 2008, Rabea et al., 2003). Antibiotics are antimicrobials used to inhibit the growth of pathogenic bacteria, and may encourage beneficial bacterial growth (Donoghue, 2003). Antibiotics are administered to

humans and animals at therapeutic levels to treat disease and at subtherapeutic levels to animals to promote growth (Donoghue, 2003, Barton, 2000).

Meat producers routinely use antibiotics in poultry feed to improve health and productivity of the flock (Donoghue, 2003, Sarmah et al., 2006). Antibiotics promote growth by reducing pathogenic bacterial colonization, and help to increase beneficial bacterial growth (Lu et al., 2008). The more commonly used antibiotics in the poultry industry include bacitracin, chlortetracycline, narasin, nicarbazin, salinomycin, and virginiamycin (Furtula et al., 2010). These are typically delivered to the birds as feed additives (Furtula et al., 2010). There is evidence that use of antibiotics in animal feed for an extended period can lead to the development of antibiotic resistant bacteria (Phillips et al., 2004) when subtherapeutic levels are used (Gilchrist et al., 2007). For instance, avoparcin, a glycopeptide antibiotic used as a feed additive for growth promotion in farm animals, may cause the occurrence of vancomycin-resistant enterococci (Wegener, 2003). Bacteria attain resistance through mutation (deletions, inversions and insertions within the bacteria genome) and transmission of resistance can be horizontal, whereby the transfer of genes between different bacteria species occurs (Normark and Normark, 2002, Palmer et al., 2010). Another mechanism of bacteria building resistance is through biofilm production on bacteria. Biofilm is an accumulation of microorganisms and their extracellular products form a structured group on a surface (Normark and Normark, 2002, Tenke et al., 2004). Antibiotic resistant bacteria enter the food chain and can be transferred to humans (Van Looveren et al., 2001) when contaminated meat is consumed (Kilonzo-Nthenge et al., 2008).

2.7 ALTERNATIVES TO ANTIBIOTICS

Humans have relied on antibiotics for the treatment of pathogenic bacterial diseases. The emergence of resistant bacterial strains has made antibiotics less effective in clinical, veterinary and farm animal use (Parisien et al., 2008, Heuer et al., 2006). Also, organic livestock producers are not permitted to use antibiotics in animal feed to control diseases unless the disease cannot be controlled by non-antibiotic treatment (La Ragione et al., 2001). However, when organic farmers use antibiotics, their products are no longer deemed organic (La Ragione et al., 2001). In the United Kingdom, the problems associated with antibiotic use have led to the ban of antibiotic use in poultry feed (Barton, 2000). In the United States, consumer demand for antibiotic-free products is prompting producers to rear animals without using antibiotics (Dibner and Richards, 2005). It is therefore, a priority to find alternatives to antibiotics (Hume, 2011). Common alternatives to antibiotics under investigation as poultry feed additives include prebiotics, probiotics, plant extracts and organic acids (Leusink et al., 2010, Joerger, 2003, Huyghebaert et al., 2011, La Ragione et al., 2001). Prebiotics are non-digestible short chain carbohydrates that are resistant to gastric acids in the GIT (Bailey et al., 1991, Cummings et al., 2001). Prebiotics belong to the dietary fiber group which are "usually poorly metabolized polysaccharides and oligosaccharides" and increase growth of beneficial bacteria (Saier and Mansour, 2005). Examples of prebiotics include inulin, fructose-derived oligosaccharides (Collins and Gibson, 1999, Tomasik and Tomasik, 2003) and Bio-Mos® which is referred to as a mannan oligosaccharide. Bio-Mos® is a carbohydrate derived from the cell wall of yeast (Baurhoo et al., 2007). Bio-Mos® control pathogenic bacteria numbers in the GIT of market-aged broilers through bacteria

competitive exclusion. The pathogenic bacteria adhere to the Bio-Mos® and the bacteria are expelled out of the GIT (Baurhoo et al., 2007). Probiotics are live cultures made up of single or a mixture of microorganisms of different strains (Patterson and Burkholder, 2003, Guarner et al., 2005). The organisms used as commercial probiotics typically belong to the bacterial genus *Bifidobacterium*, *Lactobacillus* and the yeast, *Saccharomyces* (Tomasik and Tomasik, 2003). The presence of prebiotics and probiotics in poultry diets encourages the growth of beneficial organisms in the GIT (Patterson and Burkholder, 2003). Prebiotics and probiotics act in several ways to prevent pathogenic bacterial growth. Prebiotics support the growth of beneficial bacteria in the GIT of the poultry and inhibit pathogenic bacterial growth (Corrigan et al., 2011). Probiotics act against pathogens through antagonistic action by producing inhibitory substances such as bacteriocin, organic acids and hydrogen peroxide. These adhere to the mucosal wall of the intestinal tract and prevent colonization of pathogenic bacterial and compete with pathogenic bacteria for nutrients (Callaway et al., 2008, Hume, 2011).

Naturally occurring compounds found in plants possess antimicrobial activity and serve as antimicrobial agents against foodborne pathogens (Cutter, 2000). Plant extracts from products such as pepper, cinnamon, onion, clove buds, red thyme and garlic have been used as alternative antimicrobials in poultry production research (Wilson et al., 1997, Hernandez et al., 2004). Plant extracts from *Acacia farnesiana*, *Artemisia ludoviciana*, *Opuntia ficus-indica*, and *Cynara scolymus* have been tested against *Campylobacter coli* and *Campylobacter jejuni* at minimal bactericidal concentrations of 0.3, 0.5, 0.4, and 2.0 $mg.ml^{-1}$ and were found to be effective against the pathogens tested (Castillo et al., 2011). Also, extracts of Tin Men Chu and Siu Mao Houng, Chinese

medicinal plants, have been shown to control growth of *Listeria monocytogenes* present in cabbage juice (Chung et al., 1990). Plant extracts from oregano, sugar cane and other plants have been used in broiler chicken production to improve feed conversion, GIT development and weight gain (Schiavone et al., 2008, Bampidis et al., 2005), as well as improving villi height, which is a parameter for measuring broiler health (Yamauchi et al., 2006).

Organic acids are used as feed additives in poultry feed to boost the immune response and growth performance (Pirgozliev et al., 2008). Often organic acids are fed to poultry in an effort to control *Salmonella* (Van Immerseel et al., 2005). Examples of organic acids used in broiler diets include butyric acid, fumaric acid and lactic acid (Adil et al., 2010). Diets with organic acid concentrations of 2% and 3% had beneficial effects on broiler body weight gain and feed conversion, compared with a control diet treatment (Adil et al., 2010). Incorporating formic acid, acetic acid and propionic acid into poultry feed has been found to reduce *Salmonella* numbers in the GIT of broilers (Van Immerseel et al., 2006). Organic acids inhibit bacteria growth by reducing the pH in the GIT of the broilers. However growth of acid-intolerant species such as *E. coli*, *Salmonella* and *Campylobacter* are inhibited (Dibner and Buttin, 2002).

2.8 USING ALTERNATIVE ANTIMICROBIALS TO CONTROL BACTERIA

2.8.1 Garlic Extracts and Allicin

Garlic (*Allium sativa*), onions, chives and leeks are part of the family Lilliaceae. *Allium sativa* has been used in human foods and as a feed supplement throughout the world for about 5000 years (Staba et al., 2001). Garlic extracts are prepared by blending the garlic clove and then squeezed through a gauze to remove the larger particles. The blended garlic clove is centrifuged at 4500 *x g* for 45 min. The supernate of the garlic

extract is filtered, sterilized and kept at -70°C for antimicrobial testing (Cellini et al., 1996). Many chemical substances have been found in fresh or dried garlic extract (Goncagul and Ayaz, 2010).

Allicin is produced as a result of the substrate activity of the enzyme allinase, which is released from the garlic cell vacuoles when the garlic bulb is cleaved (Shimon et al., 2007). Allinase acts on a volatile sulfur compound, alliin (S-allyl-L-cysteine sulfoxide) and converts alliin to allicin (Benkeblia and Lanzotti, 2007). Allicin is mainly composed of allyl sulfides, which are responsible for the typical garlic odor (Staba et al., 2001, Van Damme et al., 1992, Rabinkov et al., 1994).

More studies have been carried out using garlic extracts to control bacteria than with allicin (Rees et al., 1993, Rahman, 2003, Borek, 2001, Khan et al., 2007, Chowdhury et al., 2002). In the food industry, garlic extract has been used as a food preservative. An example of this is the use of garlic extract as an antimicrobial in cheese (Leuschner and Ielsch, 2003). Recently, Sarma (2004) showed that skinless chicken legs contaminated with *Salmonella* and *E.coli* had an increased shelf life when treated with garlic extract diluted with distilled water in a ratio of 1:5, 1:10, 1:20 or 1:40 before packaging. Kumar and Berwal (1998) investigated the effects of garlic extract concentrations of 1, 5 and 10% on *E.coli* and *Listeria monocytogenes* in a nutrient broth for 24 h at 37°C and inhibition was measured using turbidity. The authors indicated that ground garlic is a potent antimicrobial against these pathogenic bacteria: *E. coli* and *Listeria monocytogenes.* Kumar and Berwal (1998) suggested that the use of garlic extracts in processed food products can help reduce the possibility of food spoilage.

Garlic extract has been used as an antioxidant and has been scientifically proven to have antimicrobial properties when consumed by humans (Rees et al., 1993). In terms of human health, the antioxidant properties of garlic have been reported to aid in slowing down the aging process (Rahman, 2003). Garlic extracts may help control cardiovascular, neurodegenerative and inflammatory diseases, as well as cancer (Youdim and Joseph, 2001). Borek (2001) determined that all the benefits from garlic extracts are as a result of the presence of important water and lipid-soluble organosulphur components, selenium and flavanoids.

2.8.1.1 Antimicrobial Properties of Allicin

Allicin is a major component of garlic extracts (Ariga and Seki, 2006). Both garlic extracts and allicin have been used to control bacterial growth. Allicin is an antimicrobial that can inhibit a wide range of Gram-negative and Gram-positive bacteria (Ankri and Mirelman, 1999). Elsom et al. (2000) evaluated the antimicrobial properties of garlic on nine species of bacteria in an aqueous extract. Of those pathogenic and spoilage bacterial tested, garlic extract inhibited the growth of methicillin-resistant *Staphylococcus aureus* . They reported that *Bacteriodes fragilis*, *Clostridium perfringens*, *Enterobacter cloacae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Staphylococcus aureus* were less resistant to garlic extract treatment at concentrations less than 0.0256 $mg.ml^{-1}$ compared to *Leuconostoc mesenteroides*. Most microorganisms such as fungi, yeast, viruses and bacteria are sensitive to the effects of allicin (Rees et al., 1993). One feature of allicin as an antimicrobial is that it is often more effective at limiting growth of pathogenic bacteria compared to those that are considered beneficial. An example of a bacteria that is resistant to the effect of allicin is

Lactobacillus acidophilus. This organism is considered a beneficial lactic acid bacterium commonly found in the GIT of animals (Rees et al., 1993). Lactic acid bacteria in general have been shown to be resistant to garlic extracts at low concentrations, but are sensitive at concentrations between 12.5 and 40 $mg.ml^{-1}$ in broth (Rees et al., 1993). Shobana et al. (2009) found garlic extracts to be effective against all enteric pathogens tested at concentrations of 200, 300, 400 and 500 $mg.l^{-1}$ in nutrient broth. These pathogens included *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhi, Shigella flexineri* and *Enterobacter aerogenes.*

2.8.1.2 Factors that Affect the Activities of Allicin

2.8.1.2.1 Temperature

Research indicates that the optimum temperature range to generate the inhibitory effect of allicin against Gram-negative and Gram-positive bacteria is 30 to 50°C. Higher temperatures (70 to 100°C) can denature the allinase present in garlic extract responsible for converting alliin (S-alkyl-L-cysteine sulfoxide) materials to allicin (El Astal, 2004). Rahman et al. (2006) investigated the effects of different garlic products (dried garlic powder, garlic extracts and garlic oil) on pathogenic bacteria. Rahman et al. (2006) reported that temperatures higher than 75°C decreased the inhibitory activity of moist garlic during moist heating activities. Adler and Beuchat (2002) observed that *Salmonella, Escherichia coli* O157:H7 and *Listeria monocytogenes* retained their viability at 4.4°C, regardless of the presence of garlic extract. However, addition of garlic extract at 4/1, butter/garlic (wt/wt) ratio at 21°C and 37°C increased the inactivation of these pathogens.

2.8.1.3 Importance of Garlic and Allicin to the Poultry Industry

Allicin has been used in laying hen diets to improve growth and laying performance (Raeesi et al., 2010, Khan et al., 2007). There are no published works related to the dietary use of allicin to control bacteria in broilers or layers. However, allicin has been proven to be useful feed ingredient in the poultry industry. Raeesi et al. (2010) reported that broilers had improved body weight gain and carcass parameters when the basal diet was supplemented with 0.5, 1 and 3% allicin. Khan et al. (2007) found dietary allicin reduced blood serum and egg yolk cholesterol levels in eggs from layers fed at 2, 6 and 8% for 6 weeks. Chowdhury et al. (2002) fed garlic paste at 2, 4, 6, 8 or 10% for a period of six weeks to different commercial strains of chicken (Hisex Brown, Isa Brown, Lohmann, Starcross, Babcock and Starcross-579) and found no differences in feed consumption, feed efficiency or layer performance for all the commercial strains. However, there was a reduction in serum and yolk cholesterol.

2.8.2 Lysozyme

Lysozyme is an enzyme naturally present in hen egg white, human saliva and breast milk (Deckers et al., 2008). The enzymatic activity of this enzyme is directed against the β (1-4) linkages between N-acetylmuramic and N-acetylglucosamine of peptidoglycan of Gram-positive (Cunningham et al., 1991). This is a key component of the Gram-positive bacteria cell wall. Lysozyme inhibits the growth of bacteria by breaking down the cell wall, particularly of Gram-positive bacteria and to some extent of Gram-negative bacteria. However, lysozyme is considered largely ineffective against Gram-negative bacteria because the outer membranes of these bacteria act as a physical barrier preventing access by the enzyme (Masschalck and Michiels, 2003). Gram-

negative bacteria have complex cell walls that consist of a thin layer of peptidoglycan which in turn is surrounded by lipopolysaccharide. By comparison, Gram-positive bacteria lack an outer membrane but have many layers of peptidoglycan (Silhavy et al., 2010). Studies on bacterial inhibition by lysozyme report that lysozyme is most active against Gram-positive bacteria such as *Staphylococcus aureus, Micrococcus luteus, Bacillus cereus, Bacillus stearothermophilus* and clostridia species (Cunningham et al., 1991, Liu et al., 2010).

Several strategies have been developed to extend the action of lysozyme to Gram-negative bacteria. These include thermal modification of lysozyme, modification of lysozyme by covalent attachment of polysaccharides, fatty acid modification, and combining lysozyme with the use of an outer membrane permeabilizing agent such as ethylenediaminetetraacetic acid (EDTA) or a permeabilizing treatment such as high hydrostatic pressure treatment (Masschalck and Michiels, 2003).

Ibrahim et al. (1996) reported that it is possibile to extend the activity of lysozyme through thermal modification. The process they used was the heating of egg white lysozyme to 80°C for 20 min at a pH of 4-8. Following the heating process, the lysozyme was dried using a spray drier (Lesnierowski et al., 2001, Ibrahim et al. 1996). Although, heating of enzymes result in denaturation of the protein, Lesnierowski et al. (2001) found that the thermally modified lysozyme had increased inhibitory activity against *E. coli* and *Micrococcus luteus* compared to the unmodified lysozyme. Others have modified lysozyme through covalent attachment of molecules to produce lysozyme conjugates (Rawel et al., 2001). One example is the production of lysozyme-galactomannan conjugate prepared through a controlled maillard reaction (Nakamura et al., 1996).

Another example is lysozyme-palmitic acid conjugate produced by base-catalyzed ester exchange using *N*-hydroxysuccinimide ester of palmitic acid (Ibrahim et al., 1991). These lysozyme conjugates improve the efficacy of lysozyme action against Gram-negative bacteria (Nakamura et al., 1996). The easiest way to modify lysozyme is to add EDTA (Boland et al., 2003). EDTA is a permeabilizing agent that helps to breakdown the complex cell wall of Gram-negative bacteria (Ko et al., 2009). It has been shown that the mixture of EDTA and lysozyme enhances the antimicrobial activity of lysozyme against Gram-negative bacteria (Boland et al., 2003). Recently, Lesnierowski et al. (2009) investigated ultrafiltration-modified chicken egg white lysozyme and its antimicrobial action on bacteria. Ultrafiltration (UF) of lysozyme is carried out by mixing 2% water solutions of lysozyme monomer. The enzyme is then modified by the membrane technique in a LAB UF plate module under controlled pressure of less than 30 bars and a temperature of 50°C for 5 h. The lysozyme solution is then dried with a spray dryer. This is performed to increase the polymeric forms of lysozyme. Lesnierowski et al. (2009) found that bacteriostatic activities against Gram-negative bacteria such as *Pseudomonas fluorescens*, increased when egg white lysozyme was modified by UF.

2.8.2.1 Importance of Lysozyme to the Poultry Industry

2.8.2.1.1 Enteropathogen Control

There is interest in conducting research with natural antimicrobials that are effective in controlling bacteria in commercial poultry. One of the key bacterium that is often the target of these studies is *Clostridium perfringens* as it is associated with necrotic enteritis (Zhang et al., 2006). Zhang et al. (2006) studied the control of alpha toxin production by *Clostridium perfringens* type A with lysozyme. The authors found that

treatment with 156 μg.ml^{-1} lysozyme from hen egg white inhibited *Clostridium perfringens* in an overnight micro-broth assay, as well as inhibiting α-toxin production at a low level of 50 μg.ml^{-1}. In a more recent study, lysozyme fed to 1-day-old male Arbor Acre broilers at 40 mg.kg^{-1} of lysozyme diet for 28 days was found to inhibit the growth of *Clostridium perfringens*, and reduce intestinal lesion scores in broiler chicken (Liu et al., 2010). Lysozyme from hen egg white (Invitrogen Life Technologies, Carlsbad, California, USA) inhibited the growth of *Escherichia coli* and *Lactobacillus* in the ileum of broiler chickens when included at levels of 40 mg.kg^{-1} of soybean-based diet. In addition to the antimicrobial effects, lysozyme improved feed conversion efficiency and growth performance of broiler chickens (Liu et al., 2010).

2.9 METHODS OF EVALUATING ANTIMICROBIALS

Antimicrobials have been used in poultry feed and drinking water to help reduce the spread of disease and control enteropathogens (Griggs and Jacob, 2005). To determine the effectiveness of these products, screening is often conducted to ensure that effective dosages are administered (Jacobsen et al., 1999). *In vitro* methods for evaluating antimicrobials save time labor and minimize the number of animals used to screen antimicrobials (Morelli, 2000). Common *in vitro* methods used for antimicrobial evaluation include growth inhibition assays in a micro-broth (Zhang et al., 2006) and the *in vitro* fermentation method (Sunvold et al., 1995). The microbial fermentation *in vitro* method is normally developed by mimicking the conditions in the animal GIT (Williams et al., 2005).

The bacterial growth inhibition method is performed by isolating bacteria from the GIT of the animal. Bacterial strains are cultured overnight at an optimal temperature

for growth (Koenen et al., 2004). Zhang et al. (2006), for example, used the inhibition method to measure *Clostridium perfringens* inhibition by lysozyme in a micro-broth. The authors found that lysozyme has an inhibitory effect on *Clostridium perfringens.*

In vitro fermentation is another method used to evaluate antimicrobials. Partanen and Jalava (2005) used this method with pig digestive tract contents to prepare culture medium consisting of 53% buffer, 45% frozen ileal digesta and 2% fresh digesta from pigs. Forty ml of the culture containing 0.5 g of basal feed, 20 µl of liquid or 20 mg of solid acidifier (formic acid, calcium formate, citric acid, and potassium sorbate) were added to 5 ml of fermentation buffer and the mixture dispensed into 100 ml vessels and stirred. Samples were incubated for 24 h at 39°C and for every 15 min; acid production was measured and analyzed using the Gompertz bacterial growth model (Partanen and Jalava, 2005). A similar method was used by Rycroft et al. (2001) to evaluate the properties of prebiotic oligosaccharides in human digesta, in which the authors used fluorescent in-situ hybridization for bacterial population analysis. Zhu et al. (2003) used the same method to analyze bacterial population in piglets. This method was modified and used in the current study.

2.10 FEEDING CARBOHYDRATE BASED DIETS DURING THE FEED WITHDRAWAL PERIOD

Some feed management practices may help retain body weight of broilers and reduce the rate of foraging in the litter prior to processing. A potential example of this is providing a carbohydrate-based diet, such as cocktail supplement containing glucose or sucrose to broilers prior to processing (Hinton et al., 2002). The carbohydrate-based cocktail diet described by Hinton et al. (2002) contained protease peptone, beef extract,

yeast extract, polyoxyethylene-sorbitan monooleate (Tween 80), magnesium sulfate, heptahydrate, manganese sulfate, monohydrate and supplemented with glucose or sucrose. Maltodextrin is another feed supplement that has been fed to poultry prior to slaughter. A maltodextrin feed used to feed turkeys by Rathgeber et al. (2007) contained maltodextrin, corn germ, mono- and diglycerides, NaCl, caramel color and tallow. Maltodextrin-based feed could be supplemented with a protein source such as dehydrated egg white (Farhat et al., 2002). Hinton et al. (2002) reported that the inclusion of sucrose in either a cocktail supplement or water resulted in fewer *S. typhimurium* and *Campylobacter* in the crop. The authors suggested that the presence of the carbohydrate-based cocktail in the GIT of chickens caused a reduction in crop pH (5.93-6.03). The decrease likely encouraged growth of lactic acid bacteria and displaced harmful enteropathogens (Hinton et al., 2002). Providing birds with carbohydrate diets such as glucose, sucrose cocktail or maltodextrin feed supplement can help reduce the amount of pathogenic bacteria that birds may carry in the GIT to the processing plant (Hinton et al., 2002, Farhat et al., 2002). As birds normally forage in the litter when they are stressed, this can increase bacterial load in the GIT (Delezie et al., 2007). Feed withdrawal diets provide birds with needed nutrients, result in body weight maintenance in preparation for slaughter, and enhance evacuation of the GIT (Farhat et al., 2002, Rathgeber et al., 2007).

2.10.1 Maltodextrin

Maltodextrin, which is available in the form of a dried powder or concentrated liquid, is a hydrolyzed starch product with a dextrose equivalent (DE) of less than or equal to 20 (Chronakis, 1998). Dextrose equivalent is defined as a measure of the reducing power of starch-derived polysaccharides/oligosaccharides compared with D-

glucose on a dry-weight basis. DE is used to determine the extent to which starch is hydrolyzed. A higher DE value indicates a higher level of hydrolysis (Chronakis, 1998). According to Wang and Wang (2000), commercial maltodextrin is derived from three main sources, namely corn, potato and rice starches. In general, corn starch is used for commercial production of maltodextrin (Bhatnagar and Hanna, 1994). Maltodextrin is mainly produced through enzymatic hydrolysis using α-amylase and acid (Wang and Wang, 2000).

Maltodextrin is more soluble in water than native starch (Dokic-Baucal et al., 2004). The wetting property of maltodextrin is due to its hydrophyllic surfaces. It can absorb up to seven times its weight in water and forms a gel (Shogren et al., 2010). Morphologically, commercial maltodextrin is a mixture of spherical, cylindrical and filamentous particles (Takeiti et al., 2010). It has a tubular open surface depending on the DE value. It can be voluminous (DE=5), has a smoother surface (DE=10), or can consist of predominantly globular shaped and partially encapsulated particles (DE=20). Corn starch maltodextrin consists of particles of large, irregular bodies which are entangled by thin filaments (Takeiti et al., 2010). Boutboul et al. (2002) observed that maltodextrin with DE values of 11-14 has strong fragmented structures.

Maltodextrin has been used in experiments as the primary ingredient for diets fed to poultry prior to shipping when birds would normally be denied access to feed (Rathgeber et al., 2007, Farhat et al., 2002). Rathgeber et al. (2007) suggested that maltodextrin-based feed may help deflect birds' attention from foraging in the litter, and facilitate evacuation of the GIT as birds wait to be shipped. The acceptance of maltodextrin feed for poultry is unpredictable. Farhat et al. (2002) coaxed broilers to the

maltodextrin feed supplement by supplying a 50:50 mixture commercial feed and maltodextrin feed for 4 h followed by 25:75 mixture for another 4h before offering 100% of the maltodextrin-based feed treatment for 9 h. Farhat et al. (2002) reported that broilers consumed more commercial diet compared with maltodextrin feed. Farhat et al. (2002) failed to report the feed consumption for the control treatment but the author indicated that broilers consumed approximately 31 g of maltodextrin per bird for 9 h. Similarly, Rathgeber et al. (2007) supplied maltodextrin feed directly to turkeys without initially coaxing the birds to the maltodextrin feed. Rathgeber et al. (2007) reported that turkeys consumed the maltodextrin feed supplement of approximately 10 $g.kg^{-1}$ of their body weight (5.8kg).

2.11 SUMMARY

Broilers are normally denied access to feed prior to slaughter. This practice is initiated to ensure that broilers empty their GIT prior to slaughter. Feed withdrawal does not always result in complete evacuation of the GIT of broilers. Additionally, this practice can be associated with negative consequences including, increased bird stress, increased body weight loss and an increase in bacteria load in the GIT as birds forage in manure-laden litter. An alternative to the practice of feed withdrawal is to provide a maltodextrin feed supplement to broilers prior to slaughter. The maltodextrin-based feed supplement helps to evacuate the GIT of broilers, maintain body weight and increase carcass yield of poultry. However, it is anticipated that incorporating the antimicrobials, allicin and lysozyme will provide the benefit of controlling bacterial numbers in the GIT. Allicin is an enzyme product (alliin+allinase) from garlic that has antimicrobial properties. It has been used in micro-broth system and packaged meat to control bacterial

numbers. Allicin has been used in poultry feed to improve feed conversion efficiency, reduce blood serum cholesterol and carcass parameters in laying hens. Lysozyme is an enzyme from hen egg whites and has antimicrobial properties against Gram-negative and Gram-positive bacteria. Lysozyme has been evaluated *in vitro* and in broiler feed throughout the production period to control bacterial growth. According to the literature, allicin and lysozyme have not been evaluated in a feed delivered in the short time for market-aged broilers in preparation prior to shipping. It is anticipated that feeding these ingredients to broilers may shift bacterial population from harmful to less harmful, providing the benefit of improved safety of the digestive tract contents that remain and come in contact with the processed carcass. Consequently, there is a need to determine potentially effective levels of allicin and lysozyme for incorporation in a maltodextrin-based feed. This may be best accomplished by first evaluating effective levels of these antimicrobials in an *in vitro* model followed by full scale evaluation in live broiler chickens.

2.12 OBJECTIVES

The objectives of this study were:

1. To develop an *in vitro* method for evaluating the use of alternative antimicrobials (allicin and lysozyme) to control bacteria in the GIT of market-aged broilers prior to shipping.

2. To evaluate the influence of consumption of maltodextrin on bacteria numbers in the GIT of market-aged broilers.

3. To evaluate the inhibitory effect of allicin or lysozyme on bacteria in the ileum of market-aged broilers fed maltodextrin for a short period prior to shipping.

2.13 HYPOTHESIS

1. An *in vitro* assay using the contents of the small intestine of broilers fed a maltodextrin-based feed can be used to determine effective levels of antimicrobials for reducing bacterial numbers.

2. Providing maltodextrin to market-aged broilers reduces the number of bacteria associated with food borne illness present in the GIT contents.

3. Including lysozyme in maltodextrin-based feed reduces the number of bacteria associated with food borne illness present in the small intestinal contents of broilers.

CHAPTER 3: DEVELOPING AN *IN VITRO* METHOD TO EVALUATE ALLICIN AND LYSOZYME IN A DIGESTA FROM MALTODEXTRIN FED MARKET-AGED BROILERS

3.1 ABSTRACT

Normally feed is withdrawn from poultry several hours before catching and shipping of market-aged broilers. Feed withdrawal is initiated to reduce digesta volume in the GIT and lower the incidence of carcass contamination from gut contents. It has been shown that a highly digestible feed supplement offered during preslaughter feed withdrawal reduced live weight loss, improved carcass yield and reduced digesta volume. It was anticipated that an additional benefit can be accomplished by incorporating allicin or lysozyme to reducing bacteria in the GIT. An *in vitro* method was developed to evaluate the levels of allicin and lysozyme using digesta from maltodextrin fed market-aged broilers for trials 1, 2 and to determine the effect of allicin and lysozyme on *Salmonella enteritidis* inoculated digesta for trial 3. One hundred and twenty market-aged broilers (37 d) were randomly allocated to three treatments: maltodextrin fed, full fed (fed finisher diet) and feed withdrawal (denied access to feed) in floor pens. In trial 3, 225 birds were randomly allocated to the same treatments with three replicate pens. Broilers were assigned to the various treatments for 3, 6 or 9 h for trials 1, 2 and 9 h for trial 3. Following this, broilers were randomly selected and euthanized by cervical dislocation. The jejunum and ileum were harvested. Ten gram digesta samples from each pen fed maltodextrin were treated in duplicate with antimicrobial, at 0.0, 1.5, 3.0, 4.5 or 6.0 mg for trial 1, 0, 5, 12.5 or 20 mg for trial 2 and 0, 10, 20, 30, 40 or 50 mg for trial 3. All samples were incubated for the same period of time for which the birds were on the feed treatments. These samples were analyzed for aerobic bacteria*, E. coli*, coliforms, Enterobacteriaceae on 3M petrifilm plates. *Salmonella* and *Clostridium perfringens* enumeration were performed by pour plate. All bacteria counts were transformed to $\log_{10}$ values and analysis of variance was performed using Proc Mixed of SAS. Feeding maltodextrin feed to broilers prior to shipping had no effect on coliforms, Enterobacteriaceae, *Salmonella* and *Clostridium perfringens* ($P>0.05$) but reduced aerobic bacteria by 1.76 $\log_{10}$ cfu. Lysozyme reduced *Clostridium perfringens* numbers in the digesta at 5 and 12.5 mg. Allicin reduced Enterobacteriaceae numbers at levels of 5 and 12.5 mg. These results provided in sight for further evaluation of allicin and lysozyme *in vitro*.

Key Words: Market-aged broilers, maltodextrin, digesta, allicin, lysozyme and bacteria

3.2 INTRODUCTION

Poultry meat has been identified as a primary source of pathogenic bacteria. These pathogens can be transferred to humans through improper cooking and handling of the meat (Johnson et al., 2009). Rules and regulations of the Canadian Food Inspection Agency are followed to prevent meat contamination. The second principle of the Canadian Food Inspection Agency (CFIA)'s hazard analysis critical control points (HACCP) program seeks to identify critical control points at bird processing. These points are identified areas where measures need to be taken in order to reduce contamination of food products (CFIA, 2011). During poultry processing, the most likely source of poultry meat contamination is digesta leakage (Smith et al., 2007, Park et al., 2007) as this material may be a potential source of pathogenic bacteria. *Clostridium perfringens* and *Salmonella* are the major cause of foodborne illness in the United States of America (Scallan et al., 2011). Controlling these bacteria in the digestive tract prior to processing may help to minimize pathogenic bacterial contamination of carcasses (Hinton et al., 2002).

Digesta volume is commonly reduced by feed withdrawal while birds are waiting to be shipped. Another strategy of ensuring GIT emptying is to provide a highly digestible carbohydrate-based feed supplement prior to processing (Farhat et al., 2002, Rathgeber et al., 2007, Hinton et al., 2002). It has been shown that providing carbohydrate-based sucrose supplement to market-aged broilers through drinking water or feed for 12 h reduces pH and increase lactic acid bacteria (Hinton et al., 2002). A reduction in GIT pH can encourage beneficial bacterial growth and inhibit pathogenic bacteria (Hinton et al., 2002). An attempt has been made to reduce bacteria population in

the GIT by supplying a carbohydrate-based diet (maltodextrin). Northcutt et al. (2003a) fed maltodextrin feed to broilers for 8 h and subjected the birds to 0, 4, 8 and 12 h of feed withdrawal. The practice did not have any effect on the number of *Campylobacter*, coliforms or *Escherichia coli* for pre-eviscerated or eviscerated carcass rinse but increased body weights of the broilers as compared to birds denied access to feed for the same feed withdrawal times. The whole carcass rinse procedure was performed by placing a whole chicken into a clean plastic bag with 100 ml of BPW and shaken vigorously by hand in a 30 cm arc for 60 s. Similarly, Farhat et al. (2002) have also shown that maltodextrin feed helps to maintain poultry weight prior to slaughter.

In addition to reducing weight loss, this withdrawal feed could be used as a method to deliver ingredients to control GIT bacteria just prior to processing. Normally, antibiotics are used as an ingredient in poultry feed to control bacteria in the GIT (Donoghue, 2003). Traditional antibiotics may be hard to market at this stage of production with potential withdrawal times needed (Donoghue, 2003) and the current philosophy that the poultry industry should make use of alternatives to antibiotics (Reid and Friendship, 2002). Numerous plant and animal products provide natural alternatives to currently used antibiotics (Hume, 2011). An example of an antimicrobial for this purpose is allicin extracted from garlic. Allicin is an enzyme product that is active against a wide range of both Gram-negative and Gram-positive bacteria (Fujisawa et al., 2009, Ankri and Mirelman, 1999). Lysozyme is an enzyme with antimicrobial properties against wide range of bacteria (Masschalck and Michiels, 2003, Mine et al., 2004). It is commonly isolated from chicken egg white (Pellegrini et al., 1997).

In order to determine the effective levels of allicin and lysozyme, an *in vitro* method was developed as a modification of a microbial fermentation method used by Partanen and Javala (2005) to mimic the digestive tract of piglets. Partanen and Javala (2005) used ileal digesta as the fermentation media, feed as the substrate and feces from young piglets as the inoculum. Rycroft et al. (2001) evaluated bacterial populations in human feces using a similar method. It was essential to develop an *in vitro* procedure before evaluating the antimicrobials *in vivo* as direct application of the lysozyme and allicin *in vivo* would require more time, more birds and only a limited number of antimicrobial levels could be tested (Kloas et al., 1999). To determine the appropriate levels of antimicrobials to incorporate in maltodextrin feed, several *in vitro* trials with digesta harvested from maltodextrin fed market-aged broilers.

3.3 OBJECTIVES

The objectives of this study were:

1. To evaluate the effects of the three pre-shipping preparations (namely full fed, feed withdrawal and maltodextrin fed) on bacterial numbers in the GIT of market-aged broilers.

2. To develop an *in vitro* procedure used to evaluate the effect of bacterial numbers in the GIT of maltodextrin fed market-aged broilers digesta.

3. To determine the effect of allicin or lysozyme as alternative antimicrobials on *Salmonella enteritidis* in digesta from maltodextrin fed market-aged broilers.

3.4 HYPOTHESIS

1. The maltodextrin feed may reduce bacterial numbers in the digesta of market-aged broilers.

2. The developed *in vitro* method can be used effectively to evaluate allicin and lysozyme in digesta from maltodextrin fed market-aged broilers.

3. The allicin and lysozyme can inhibit *Salmonella* populations in the digesta of maltodextrin fed market-aged broilers.

3.5 MATERIALS AND METHODS

3.5.1 Maltodextrin Feed Formulation

In the first trial, a supplement was formulated to feed market-aged broilers in preparation for shipping. The feed formulation was based on the description of Farhat et al. (2002) and Rathgeber et al. (2007) with some modifications in the types and composition of the ingredients. The supplement consisted of 78.5% corn maltodextrin (Grain Processing Corporation, Muscatine, IA, U.S.A) with a dextrose equivalent of 11.0, 19.1% broiler finisher diet (Table 3.1), 0.4% NaCl and 2.0% water. To facilitate pelleting, water was added to the finisher feed component before mixing with maltodextrin. Preliminary attempts to use steam were unsuccessful as direct application of water in the form of steam resulted in a plugged die of the pelleter. The ingredients were thoroughly mixed and passed through the pelleter (California Pellet Mill Co. CPM, Crawfordsville, IN 47933, United States) with die size of 3 mm at a temperature of 70°C

Table 3.1
Composition of the experimental finisher feed.

Ingredients	**g.100g^{-1}**
Corn	56.67
Soybean meal	26.56
Wheat	10.00
Poultry fat-M	3.54
Limestone	1.63
Mono-Dicalcium phosphate	0.59
Vitamin and mineral premix[x]	0.50
Iodized salt	0.41
Methionine premix[y]	0.10
Total	100.00
Calculated analysis	
ME (kcalkg^{-1})	3200
Calcium	0.9
Crude protein	18.0
Available phosphorus	0.49
Lysine	1.01
Methionine	0.37
Methionine+cystine	0.66

[x]Vitamin supplied per kg of diet: Vitamin A (15 mg), Vitamin D3 premix (40 mg), Vitamin E (50 mg), Vitamin K (9 mg), Riboflavin (8 mg), DL Ca-pantothenate (30 mg), Vitamin B12 (12 mg), Niacin (30 mg), Folic acid (33 mg), Biotin (750 mg), Pyridoxine (5 mg) and Thiamine (3 mg), Manganous Oxide (100 mg), Zinc Oxide (117 mg), Copper Oxide (100 mg), Selenium premix (220 mg), Ethoxyquin (100 mg), Choline chloride (1335 mg), Wheat middlings (1543 mg) and Ground limestone (500 mg).
[y]Methionine premix supplied/kg premix: DL-methionine 0.5 kg, wheat middlings 0.5 kg.

In trial 2, the diet was formulated the same as the first trial, except in the maltodextrin treatment, the finisher feed was replaced by corn germ to facilitate pelleting. Water was added to the corn germ, mixed thoroughly and then added to the maltodextrin to facilitate ease of mixing and pelleting success.

In trial 3, all the ingredients described by Farhat et al. (2002) and Rathgeber et al. (2007) were available but finisher feed was added due to the difficulty of forming pellets from the ingredients. The maltodextrin feed formulation was 80% maltodextrin (Grain Product International), 17% broiler finisher diet, 2.0% corn germ, 0.4% NaCl and 0.6% caramel color (Sethness Caramel Color, W. Touhy Avenue, Lincolnwood, IL, U.S.A).

The finisher feed which was formulated to meet the requirements of the National Research Council (NRC, 1994) was provided to the full fed treatment on each occasion.

3.5.2 Feeding and Allocation of Market-aged Broilers

In trials 1 and 2, one hundred and twenty Ross 508×Ross 508 male broilers raised in the Atlantic Poultry Research Institute facility were fed with standard broiler diets until 37 days of age (Table 3.1). Forty birds were randomly allocated to three pens.

In trial 3, 225, 37-day-old market-aged male (Ross 508×Ross 508) broilers were used. Twenty-five birds were randomly allocated to each of the 9 floor pens. Broiler chickens are meal feeders who tend to feed every four hours (Zuidhof et al., 2004). Therefore, the feed consumption pattern of the broilers was synchronized by denying access to feed for 4 h then returning access to finisher feed for 2 h (Zuidhof et al., 2004). Following feed synchronization, each pen was assigned to one of the following pre-shipping preparation treatments; full fed where finisher feed remained available, feed withdrawal, where feeders were raised out of reach of the birds and full fed with the maltodextrin diet. The birds were subjected to the pre-shipping preparation treatments for times of 3, 6 or 9 h for trials 1 and 2 and 9 h for trial 3. All animal use was approved by the Local Animal Care and Use Committee following the guidelines of the Canadian Council of Animal Care (CCAC, 2009).

3.5.3 Digesta Sampling

In trials 1 and 2, to monitor changes in the bacteria associated with the three pre-shipping preparation treatments, birds were sampled at 3, 6 and 9 h following the start of dietary treatments. It was anticipated that birds in the feed withdrawal and maltodextrin treatments would have diminishing quantities of intestinal digesta over time. Therefore,

an increasing number of birds were sampled from these treatments, as time progressed. The number of full fed birds sampled remained at 4 birds per time period, whereas for the feed withdrawal treatment, 4, 8 and 12 birds were utilized and for the maltodextrin treatment, 8, 12 and 16 birds were sampled for the 3, 6 and 9 h sampling times respectively (trials 1 and 2).

For trial 3, 20 market-aged broilers were randomly selected from each maltodextrin fed treatment pen, 2 birds were selected from the full fed treatment pens and 4 birds from the feed withdrawal treatment. All randomly selected birds at each feeding time were euthanized by cervical dislocation and a segment of the small intestine was aseptically harvested that included the entire jejunum and the ileum (Buhr et al., 1998). The harvested intestines were placed in sterile plastic bags and sent to the laboratory on ice. The samples were kept overnight at 4°C.

3.5.4 Laboratory Analysis

For trial 1, 15 h after sampling, the contents of harvested GIT samples were gently squeezed into a stomacher bag. Samples within a dietary treatment for each treatment time were pooled and blended for 60 s. Ten subsamples (10±0.05 g) were generated from the maltodextrin-pooled digesta at each treatment time and placed into 15 ml plastic tubes. The feed withdrawal and the full fed samples were divided into four subsamples (10±0.05 g) each. EDTA-modified lysozyme (Neova Technologies, Abbotsford BC, Canada) was added to maltodextrin digesta samples at 0.0, 1.5, 3.0, 4.5 and 6.0 mg in duplicate. To maintain a standard digesta dilution, 6.0 mg of maltodextrin was added to the 0.0 mg (control) and an amount equal to the weight of the lysozyme was subtracted from these for each of the treatments. The selection of the lysozyme levels was

based on the manufacturer of lysozyme's recommendation (Neova Technologies, Abbotsford BC, Canada).

In trial 2, the maltodextrin digesta was divided into 14 subsamples (10±0.05 g) and the full fed and feed withdrawal digesta were divided into two subsamples (10±0.05 g) each. Allicin (Allimax®, Toronto, ON, Canada) and EDTA modified lysozyme (Neova Technologies Inc., Abbotsford BC, Canada) were prepared by mixing 1 g of either allicin or lysozyme in 9 g of celite. This stock was used to deliver 5, 12.5 or 20 mg of lysozyme or allicin in duplicate for each 10 g sample.

In trial 3, the digesta from full fed and feed withdrawal treatments were weighed and divided into two subsamples of 10±0.05 g into plastic tubes. The digesta samples from the maltodextrin treatment were divided into twenty-two subsamples of 10±0.05 g. The first two digesta sample represented the control for maltodextrin treatment and 50 mg of celite was added and vortexed. Five different levels of allicin and lysozyme were added to the rest of the maltodextrin digesta samples in duplicate at 10, 20, 30, 40 and 50 mg per 10 g digesta. Celite levels of 40, 30, 20, 10 or 0 mg were added to each of these digesta treatments respectively, to ensure equal volume of material in each 15 ml tube. All samples were mixed thoroughly by vortexing for 30 s. Each sample was challenged with *Salmonella enteritidis* (CFIA) of 10^4 cells per 10 g of digesta from an overnight culture. Twenty ml of buffered peptone water (BPW) was added to each 10 g digesta sample and blended for 60 s. All the samples were incubated in an unmodified atmosphere for trial 1 and in an anaerobic incubator for trials 2 and 3 at 40°C for the same time that the birds were on the dietary treatments: 3, 6 or 9 h for trials 1, 2 and 9 h

for trial 3. These incubation methods were carried out to mimic the digestive tract of market-aged broilers.

This method was a modification of an *in vitro* fermentation method of evaluating antibiotics (Partanen and Jalava, 2005; Rycroft et al., 2001, Zhu et al., 2003). Following incubation, 70 ml of buffered peptone water (BPW) was added and serial dilutions were performed prior to plating on 3M petrifilm plates and incubated on 3M aerobic, *E. coli*/coliform and Enterobacteriacae petrifilm (3M petrifilm^{TM} Minnesota, MN) plates in duplicate. The determinations of dilutions to plate were based on an estimate of bacterial numbers in freshly harvested chicken digesta: 1×10^{6} to 1×10^{9} (Carvalho et al., 2010; Rosenquist et al., 2006). The aerobic plates were incubated at 35±1°C for 24 h and all red colonies were counted (AOAC 940.36, 2005). Enterobacteriaceae plates were incubated for 24 h at 35±1°C and red colonies with yellow zones with or without gas production were enumerated as Enterobacteriaceae (AOAC 940.37, 2005). All red colonies on the 3M *E.coli*/coliform petrifilm plates were enumerated as coliforms following 24 h of incubation at 35±1°C. Plates were reincubated for another 24 h at 35±1°C and all blue colonies with gas production were counted as *E.coli* (AOAC 989.10, 2005) for trials 1 and 2.

In trial 2, additional plating was carried out on *Clostridium perfringens* pour plates. The methodology of Bolder et al. (1999) was followed for *Clostridium perfringens* isolation. *Clostridium perfringens* agar was used to determine the allicin and lysozyme effect on this pathogenic bacterium. The first three dilutions of the 6 and 9 h treatments were plated on Perfringens tryptose sulfite cycloserine (TSC) agar base with Perfringens (TSC) supplement (Oxoid Ltd., Nepean, ON) using the pour plate method. The

Clostridium perfringens plates were incubated for 48 h at 36±1°C under anaerobic conditions and all black colonies characteristic of *Clostridium perfringens* were counted.

In trial 3, allicin and lysozyme were evaluated on aerobic bacteria, *Clostridium perfringens* and *salmonella*. Serial dilutions were plated on Xylose-Lysine-Tergitol 4 (XLT4) agar pour plates to count *Salmonella*. The XLT4 plates were incubated at 35±1°C for 24 h and all black colonies were enumerated as presumptive *Salmonella* (AOAC 967.26, 2005).

3.5.5 Statistical Analysis

All bacteria counts were transformed to $\log_{10}$ values before subjecting data to analysis of variance. The antimicrobial data were run as a 3×5 for trial 1, 3×4 for trial 2 (time×level) and 2×5 (antimicrobials×levels) factorial design for trial 3. The data from pre-shipping preparation treatments was analyzed as a 3×3 factorial (time×pre-shipping treatments) for trials 1, 2 and as a completely randomized design for trial 3. Proc Mixed of SAS (SAS Institute Inc. 2003) was used to analyze all the data. The model for the factorial design analysis was $\gamma_{ijk} = \mu + \tau_i + \beta_j + \tau\beta_{ij} + \varepsilon_{ijk}$ where γ_{ijk} was for the response variable (aerobic bacteria, *E. coli*/coliform, Enterobacteriaceae, *Salmonella* and *Clostridium perfringens*), μ was for the overall mean, τ_i was for the treatment effect of hours (i=1,2,3), β_j was for the treatment effects of the various levels of lysozyme treatments (j=1,2,3,4,5 or 1,2,3,4), $\tau\beta_{ij}$ was for the interaction effect of the treatment times and the various levels. ε_{ijk} was for the error term. The model for the complete randomized design was $\gamma_{ij} = \mu + \tau_i + \varepsilon_{ij}$, where μ was for the overall mean, τ_i was for the treatment effect of the pre-shipping treatments (i=1,2,3) and ε_{ij} was for the error term.

Significant differences among treatments were separated using the mean separation test of Tukey-Kramer ($P \leq 0.05$).

3.6 RESULTS AND DISCUSSION

3.6.1 Aerobic Bacteria

3.6.1.1 The Effects of the Pre-shipping Preparations on Aerobic Bacteria

Three pre-shipping preparations were used to evaluate the effect on bacterial numbers in the GIT of market-aged broilers. Preparing market-aged broilers for shipping by supplying maltodextrin feed, finisher feed or feed withdrawal for 3, 6 and 9 h resulted in differences in bacterial numbers ($P<0.05$) (Table 3.2). The broilers fed with maltodextrin-based feed for 9 h had reduced aerobic bacterial numbers. This confirmed the hypothesis that the maltodextrin feed reduces the aerobic bacterial numbers in the digesta. Similarly, Hinton et al. (2002) indicated that broilers fed with carbohydrate-based cocktail for 12 h had fewer *S. typhimurium* and *Campylobacter* in the crop. The authors explained that the sucrose cocktail feed reduced the pH in the crop and led to the reduction of the pathogenic bacteria. Although, different carbohydrate-based diets were used in their study and this current study, it is possible that the maltodextrin feed reduced the pH in the digestive tracts of these birds and reduced the aerobic bacterial numbers although, the pH was not determined in this current study.

The full fed (birds fed with the finisher diet) and feed withdrawal treatments had no effect on aerobic bacterial numbers ($P>0.05$). Hinton et al. (2000) indicated that feed withdrawal period of 12 to 24 h reduced Enterobacteriaceae and *S. typhimurium* populations in the crop of broilers as compared to broilers fed with finisher feed for the same period of time.

Table 3.2 The *in vitro* effects of the pre-shipping preparation on aerobic bacteria in the digesta of market-aged broilers (Trial 1).

Pre-shipping preparation (Diet)	Treatment time (h)	Aerobes (Log_{10} cfu.g^{-1})
Full fed	3	8.94 ± 0.48^{ab}†
Feed withdrawal	3	10.30 ± 0.48^{a}
Maltodextrin	3	8.59 ± 0.31^{ab}
Full fed	6	8.44 ± 0.48^{ab}
Feed withdrawal	6	8.25 ± 0.48^{ab}
Maltodextrin	6	9.53 ± 0.31^{a}
Full fed	9	9.18 ± 0.48^{ab}
Feed withdrawal	9	9.32 ± 0.48^{ab}
Maltodextrin	9	7.77 ± 0.31^{b}

Anova	P-value
Time	0.15
Diet	0.24
Time × diet	0.001

Means with the same superscript are not significantly different (P>0.05).
† Mean ± Standard error of the mean.
Cfu - colony forming units.

Research has indicated that feed withdrawal purges the crop of broilers, which may reduce the pathogenic bacteria in the crop (Hinton et al., 2000). In this study, the feed withdrawal period was shorter than what has been conducted previously. The insignificant effect of feed withdrawal and full fed observed in this study might be that the lower GIT may need a longer period of time for evacuation leading to bacterial numbers reduction than what was used in this current study.

In trial 2**,** the methods of the pre-shipping preparation influenced the aerobic bacterial numbers. The 9 h full fed treatment had a reduced number of aerobic bacteria as compared with the other treatments (Table 3.3). This is in contrast to trial 1 and the study of Northcutt et al. (2008). Full fed birds had high bacterial numbers in the GIT compared with feed withdrawal birds in their study. Northcutt et al. (2008) reported high mucosal

microbiota similarities in the ileum of full fed birds compared to birds withdrawn from feed for 8, 12 and 24 h. This can be explained that the presence of some carbohydrate sources in the feed provided substrate for the lactic acid bacteria that were present and led to the inhibition of aerobic pathogenic bacteria which led to the reduction in aerobic bacterial numbers (Hinton et al., 2002). .

Feeding maltodextrin feed to market-aged broilers for 3 and 6 h had no effect on aerobic bacteria (P>0.05) but increased aerobic bacterial numbers at 9 h (P>0.05). This is in contrast to the results in trial 1 but similar to the findings of Rathgeber et al. (2007). Rathgeber et al. (2007) indicated that maltodextrin feed had no effect on aerobic bacteria numbers after feeding it to turkeys for 5 h.

Table 3.3 The *in vitro* effects of the pre-shipping preparations on aerobic bacteria numbers in market-aged broilers digesta (Trial 2).

Pre-shipping preparation (Diet)	**Treatment time (h)**	**Aerobes (log_{10} cfu.g^{-1})**
Full fed	3	$9.29\pm0.23^{b\dagger}$
Feed withdrawal	3	9.85 ± 0.23^{ab}
Maltodextrin	3	9.32 ± 0.23^{b}
Full fed	6	9.28 ± 0.23^{b}
Feed withdrawal	6	9.50 ± 0.23^{ab}
Maltodextrin	6	9.30 ± 0.23^{b}
Full fed	9	7.57 ± 0.23^{c}
Feed withdrawal	9	9.60 ± 0.23^{ab}
Maltodextrin	9	10.14 ± 0.23^{a}

Anova	**P-value**
Time	0.001
Diet	0.16
Diet ×Time	<0.002

Means with the same superscript are not significantly different (P>0.05).
† Mean ± Standard error of the mean.
Cfu - colony forming units.

It is possible that the incubation of the samples in an aerobic atmosphere for trial 1 and in anaerobic atmosphere for trial 2 accounted for the differences between the results of trials 1 and 2. The differences in incubation environment might have favored some bacteria over the others.

The high aerobic bacterial number in the digesta from maltodextrin fed birds at 9 h might be explained if maltodextrin fed birds lost interest in the maltodextrin feed. The aim of providing this feed is to evacuate the digestive tract of broilers prior to slaughter (Farhat et al., 2002). It is possible that the birds did not consume the maltodextrin feed. The feed consumption was not determined because maltodextrin weight increased with time due to moisture absorption from the atmosphere.

Withdrawing feed from broilers for 3, 6 and 9 h prior to slaughter did not affect bacterial numbers as feed withdrawal times increased ($P>0.05$). Other studies have investigated the effect of feed withdrawal times on bacteria in other parts of the GIT. Sengor et al. (2006) found no difference in aerobic bacteria counts in the ceca of broilers for feed withdrawal times of 0, 6, 12 and 18 h. Similarly, Hinton et al. (2000) conducted a study on the effect of feed withdrawal times of 0, 12 and 24 h with the intension that broilers may consume litter and this may lead to an increase in bacterial numbers. Crop contents from broilers placed on either litter or in crates were collected and blended in distilled water and cultured for aerobic bacteria, *Salmonella* and Enterobacteriaceae. However, no differences were observed between placing broilers in crates and on litter. The results of these studies are similar to the results from this study although, the digesta were harvested from different sections of the digestive tract.

In trial 3, aerobic bacteria numbers for feed withdrawal and maltodextrin treatments for 9 h were different ($P<0.05$). The maltodextrin-fed birds had higher aerobic bacterial numbers than the full fed birds ($P>0.05$). It is possible that the maltodextrin treated birds did not consume the maltodextrin feed.

Full fed treatment had a reduced aerobic bacterial number as compared with the other two treatments ($P<0.05$) (Table 3.4). This is an indication that pre-shipping treatments manipulated the bacterial numbers in the GIT. Normally, studies on feed withdrawals are initiated after supplying birds with dietary treatments. Thompson et al. (2008) fed a control diet, 250 ppm of $CuSO_4$, or bacitracin to 42 day old broilers and subjected the birds to 0, 10 and 24 h feed withdrawal times. Thompson et al. (2008) found that feed withdrawal and dietary treatments: control diet, 250 ppm of $CuSO_4$, or bacitracin changed the microbial community in the GIT by reducing bacterial diversity in the ileum with an increase in feed withdrawal times.

The 9 h full fed result was similar to the 9 h full fed treatment in trial 2. These may be due to the fact that the samples in trials 2 and 3 were incubated in an anaerobic chamber compared to trial 1 where the samples were incubated in aerobic incubator.

Table 3.4 The *in vitro* effects of the three pre-shipping preparations on aerobic bacteria numbers in market-aged broiler digesta incubated for 9h (Trial 3).

Pre-shipping preparations (Diet)	**Treatment time (h)**	**Aerobes (log_{10} cfu.g^{-1})**
Full fed	9	8.43 ± 0.28^{c}†
Feed withdrawal	9	9.74 ± 0.28^{a}
Maltodextrin	9	9.18 ± 0.24^{b}

Anova	**P-value**
Diet	<0.001

Means with the same superscript are not significantly different ($P>0.05$).
† Mean ± Standard error of the mean.
Cfu - colony forming units.

The high aerobic bacterial numbers for feed withdrawal treatments could be explained that the litter might contain bacteria. Birds are stressed in the absence of feed (Delezie et al., 2007) and pick in the litter when feed is denied (Hinton et al., 2000). Others have suspected birds may consume litter during pre-shipping feed withdrawal period. Hinton et al. (2000) subjected birds to feed withdrawal times of 0 to 24 h either on litter covered floors or in crates. The practice did not affect *Salmonella typhimurium* or Enterobacteriaceae numbers in sampled crop. The variation in results for aerobic bacteria between this trial and other publications may be attributed to the fact that the digesta was incubated outside the GIT of the birds and the differences in methods used for bacterial enumeration.

3.6.1.2 The Effects of Alternative Antimicrobials on Aerobic Bacteria *In Vitro*

In general, the aerobic bacteria numbers in the GIT of market-aged broilers were reduced for the various levels of lysozyme as the feed withdrawal times increased ($P<0.05$) (Table 3.5) from 3 to 9 h but the bacterial numbers at 3.0, 4.5 and 6.0 mg of the lysozyme levels at 6h treatments grew beyond countable range. There was an interaction between treatment time and lysozyme levels. This was a result of 3 highest levels of lysozyme being too numerous to count (TNTC) for 3.0, 4.5 and 6.0 mg of lysozyme at 6 h treatment. The various lysozyme levels did not affect the bacterial numbers within a particular time. Research investigations on lysozyme inhibition in nutrient broth showed that lysozyme reduced growth of pathogenic bacteria (Johansen et al., 1994). According to Johansen et al. (1994), egg white lysozyme inhibited growth of *Listeria monocytogenes* Scott A in tryptic soy broth at temperatures of 5°C and 25°C. However, other studies have reported a lack of response to lysozyme of certain types of bacteria.

The inability of lysozyme to extend its antimicrobials effects to some Gram-negative bacteria was mostly solved by modifying the lysozyme by adding chelating substance such as ethylenediaminetetraacetic acid (EDTA) (Masschalck and Michiels, 2003).

Table 3.5 The *in vitro* effects of lysozyme on aerobic bacteria numbers in market-aged broilers digesta (Trial 1).

Lysozyme levels (mg) per 10 g maltodextrin digesta	**Treatment time (h)**	**Aerobes (log_{10} cfu.g^{-1})**
0.0	3	9.43±0.25^{ab†}
1.5	3	8.08±0.25^{bc}
3.0	3	8.65±0.25^{bc}
4.5	3	8.40±0.25^{bc}
6.0	3	8.41±0.25^{bc}
0.0	6	8.43±0.25^{bc}
1.5	6	8.34±0.25^{bc}
3.0	6	10.30±0.25^{a*}
4.5	6	10.30±0.25^{a*}
6.0	6	10.30±0.25^{a*}
0	9	7.51±0.25^{c}
1.5	9	7.60±0.25^{c}
3.0	9	7.92±0.25^{c}
4.5	9	7.92±0.25^{c}
6.0	9	7.92±0.25^{c}

Anova	**P-value**
Time	0.003
Lysozyme	<0.001
Time×Lysozyme	0.004

Means with the same superscript are not significantly different (P>0.05).
† Mean ± Standard error of the mean.
Cfu - colony forming units.
*TNTC – too numerous to count (this was replaced with 1720 at the 7th dilution which was 10.24 log_{10} cfu).

Combinations of EDTA or apo-lactoferrin (Apo-Lf) and lysozyme (Sigma Chemical Co., St Louis, MO) were evaluated for their antimicrobial effects on ultra-high temperature pasteurized milk against *Pseudomonas fluorescens, Salmonella typhimurium, Escherichia*

coli O157:H7, and *Listeria monocytogenes*. None of the bacteria were influenced by EDTA, Apo-Lf or lysozyme alone or in combination (Payne et al., 1994). This is similar to the results of this current study, where the lysozyme used had EDTA in it but it did not affect aerobic bacterial numbers. The lack of significant differences observed in this study could be that the lysozyme killed some bacteria in the digesta and some lysozyme resistant aerobic bacteria grew on the plate. It has been indicated that the GIT of broilers is populated by hundreds of different species (Apajalahti et al. 2004). Another reason for this could be that the lysozyme levels used were too low. Other factors may also be responsible to the inability of lysozyme to inhibit bacteria may be associated to the pH of the medium and the cations in the buffer. Although, pH and the cations in the samples were not measured in this study, Smolelis and Hartsell (1952) indicated that lysozyme lysis activity increases at a pH of 6.6 and the salt concentration for maximum lysis depends on the increasing power of the cation. The cationic increasing efficiency is as follows: calcium, magnesium, ammonium, potassium, and sodium at atemperature between 37 and 75°C.

In trial 2, the digesta harvested from the maltodextrin fed broilers was analyzed for aerobic bacteria following the addition of four levels of 0.0, 5.0, 12.5 or 20.0 mg antimicrobials (allicin or lysozyme) to 10 g digesta. These levels of lysozyme were higher than the levels used in trial 1. Although, the allicin and lysozyme levels were increased, they did not have an inhibitory effect at any level on the aerobic bacteria load at 3, 6 or 9 h, (Table 3.6). The number of bacteria cultured from the 9 h control treatment was higher than for the lysozyme treatments ($P<0.05$). There was no difference among

the control treatments and lysozyme treatments for the other two time periods except when treated for 9 h, then all levels of lysozyme had fewer bacteria than the control.

Table 3.6 The *in vitro* effects of antimicrobials on aerobic bacteria in maltodextrin fed market-aged broilers digesta (Trial 2).

Antimicrobials level (mg)/ 10 g maltodextrin digesta	**Treatment time (h)**	**Aerobes (log_{10} cfu.g^{-1})**	
		Allicin	Lysozyme
0.0	3	9.32±0.23	9.32±0.13^{bc}
5.0	3	10.01±0.23	9.55±0.13^{bc}
12.5	3	9.36±0.23	9.57±0.13^{b}
20.0	3	9.65±0.23	9.59±0.13^{b}
0.0	6	9.30±0.23	9.30±0.13^{bc}
5.0	6	9.49±0.23	9.15±0.13^{c}
12.5	6	9.40±0.23	9.31±0.13^{bc}
20.0	6	9.44±0.23	9.53±0.13^{bc}
0.0	9	10.14±0.23	10.14±0.13^{a}
5.0	9	9.68±0.23	9.49±0.13^{bc}
12.5	9	9.55±0.23	9.34±0.13^{bc}
20.0	9	9.46±0.23	9.34±0.13^{bc}

Anova	**P-value**	**P-value**
Time	0.20	0.05
Level	0.45	0.34
Time×Level	0.26	0.01

Means in a column with the same superscript are not significantly different (P>0.05).
† Mean ± Standard error of the mean.
Cfu - colony forming units.

Garlic extract inhibition on specific bacteria has been evaluated by Bakri and Douglas (2005). Bakri and Douglas (2005) reported that garlic extract had an effect on oral Gram-negative (garlic extract MIC range 1.1 to 35.7 mg.ml^{-1}) and Gram-positive bacteria (garlic extract MIC range 35.7 to 142.7 mg.ml^{-1}) extracted from humans. The inhibitory activity by allicin was dependent on the level of allicin and type of bacteria evaluated. Bakri and Douglas (2005) found that Gram-negative pathogenic bacteria are more sensitive to garlic extract, lysing at lower levels than the Gram-positive pathogens.

The inhibitory activity of allicin was not observed in this research. It is possible that garlic extracts may be more effective than allicin. This may be associated with the medium in which the allicin was examined. The stability of allicin activity varied across different solutions. Fujisawa et al. (2008) investigated the stability of allicin in various aqueous extracts and ethanolic solutions as well as in vegetable oil. It was found that the allicin was very unstable in vegetable oil but was 20% more stable in ethanol than in water. It is possible that the allicin lost its stability in the media (BPW and digesta) used in this study. Garlic extract has been use in several studies to determine its effects on bacteria in a nutrient broth more than allicin. Although, pH was not determined in this study, it has been shown that the stability of garlic extracts in a nutrient broth is affected by the pH (Tynecka and Gos, 1975, Durairaj et al., 2009). Durairaj et al. (2009) indicated that garlic extracts are very stable at a pH of 5.8, which is the original pH in garlic but may moderately inhibit bacterial growth at a pH of 7 to 9. However, garlic extract activity decreases with increase in pH (Tynecka and Gos, 1975).

In trial 3, the inclusion of either allicin or lysozyme did not reduce the number of bacteria in the digesta of maltodextrin fed broilers at the different times used ($P>0.05$) (Table 3.7). Despite the lack of response in our study, recent research has shown the antimicrobial effect of allicin. Perez-Giraldo et al. (2003) reported that allicin had inhibitory effect on 28 *Staphylococcus* species at a concentration of 8 $mg.l^{-1}$ using a standard broth microdilution technique. The authors found that allicin reduced biofilm formation in *Staphylococcus epidermidis* at a minimum inhibition concentration of 8 $mg.l^{-1}$. There are examples of investigators attempting to reduce GIT bacteria numbers with garlic products. Ross et al. (2001) studied the effect of garlic powder and garlic oil

on human enteric bacteria and found that garlic powder levels of 6.25 to 12.5 $mg.ml^{-1}$ reduced enteric bacteria more than garlic oil levels of 0.02 to 5.5 $mg.ml^{-1}$. Viability time

Table 3.7 The *in vitro* effects of antimicrobials on aerobic bacterial numbers in market-aged broiler digesta incubated for 9h (Trial 3).

Antimicrobials	**Antimicrobials levels (mg) per 10 g maltodextrin digesta**	**Aerobes (log_{10} $cfu.g^{-1}$)**
Allicin	0	9.42±0.24†
	10	9.70±0.22
	20	9.32±0.22
	30	9.16±0.22
	40	9.14±0.22
	50	9.10±0.22
Lysozyme	0	9.42±0.24
	10	9.62±0.26
	20	9.55±0.22
	30	9.19±0.24
	40	9.48±0.19
	50	9.50±0.26

Anova	**P-value**
Antimicrobial	0.24
Level	0.42
Antimicrobial×level	0.87

† Mean ± Standard error of the mean.
Cfu - colony forming units.

studies of garlic oil and garlic powder against *Enterobacter aerogenes* indicated that there were time exposure and dose dependent effects (Ross et al., 2001). Similarly, variability time and dosage study on a variety of garlic products: garlic oil, garlic powder and their diallyl constituents against *Helicobacter pylori*, indicated time of exposure and dose dependent effect of garlic products on *Helicobacter pylori* (O'Gara et al., 2000). A lack of response to allicin in this trial might be due to the differences in the garlic products used, variations in the levels and exposure times used which were 3, 6 and 9 h.

Although, garlic products have effect on bacteria at a wide range of levels but the highest inhibition effect was determined at 24 h of incubation (O'Gara et al., 2000, Ross et al., 2001).

3.6.2 Coliforms

3.6.2.1 The Pre-shipping Preparation Effect on Coliforms

In trial 1, all the coliform plates were overgrown with bacteria. Therefore, no results were obtained from the first *in vitro* trial.

In trial 2, as time progressed, the feed withdrawal treatment coliform numbers increased ($P<0.05$) and full fed sample coliform numbers decreased (Table 3.8). Reports on the effects of feed withdrawal time on number of *E.coli* and coliforms in the GIT are not numerous. However, there have been reports on how varying withdrawal times in conjunction with maltodextrin influence these bacteria on the surface of a processed carcass. Northcutt et al. (2003a) fed replacement finisher diet (maltodextrin feed) to market-aged broilers for 8 h and subjected the birds to 0, 4, 8 and 12 h of feed withdrawal periods. *E.coli* and coliform numbers were evaluated after pre-eviscerated (feathers, feet, and heads removed) and eviscerated whole carcasses rinses in BPW. Northcutt et al. (2003a) found that the feed withdrawal length did not affect *E.coli* and coliform numbers. Similarly, Northcutt et al. (2003b) subjected different aged (41, 49 and 56 day) broilers to either 0 (full fed) or 12 h of feed withdrawal and measured bacteria recovery before and after immersion chilling with 20 ppm sodium hypochlorite in whole carcass rinses in BPW. Feed withdrawal period had no effect on the number of *E.coli* and coliforms recovered. In this *in vitro* study, the samples were taken from digesta but Northcutt et al. (2003a, 2003b) based their microbial analysis on whole carcass rinses. It could be that the

carcasses were not contaminated by the digesta, as cotton plugs were inserted in the cloaca of each carcass during bleeding (Northcutt et al., 2003b). Feeding a maltodextrin

Table 3.8 The *in vitro* effects of the pre-shipping preparations on coliform numbers in market-aged broilers digesta (Trial 2).

Pre-shipping preparations (Diet)	**Pre-shipping preparation times (h)**	**coliform (log_{10} cfu.g^{-1})**
Full fed	3	8.76±0.15^{b†}
Feed withdrawal	3	7.67±0.15^{c}
Maltodextrin	3	8.98±0.15^{ab}
Full fed	6	8.98±0.15^{ab}
Feed withdrawal	6	9.08±0.15^{ab}
Maltodextrin	6	8.91±0.15^{ab}
Full fed	9	7.49±0.11^{c}
Feed withdrawal	9	9.24±0.15^{a}
Maltodextrin	9	8.94±0.15^{ab}

Anova	**P-value**
Time	0.004
Diet	0.01
Time×Diet	0.001

Means with the same superscript are not significantly different (P>0.05).
† Mean ± Standard error of the mean.
Cfu - colony forming units.

supplement to market-aged broilers did not change coliform numbers as feeding times increased. Similar attempts have been made by researchers to control bacteria populations by feeding a maltodextrin feed to poultry but the treatment did affect bacterial numbers (Rathgeber et al., 2007, Northcutt et al., 2003a). Northcutt et al. (2003a) reported that feeding a maltodextrin feed to market-aged broilers for 8 h followed by feed withdrawal periods of 0, 4, 8 and 12 h did not affect *E. coli*, coliforms and Enterobacteriaceae. On the other hand, Rathgeber et al. (2007) observed a reduction in coliform numbers in the crop of turkeys after feeding them maltodextrin feed for 5 h. Rathgeber et al. (2007) attributed the reduction of coliform numbers in the crop to the fact that the maltodextrin may have

reduced the pH in the GIT of the turkeys. The reason for not observing reduction in coliform number in this current study could be that the maltodextrin did not effectively evacuate the GIT or the maltodextrin did not reduce the pH in the GIT to encourage the multiplication of lactic acid bacteria.

The coliform numbers were reduced for the full fed treatment as feeding time increased from 6 to 9 h ($P<0.05$). It has been explained earlier that for the full fed treatment effect on aerobic bacteria, it could be that the full fed birds had some carbohydrate substrate in the digesta that reduced the pH in the GIT and led to the reduction in coliform numbers.

3.6.2.2 The Effect of Alternative Antimicrobials on Coliform Numbers in Maltodextrin Fed Market-aged Broiler Digesta

In trial 1, the coliform plates were too numerous to count (TNTC) for all dilutions and treatments, there was no data available for trial 1.

In trial 2, petrifilm plates were countable. The incorporation of allicin and lysozyme did not change the coliform numbers ($P>0.05$) (Table 3.9).

Allicin from garlic has been reported to have antimicrobial effects on Gram-negative and Gram-positive bacteria (Ankri and Mirelman, 1999). Other commercial extracts from garlic namely Proallium-S-DMC and Proallium-SO-DMC have previously been shown to have inhibitory effects on total aerobes, anaerobes, lactobacilli, bifidobacteria, coliforms, enterobacteria, bacteroides and clostridia in the feces of swine *in vitro* (Ruiz et al., 2010). Ruiz et al. (2010) indicated that 0.47, 1.88 and 3.76 $mg.ml^{-1}$ of industrial extracts from garlic decreased *Salmonella* and Enterobacteriaceae numbers as incubation time increased to 25 h. Although, the levels used by Ruiz et al. (2010) were

Table 3.9 The *in vitro* effects of antimicrobials on coliform numbers in maltodextrin fed market-aged broilers digesta (Trial 2).

Antimicrobials level (mg) per 10 g maltodextrin digesta	**Treatment time (h)**	**Coliforms (log_{10} cfu.g^{-1})**	
		Allicin	Lysozyme
0.0	3	8.98±0.10	8.98±0.11†
5.0	3	8.96±0.10	8.92±0.11
12.5	3	8.85±0.10	9.01±0.11
20.0	3	8.92±0.10	8.95±0.11
0.0	6	8.92±0.10	8.92±0.11
5.0	6	9.04±0.10	8.95±0.11
12.5	6	9.07±0.10	8.93±0.11
20.0	6	9.16±0.10	9.04±0.11
0.0	9	8.94±0.10	8.94±0.11
5.0	9	9.17±0.10	9.09±0.11
12.5	9	9.08±0.10	8.96±0.11
20.0	9	9.13±0.10	9.01±0.11

Anova	**P-value**	**P-value**
Time	0.14	0.85
Level	0.47	0.94
Time×Level	0.70	0.93

† Mean ± Standard error of the mean.
Cfu - colony forming units.

similar to the levels used in this study, the incubation time may have caused the differences in the results of their study and this current study.

Lysozyme has been found to be an effective antimicrobial. Nattress et al. (2001) found that lysozyme (>22,800 Shugar units rmg, Canadian Inovatech, Abbotsford, BC, Canada) or 1:3 nisin/lysozyme mixture inhibited *Carnobacterium* species on fat tissues following 21 days storage in vacuum packages at 2°C. Pellegrini et al. (2000) indicated that lysozyme from hen egg white was effective on Gram-negative and Gram-positive bacteria. Lysozyme inhibited *Escherichia coli* with evidence from electron microscopy.

Images revealed a condensed cytoplasm and rippled bacterial membrane in treated bacteria (Pellegrini et al., 2000).

Although, there is strong evidence of antimicrobial effects of these two antimicrobials, the current study did not indicate the antimicrobial activity on the bacterial numbers in the digesta. The reason could be associated with the fact that the BPW was added to the digesta and incubated in an anaerobic atmosphere for 3, 6 or 9 h. This approach may have supported rapid multiplication of some bacteria to overgrow on the *E.coli*/coliform plates. Baylis et al. (2000) reported that heat-injured *Salmonella* cells can be recovered in BPW after 24 h of incubation.

3.6.3 Enterobacteriaceae

The Enterobacteriaceae family includes *Salmonella*, *Yersinia*, *Shigella* and *Escherichia* species. These bacteria have been well studied in humans due to their relationship with health related problems (Paterson, 2006, Spanu et al., 2002).

3.6.3.1 The Effect of the Pre-shipping Preparations on Enterobacteriaceae in Maltodextrin Digesta

Enterobacteriaceae plates were TNTC for all dilutions and treatments in trial 1, therefore, there was no data available from this trial.

In trial 2, there were no interaction effects between treatment times and the pre-shipping preparation used for Enterobacteriaceae ($P>0.05$) (Table 3.10). The full fed, feed withdrawal and maltodextrin fed treatments did not affect Enterobacteriaceae numbers for the 3, 6 and 9 h treatments ($P>0.05$). There were TNTC plates for full fed, feed withdrawal and maltodextrin treatments at 6 h and maltodextrin treatment at 9 h.

The levels of bacterial numbers have been evaluated for broilers subjected to full fed and feed withdrawal treatments (Hinton et al., 2000). Hinton et al. (2000) subjected broilers

Table 3.10 The *in vitro* effects of the pre-shipping preparation on Enterobacteriaceae numbers of market-aged broiler digesta (Trial 2).

Pre-shipping preparations (Diets)	**Treatment time (h)**	**Enterobacteriaceae (log_{10} $cfu.g^{-1}$)**
	3	8.59 ± 0.20^{b}†
	6	9.44 ± 0.20^{a}*
	9	9.01 ± 0.20^{ab}
Full fed	3	8.82±0.35
Feed withdrawal	3	7.74±0.35
Maltodextrin	3	9.22±0.35
Full fed	6	9.44±0.35*
Feed withdrawal	6	9.44±0.35*
Maltodextrin	6	9.44±0.35*
Full fed	9	8.46±0.35
Feed withdrawal	9	9.13±0.35
Maltodextrin	9	9.44±0.35*

Anova	**P-value**
Time	0.05
Diets	0.15
Time×Diets	0.15

Means with the same superscript are not significantly different (P≥0.05).
† Mean ± Standard error of the mean.
Cfu - colony forming units.
*TNTC-too numerous to count (this was replaced with the upper limit count of 2500 at the 6^{th} dilution which was equivalent to 9.44 log_{10} cfu).

to 0, 12 and 24 h of feed withdrawal periods and indicated that, the feed withdrawal times had no effect on Enterobacteriaceae in the crop, the average Enterobacteriaceae numbers in the crop was 5.8 log_{10} cfu (Hinton et al., 2000). Typically, the number of bacteria observed in their study was less than what was observed in this study. The high numbers of aerobic bacteria in this study may be attributed to the handling of the digesta, the part

of the digestive tract from which the sample was generated or the method of sampling. Hinton et al. (2000) took samples from the crop but in this research, samples were taken at the lower digestive tract (beginning of the jejunum to the ileocecal junction). Hinton et al. (2000) analyzed for Enterobacteriaceae right after sample collection, but these samples were placed at 4°C storage overnight and incubated in a glove box the following day before analysis of bacterial numbers. Direct comparisons of results with other studies are difficult. Studies that measured the effects of feed withdrawal or Enterobacteriaceae counts either measured bacteria at different parts of the GIT or did not use a period of time where the bacteria in the samples were allowed to grow outside the host.

3.6.3.2 The Effect of Alternative Antimicrobials on Enterobacteriaceae in Maltodextrin Digesta

In trial 1, the Enterobacteriaceae plates were overgrown in all treatments therefore, there was no data for analysis for trial 1.

In trial 2, the addition of allicin and lysozyme at four different levels to maltodextrin digesta at the three treatment times had no effect on Enterobacteriaceae numbers (Table 3.11). Antimicrobials have been used in an attempt to inhibit growth of bacteria. Lactoferrin (LF), lysozyme (LZ), the lactoperoxidase system (LPOS), and edible whey protein isolate were tested against *Salmonella enterica* and *Escherichia coli* O157:H7 in tryptic soy broth (Min et al., 2005). The lactoperoxidase system (LPOS) reduced *Salmonella enterica* and *Escherichia coli* O157:H7 but lysozyme (Sigma-Aldrich) at ≥6 and ≥20 $mg.ml^{-1}$ had no effects on the number of cultured bacteria (Min et al., 2005). Although, the authors analyzed for specifically for Enterobacteriaceae family, similar results were observed in this current study. The Lactoferrin (LF), lysozyme (LZ)

Table 3.11 The *in vitro* effects of the antimicrobials on Enterobacteriaceae numbers in market-aged broilers' digesta (Trial 2).

Antimicrobials level (mg) per 10 g maltodextrin digesta	Treatment time (h)	Enterobacteriaceae (log_{10} cfu.g^{-1})	
		Allicin	**Lysozyme**
	3		9.10±0.06^{b}†
	6		9.44±0.06^{a}
	9		9.31±0.06^{a}
0.0		9.37±0.07^{a}	
5.0		9.11±0.07^{b}	
12.5		9.01±0.07^{b}	
20.0		9.20±0.07^{ab}	
0.0	3	9.22±0.12	9.22±0.12
5.0	3	9.09±0.12	9.04±0.12
12.5	3	8.93±0.12	9.05±0.12
20.0	3	9.30±0.12	9.09±0.12
0.0	6	9.44±0.12*	9.44±0.12*
5.0	6	9.07±0.12	9.44±0.12*
12.5	6	9.01±0.12	9.44±0.12*
20.0	6	9.15±0.12	9.44±0.12*
0.0	9	9.44±0.12*	9.44±0.12*
5.0	9	9.18±0.12	9.20±0.12
12.5	9	9.07±0.12	9.44±0.12*
20.0	9	9.14±0.12	9.17±0.12

Anova	**P-value**	**P-value**
Time	0.72	0.01
Level	0.02	0.49
Time×Level	0.73	0.49

Means within a column with the same superscript are not significantly different (P>0.05).
† Mean ± Standard error of the mean.
Cfu - colony forming units.
*TNTC –too numerous to count (this was replaced with the upper limit count of 2500 at the 6th dilution which was equivalent to 9.44 log_{10} cfu).

and the lactoperoxidase system (LPOS) had no effect on the tested bacteria. It is possible that lysozyme may not affect Enterobacteriaceae. Based on the authors' search of literature, this study is the first attempt of using allicin or lysozyme to control

Enterobacteriaceae in GIT contents of broilers. All the 6h and 9 h at 0 and 12.5 mg lysozyme treatments had overgrown plates. The 0mg allicin (control) for 6 and 9h were also overgrown however, allicin levels at 5.0 and 12.5 mg reduced Enterobacteriaceae numbers compared to the control treatments but the 20 mg treatments did not.

3.6.4 *Clostridium perfringens*

3.6.4.1 The Effect of the Pre-shipping Preparations on *Clostridium perfringens*

Market-aged broilers were subjected to three pre-shipping preparation treatments to determine the pre-shipping preparations effect on *Clostridium perfringens*. The results indicated that the three pre-shipping preparation treatments had no inhibitory effect on *Clostridium perfringens* load in the digesta of market-aged broilers ($P>0.05$) (Table 3.12). However, in this instance many of the agar pour plates had no growth regardless of the treatments. Only samples from birds fed maltodextrin contained culturable *Clostridium perfringens*. The small numbers of *Clostridium perfringens* found in this study was very surprising. Research on detection of *Clostridium perfringens* in the ileum of broilers have shown that *Clostridium perfringens* numbers increased with age (Knarreborg et al., 2002, Mitsch et al., 2004). Knarreborg et al. (2002) fed dietary fat (soy oil, and a mixture of lard and tallow) with a dietary supplementation of antibiotics (a combination of avilamycin and salinomycin) on the bacterial population in the ileum of broilers at different ages (7, 14 21 and 35 d). The authors analyzed bacterial population using DGGE and it was reported that *Clostridium perfringens* type A band numbers increased with response to age. Similar to their study, a feeding trial was performed by Mitsch et al. (2004) to determine the effects of two different blends of essential oils on *Clostridium perfringens* of broilers at 14, 21 and 35 d of age. Contents from the jejunum,

cecum, cloaca and feces were analyzed for *Clostridium perfringenes* using traditional culturing methods. The incidence of samples positive for *Clostridium perfringens* was 83.3% for feces samples, 88.0% for jejunum and cloaca samples, 82.6% for cecum samples for the total population in the control birds at 30 d. Essential oils reduced *Clostridium perfringens* numbers. The fact that the differences in bacterial isolation methods did not affect the identification in *Clostridium perfringens*, it is possible that the broilers used in this trial had low numbers of *Clostridium perfringens* in their GIT.

Table 3.12 The *in vitro* effects of pre-shipping preparations on *Clostridium perfringens* of market-aged broiler digesta (Trial 2).

Pre-shipping preparation (Diet)	**Treatment time (h)**	***Clostridium perfringens*** **(log_{10} cfu.g^{-1})**
Full fed	6	1.00±0.09†‡
Feed withdrawal	6	1.00±0.09‡
Maltodextrin	6	1.12±0.09
Full fed	9	1.00±±0.09‡
Feed withdrawal	9	1.00±±0.09‡
Maltodextrin	9	1.29±±0.09

Anova	**P-value**
Time	0.46
Diet	0.09
Time×Diet	0.56

† Mean ± Standard error of the mean.
Cfu-colony forming units.
‡TFTC-lowest detectable limit (this was replaced with 1log_{10} cfu).

There was no reduction in bacterial numbers even after feeding the maltodextrin for 9 h, it is possible that the maltodextrin did not evacuate the GIT or the time allowed for the feed treatment was too short.

In trial 3, no differences were observed among the pre-shipping preparation treatments (P>0.05) (Table 3.13). Once again, providing maltodextrin feed to market-aged broilers had no effect on *Clostridium perfringens*. However, this time the samples

were positive for *Clostridium perfringens* in all treatments. It was anticipated that, the maltodextrin feed may evacuate the GIT and reduce the concentration of *Clostridium perfringens* in the digesta but there was no effect on these bacteria this study.

Table 3.13 The *in vitro* effects of the pre-shipping preparations on *Clostridium perfringens* in market-aged broiler digesta (Trial 3).

Pre-shipping preparation (Diet)	**Treatment time (h)**	***Clostridium perfringens* (log_{10} cfu.g^{-1})**
Full fed	9	2.91±0.86†
Feed withdrawal	9	3.69±0.86
Maltodextrin	9	3.21±0.58

Anova	**P-value**
Diet	0.71

† Mean ± Standard error of the mean.
Cfu - colony forming units.

Clostridium perfringens numbers were high compared to the *Clostridium perfringens* numbers in the previous trial. This may be attributed to the the fact that different set of broilers were used.

3.6.4.2 The Effect of Alternative Antimicrobials on *Clostridium perfringens*

In trial 2, allicin did not inhibit *Clostridium perfringens* in the digesta of maltodextrin fed market-aged broilers. There were no differences in bacterial numbers ($P>0.05$) among the levels used (Table 3.14) but the levels were affected by treatment times ($P<0.05$).

Clostridium perfringens increased as time progressed from 6 to 9 h for allicin. Allicin has been found to have an inhibitory effect on a wide range of microorganisms (Harris et al., 2001) but *Clostridium perfringens* numbers increased with time. This does not reflect what others have found. Ankri and Mirelman (1999) indicated that allicin inhibited bacterial growth at a concentration range of 0.015 to >0.100 mg.ml^{-1} depending

on the type of bacteria (*Escherichia coli, Staphyloccocus aureus, Streptococcus pyogenes, Proteus mirabilis, Pseudomonas aeruginosa, Acinetobacter baumanii, Klebsiella pneumonia* and *Enterococcus faecium*). Another study was conducted with garlic extract

Table 3.14 The *in vitro* effects of antimicrobials on *Clostridium perfringens* of maltodextrin fed market-aged broilers digesta (Trial 2).

Antimicrobials Levels (mg)/10 g of digesta	**Treatment time (h)**	***Clostridium perfringens*** **(log_{10} $cfu.g^{-1}$)**	
		Allicin	**Lysozyme**
	6	1.06±0.05[b]	
	9	1.23±0.05[a]	
0.0			1.21±0.06[a†]
5.0			1.00±0.06[b]
12.5			1.00±0.06[b]
20.0			1.21±0.06[a]
0.0	6	1.12±0.10	1.12±0.08
5.0	6	1.13±0.10	1.00±0.08‡
12.5	6	1.00±0.10‡	1.00±0.08‡
20.0	6	1.00±0.10‡	1.00±0.08‡
0.0	9	1.29±0.10	1.29±0.08
5.0	9	1.36±0.10	1.00±0.08‡
12.5	9	1.11±0.10	1.00±0.08‡
20.0	9	1.45±0.10	1.43±0.08
Anova		**P-value**	**P-value**
Time		0.04	0.03
Level		0.19	0.04
Time×Level		0.94	0.08

Means within a column with the same superscript are not significantly different (P>0.05).
† Mean ± Standard error of the mean.
Cfu - colony forming units.
‡TFTC- lowest detectable limit (this was replaced with 1log_{10} cfu).

and was reported to be successful. According to Banerjee and Sarkar (2003), garlic extract had an inhibitory effect on Gram-negative and Gram-positive bacteria at a range of 6 to100 $mg.ml^{-1}$ depending on the type of bacteria. It was observed that some bacteria

that were resistant to other antimicrobials such as penicillin were susceptible to garlic extract at concentrations of 6–10 $mg.ml^{-1}$ for *Bacillus cereus*, 30–40 $mg.ml^{-1}$ for *Staphylococcus aureus*, 20–30 $mg.ml^{-1}$ for *Clostridium perfringens*, 10$mgml^{-1}$ for *Escherichia coli*, 40–100 $mg.ml^{-1}$ for *Salmonella*, and 10–40 $mg.ml^{-1}$ for *Shigella*. Although, the levels used in this current research fall within the range of level used by previous researchers but allicin did not have any inhibitory effect on *Clostridium perfringens*. It is possible that there were other factors that affected antimicrobial activity of allicin. These factors may include inoculum level, proportion of digesta and temperature (Klančnik et al., 2011).

Lysozyme affected *Clostridium perfringens* at the various levels used. A reduction in bacterial numbers for lysozyme was observed on *Clostridium perfringens* at 5 and 12.5 mg of lysozyme compared to the control ($P<0.05$). However the highest level of 20 mg was not different from the control. The inhibitory effect demonstrated the effectiveness of lysozyme on *Clostridium perfringens*. The inhibition of *Clostridium perfringens* by lysozyme in this study was similar to the findings of Zhang et al. (2006), where lysozyme from hen egg white had inhibitory effect on *Clostridium perfringens*. It has been reported that lysozyme has the ability to rapidly degrade the peptidoglycan cell wall of Gram-positive bacteria (Nash et al., 2006, Ibrahim et al., 2001). In this study since most of the plates did not show *Clostridium perfringens* growth, these data were replaced with the least detectable level of $1\log_{10}$ cfu before statistical analysis was performed.

In trial 3, the levels of antimicrobials used, were increased over trial 2, however there was no reduction in the number of *Clostridium perfringens* compared to controls ($P>0.05$)

(Table 3.15). Although, lysozyme did not affect *Clostridium perfringens* in this study, it has been confirmed that hen egg white lysozyme is effective against Gram-positive bacteria (Ibrahim et al., 1991, Pellegrini et al., 1997). A recent study by Abdou et al. (2007) indicated complete inhibition of Gram-positive bacteria (*Bacillus* species) by lysozyme (Pharma Foods International Co., Ltd Kyoto, Japan) at a concentration of 0.1 $mg.ml^{-1}$ in trypticase soy broth. It is difficult to explain the factors that may be responsible for non-inhibitory effects of these antimicrobials as the levels used were higher than the level used by Abdou et al. (2007).

Table 3.15 The *in vitro* effects of antimicrobials on *Clostridium perfringens* of market-aged broiler digesta (Trial 3).

Antimicrobials	**Antimicrobials levels (mg) per 10 g maltodextrin digesta**	***Clostridium perfringens*** **(log_{10} cfu.g^{-1})**
Allicin	0	3.98±0.98†
	10	3.51±1.28
	20	2.30±0.92
	30	1.85±0.92
	40	2.72±0.87
	50	3.67±0.87
Lysozyme	0	3.98±0.98
	10	3.98±1.28
	20	1.81±0.99
	30	3.46±0.92
	40	4.00±0.82
	50	3.23±0.99

Anova	**P-value**
Antimicrobial	0.38
Level	0.15
Antimicrobial×level	0.54

† Mean ± Standard error of the mean.
Cfu - colony forming units.

3.6.5 *Salmonella*

There were no presumptive identifications of *Salmonella* colonies on the XLT4 agar plates. Colonies that turned the media yellow grew over the plates in this study. The standard operating procedure (SOP) of the XLT4 agar indicates that *Escherichia coli* appears as yellow colonies (XLT4 agar supplement, 2012). It is possible that *Salmonella* grew on the plates but did not produce the black colonies due to bacteria failure to produce α-galactosidase which is responsible for bacterial colony color identification (Perez et al., 2003, Perry et al., 2004). All the digesta samples were inoculated with 10^4 cells *Salmonella enteritidis,* yet no *Salmonella* recovery was observed. It could also be that *Salmonella* did not grow because of the presence of competing bacteria.

3.6.6 The Developed *In Vitro* Method

The inconsistency in results has made it difficult to determine whether the *in vitro* method developed was good or not. There are some factors that may account for this bacteria count overgrowth. These factors may include: the fact that the samples were kept in the fridge overnight. The storage in the fridge overnight might have changed the profile of bacteria population. As some bacteria can grow rapidly in refrigeration temperatures while other bacteria cannot. Bacteria that can survive and grow at refrigeration temperature are referred to as psychrotrophic (Gounot, 1991). This may have caused some bacteria to overgrow the others.

Also the fact that the digesta was incubated in the anaerobic incubator for the time in which the birds were on feed had the tendency to affect the bacteria population. Under normal circumstances, the digesta in the GIT of broilers are evacuated and feed is continuously added to dilute the bacteria population. In this study, it is possible that the

bacteria made use of the nutrients available in the digesta as there was no flow system. It could be that the insufficiency of nutrients in the digesta at some point of incubation caused the inability of *Salmonella* and *Clostridium perfringens* recovery on some of the pour plates.

Bacteria population in the digestive tract of broilers has been estimated to be within the range of 1×10^6 to 1×10^9 (Carvalho et al., 2010, Rosenquist et al., 2006). Bacterial numbers evaluated from other studies in the digesta of feed withdrawal birds ranged from 5 to 7 $\log_{10}$ cfu on the average (Rathgeber et al., 2007, Hinton et al., 2002, Sengor et al., 2006). In this study, most of the plates were overgrown with bacteria above 10^9 and at some instances bacterial growth was as low as 10^1. In order to solve the unpredictable bacterial numbers problem, digesta from the experimental birds should be cultured to give an estimate on the bacteria numbers.

3.7 CONCLUSION

The addition of different levels of lysozyme to maltodextrin digesta from market-aged broilers did not affect aerobic bacteria and coliforms. Allicin and lysozyme had an inhibitory effect on Enterobacteriaceae and *Clostridium perfringens* numbers in the maltodextrin digesta from market-aged broilers at the same levels (5 and 12.5 mg) respectively. It can be concluded that the antimicrobials may not work on all the bacteria present in the maltodextrin digesta at the levels used. The aerobic bacteria numbers reduced in the maltodextrin digesta as time progressed. This agreed with the hypothesis that maltodextrin feed reduces bacteria numbers in the digesta. XLT4 plates had no indication of *Salmonella* presumptive positive colonies. The lack of response of bacteria to the treatments imposed and the inability of some bacteria growth on the culturing

plates do not allow us to determine if this *in vitro* assay is appropriate for determining the effective levels of antimicrobials for application with *in vivo* studies.

CHAPTER 4: *IN VITRO* SCREENING OF ALLICIN AND LYSOZYME ON BACTERIA CULTURED FROM MALTODEXTRIN FED MARKET-AGED BROILER'S DIGESTA AND *SALMONELLA ENTERICA* SEROVAR HEIDELBERG PURE CULTURE

4.1 ABSTRACT

An *in vitro* trial was conducted to determine the susceptibility of bacteria from the small intestine of broilers to lysozyme from hen egg white and allicin from garlic. This was performed to determine appropriate doses for inclusion in a maltodextrin-based feed offered to broilers for the time period before shipping when feed is normally removed. The *in vitro* assay involved the addition of the antimicrobials to a broth solution containing bacteria cultured from digesta of maltodextin fed broilers and/or *Salmonella enterica* serovar Heidelberg pure culture. Lysozyme was evaluated in two trials and allicin only in the second trial. The antimicrobials were added at 0, 50, 100 and 150 mg per 9 ml of buffered peptone water and replicated 6 times. Allicin was evaluated at the same inclusion levels the second trial. All samples were incubated for 6 h at 35°C and plated on 3M petrifilm aerobic plates. Aerobic bacterial numbers were transformed into $\log_{10}$ values and analyzed using Proc Mixed of SAS. Dry matter contents of the maltodextrin-based feed and the collected digesta were measured and used to develop a formula for converting antimicrobial levels used *in vitro* to inclusion levels in maltodextrin feed evaluated *in vivo*. Lysozyme reduced bacteria numbers in the *Salmonella* pure culture and in the culture originated from maltodextrin digesta. In the combined culture, the levels of allicin used had no effect on the aerobic bacteria but lysozyme reduced bacterial numbers by 1.65, 4.15 and 3.91 $\log_{10}$ cfu for 50, 100 and 150 mg respectively.
Based on the lysozyme results, dry matter percentages of maltodextrin digesta samples and the maltodextrin feed, a formula for determining inclusion levels in a maltodextrin-based withdrawal feed was devised. Lysozyme levels of 0, 10 and 20 g were selected to be incorporated in 1000 g of maltodextrin feed for an upcoming *in vivo* trial.

Key words: *Salmonella enterica* serovar Heidelberg, aerobic bacteria, maltodextrin digesta culture, *in vitro*, lysozyme and allicin.

4.2 INTRODUCTION

Alternative antimicrobials are used in poultry research to improve growth performance, and ensure the health and welfare of the bird is maintained by controlling bacteria in the GIT (Buchanan et al., 2008). Government regulation in Europe and consumer pressure in North America has led to an increased interest in producing antibiotic-free poultry meat (Fernando et al., 2009). Often the first stage in evaluation of alternatives to antibiotics is to determine the effective dosage. The Canadian Animal Care requires that the 3R (reduce, refine and replace animal use) rules be strictly observed during animal experimentation (CCAC, 2009). A strategy to comply with the 3R rules of animal use is to conduct *in vitro* experiments (Kulpa-Eddy et al., 2011).

In vitro methods of testing antimicrobials have been used on numerous occasions to determine inhibitory effects of these substances on bacterial isolates (Citron et al., 2003, Zhang et al., 2006). *In vitro* methods of evaluation are typically less expensive compared to *in vivo* methods and often require less time (Zips et al., 2005). The micro-broth dilution assay in which the wells of a microtitre plate is used as the container of the test material and bacteria, is an example of an *in vitro* method used to evaluate the minimal inhibitory concentrations of antimicrobial (Anil and Samaranayake, 2002, Hall et al., 2011). Lysozyme from egg white was tested in this way against three clinical isolates of *Clostridium perfringens* type A that were associated with severe necrotic enteritis in chickens (Zhang et al., 2006). They found the method was effective for determining dosage required for *Clostridium perfringens* inhibition.

The antimicrobial screening procedure in the previous chapter was based on evaluation of several levels of antimicrobials incubated in GIT contents from broilers fed

a maltodextrin-based feed. Results of bacterial inhibition tests were difficult to interpret and did not lead to successful determination of effective doses of allicin or lysozyme. Modifications were made to the methods used in the previous chapter in efforts to simplify the procedure to facilitate determination of the optimal inclusion level of the two test antimicrobials in the maltodextrin feed supplement. Bacteria cultured from the digesta of broilers fed maltodextrin and/or a pure culture of *Salmonella* were subjected to increasing levels of allicin and lysozyme.

4.3 OBJECTIVE

The objective of this study was to evaluate the bactericidal effect of allicin and lysozyme at increasing levels on bacteria generated from maltodextrin digesta or a *Salmonella enterica* serovar Heidelberg pure culture.

4.4 HYPOTHESIS

Increasing levels of commercial allicin or lysozyme reduce the growth of bacteria in a nutrient broth.

4.5 MATERIALS AND METHODS

4.5.1 Preparation of Bacteria Cultures

In trial 3 of the previous chapter, maltodextrin digesta from market-aged broilers fed maltodextrin feed was stored at -80°C. After storage for 8 months, one of these digesta samples was randomly selected and thawed for 3 h at room temperature. A loop full of digesta was removed and inoculated in 100 ml Luria Berthani (LB) broth (Lot No. 0082283). Similarly a loop full of *Salmonella enterica* serovar Heidelberg (CFIA, Canada) pure culture was inoculated in 5 ml of LB broth. The bacteria culture generated from maltodextrin fed market-aged broiler digesta and *Salmonella enterica* serovar

Heidelberg (Strain No. S-8) pure culture were incubated at 35±1°C overnight. Following incubation, both cultures were diluted to McFarland's standard of 1.5×10^8 cells per ml, determined by dilution to an optical density of 0.08-0.1. These cultures were further diluted so that 100 µl delivered approximately 10,000 cells.

4.5.2 Preparation and Sampling of the Antimicrobials

In two trials 4 and 5 a stock solution was prepared by adding 1 g of lysozyme from hen egg white or allicin from garlic each to 20 ml of BPW and dissolving completely. The solution was used to deliver 50, 100 and 150 mg of allicin and lysozyme. The levels for allicin and lysozyme: 50, 100 and 150 ml were equivalent to 1, 2 and 3 ml respectively. Similarly 0.105 g of tetracycline (Sigma Aldrich, St. Louis, MO) was dissolved in 13 ml of BPW for a solution of 80 $mg.ml^{-1}$. The *Salmonella* strain used in preparing the culture was resistant to this level of tetracycline.

In trial 4, there were ten treatments including negative control (tetracycline), 0 (positive control), 50, 100 and 150 mg of lysozyme for *Salmonella enterica* serovar Heidelberg or culture from maltodextrin fed broiler digesta and replicated 6 times. These treatments were added to 9 ml of BPW and 100 µl of the prepared culture was added to each of the respective treatments.

In trial 5, there were eight treatments with six replicates. These treatments include the positive control, 50, 100 and 150 mg of allicin or lysozyme. The treatments were added in 9 ml of BPW. Following this 100 µl of both *Salmonella enterica* serovar Heidelberg pure culture and culture generated from maltodextrin fed broilers digesta were inoculated in the aliquot. Since the antimicrobials were delivered into 9 ml of BPW in the form of liquid, the total volume for the 150 mg treatment tube was 12 ml. However, to

ensure equal volumes of aliquots in the tubes, the other tubes were filled up with additional BPW to 12 ml. The samples were blocked into two, where the first three replicates of the treatments represented block 1 and the last three replicates represented block 2, with 30 min between preparations of the two blocks.

All the samples were incubated aerobically at 35±1°C for 6 h and 1 ml of each sample was diluted five times and dilutions 2 to 5 were plated on aerobic 3M petrifilm plates in duplicate. Following plating, the aerobic 3M petrifilm plates were incubated for 24 h at 35±1°C. All red colonies were enumerated as aerobic bacteria (AOAC 940.37, 2005).

4.5.3 Dry Matter Determination

Maltodextrin digesta samples that were thawed overnight were weighed and refrozen at -80°C overnight. Frozen samples were placed in a freeze dryer (4K Freeze Dryer, Mano Royal, Crawleg, West Sussex, England) for 24 h. The samples were weighed and the dry matter determined. The moisture content was determined by subtracting the dry matter weight from the initial weight of the digesta. Dry matter percentages were calculated by dividing the dry matter weight by the initial weight of the sample and multiplied by 100.

4.5.4 Experimental Design

The trials were analyzed as a 2×5 and 2×4 factorial design for trials 4 and 5 respectively. The factors were the 2 cultures and the 5 levels of antimicrobials.

Trial 4 had 5 treatments which include tetracycline (negative control), positive control (0 mg), 50, 100 and 150 mg of lysozyme for either *Salmonella enterica* serovar

Heidelberg pure culture or culture generated from maltodextrin fed market-aged broiler digesta.

In trial 5, there were 4 levels of allicin and lysozyme: 0, 50, 100 and 150 mg, each treatment was replicated 6 times. All bacterial counts were transformed into $\log_{10}$ values before analysis, using Proc Mixed of SAS (SAS Institute Inc. 2003). The trials were analyzed as a factorial design. The model for the analysis was $\gamma_{ijk} = \mu + \tau_i + \beta_j + \tau\beta_{ij} + \varepsilon_{ijk}$.

In trial 4, γ_{ijk} was for the aerobic bacteria numbers, μ was for the overall mean, τ_i was for the treatment effect of the cultures (i=1,2), β_j was for the treatment effect of the antimicrobials levels (j=1,2,3,4,5), $\tau\beta_{ij}$ was for the interaction effect of the treatments (digesta and *Salmonella* cultures) and the antimicrobials levels. ε_{ijk} was for the error term. All too numerous to count (TNTC) plates were replaced with 168 at the 5^{th} dilution which were equivalent to 7.23 $\log_{10}$ cfu before the data analysis.

In trial 5, γ_{ijk} was for the aerobic bacteria numbers, μ was for the overall mean, τ_i for the treatment effect of the antimicrobials (i=1,2), β_j was for the treatment effect of the antimicrobials levels (j=1,2,3,4), $\tau\beta_{ij}$ was for the interaction effect of the treatments (bacteria culture from maltodextrin fed market-aged broilers and *Salmonella* pure culture) and the antimicrobials levels. ε_{ijk} was for the error term. All TNTC plates were replaced with the preferable upper count limit of aerobic bacteria which was 250 at the highest dilution which was 5. This was transformed to $\log_{10}$ cfu value of 7.40. Significant differences among treatments were separated using the mean separation test of Tukey-Kramer ($P \leq 0.05$)

4.6 RESULTS AND DISCUSSION

4.6.1 Aerobic Bacteria

Results from counting bacteria from the *Salmonella enterica* serovar Heidelberg pure culture and the bacteria culture generated from maltodextrin fed broiler digesta, negative control treatments without antimicrobial were not different (P>0.05). The tetracycline was added to the culture to prevent the growth of other bacteria except for *Salmonella enterica* serovar Heidelberg however, the tetracycline treatment and the culture generated from maltodextrin digesta had the same bacterial count (Table 4.1). It was suspected that the culture generated from maltodextrin digesta would have a higher level of bacteria than the tetracycline treatment because the maltodextrin digesta culture may have had different species of bacteria multiplying during the incubation period.

Table 4.1 The *in vitro* effect of lysozyme on bacteria generated from maltodextrin fed broiler's digesta and *Salmonella enterica* serovar Heidelberg pure culture (Trial 4).

Treatments (culture)	**Levels of lysozyme(mg)**	**Aerobes (log_{10} cfu.ml^{-1})**
Bacteria culture	0	7.23±0.22$^{a\dagger *}$
	50	6.61±0.22^{b}
	100	5.70±0.22^{c}
	150	4.62±0.22^{d}
	Tetracycline	6.88±0.22^{ab}
Salmonella pure culture	0	7.23±0.22^{a*}
	50	5.66±0.22^{c}
	100	4.27±0.22^{d}
	150	4.04±0.22^{d}
	Tetracycline	7.23±0.22^{a*}

Anova	**P-value**
Treatments	0.001
Levels	<0.001
Treatments×levels	0.002

Means with different superscripts are significantly different (p≤0.05).
† Mean ± Standard error of the mean.
*TNTC- too numerous to count (this was replaced with upper limit is 168 on the 5th dilution which was equivalent to 7.23 log_{10} cfu).

Since market-aged broilers have diverse microbial community in their GIT (Thompson et al., 2008). The bacteria culture generated from maltodextrin fed broiler digesta control plates were TNTC and data from these samples referred to as the upper limit count for analysis. That was an indication that there was high number of aerobic bacteria in the culture generated from maltodextrin digesta. However, when lysozyme was added at 50, 100 and 150 mg, the aerobic bacteria numbers decreased with an increase in lysozyme levels ($P<0.05$).

Lysozyme reduced *Salmonella enterica* serovar Heidelberg at 50, 100 and 150 mg per 9 ml ($P<0.05$). This demonstrates that *Salmonella enterica* serovar Heidelberg is susceptible to lysozyme. Similarly, Ellison and Giehl (1991) indicated that lysozyme level of 0.05 $mg.ml^{-1}$ had a bactericidal effect on *V. cholerae*, *S. typhimurium* and *E. coli* in 1% bacto-peptone. Interaction showed greater effect of lysozyme at 50 and 100 mg on *Salmonella enterica* serovar Heidelberg than bacteria generated from maltodextrin fed market-aged broiler digesta.

In trial 5, allicin had no effect on aerobic bacteria growth ($P>0.05$). All the allicin treatments were TNTC and assigned the upper limit of bacteria count (Table 4.2). The results from allicin treatments were in contrast to the findings of Cellini et al. (1996) findings. The authors observed 90% inhibition of *Helicobacter pylori* isolated from antral mucosal biopsies of patients at a garlic extract concentration of 5 $mg.ml^{-1}$. It could be that other bacteria that were resistant to allicin were growing on the plate. Also the differences in garlic product, concentrations and bacteria strain may account for the variation in the results. Drenkard (2003) demonstrated that some bacteria strains are

resistant to certain antimicrobials. It is possible that most bacteria present in the solution were resistant to allicin.

Table 4.2 The *in vitro* effects of allicin and lysozyme on bacteria generated from maltodextrin fed market-aged broiler's digesta (Trial 5).

Treatments (Maltodextrin digesta)	**Antimicrobials levels (mg)**	**Aerobes (log_{10} cfu.ml^{-1})**
Allicin	0	7.40±0.11$^{a\dagger *}$
	50	7.40±0.11^{a*}
	100	7.40±0.11^{a*}
	150	7.40±0.11^{a*}
Lysozyme	0	7.40±0.11^{a*}
	50	5.69±0.11^{b}
	100	3.40±0.11^{c}
	150	3.60±0.11^{c}

Anova	**P-value**
Treatments	<0.001
Levels	<0.001
Treatments×Levels	<0.001

Means with the same superscript are not significantly different (P≥0.05).
† Mean ± Standard error of the mean.
Cfu - colony forming units.
*TNTC- too numerous to count (this was replaced with 250 at the 5th dilution and was equivalent to 7.40 log_{10} cfu).

Lysozyme treatments of 100 and 150 mg were equal for the extent of bacterial inhibition and were more effective than 50 mg (P<0.05). The results revealed that lysozyme levels of 50, 100 and 150 mg reduced bacteria growth by 1.65, 4.15 and 3.91 log_{10} cfu values respectively. Similarly, previous studies on the inhibitory effects of lysozyme in a nutrient broth have been shown to be successful. Zhang et al. (2006) reported that lysozyme from hen egg white had an inhibitory effect against *Clostridium perfringens* in nutrient broth.

4.6.2 Determination of Antimicrobials Levels for the *In Vivo* Trial

The antimicrobial levels used for the screening assays were determined by taking into account, the dry matter percentages of the maltodextrin digesta samples and the maltodextrin feed for the calculations. The dry matter percentages calculated from each maltodextrin digesta sample, indicated that there was approximately 4% dry matter content in the maltodextrin feed.

The maltodextrin digesta sample from the last *in vitro* trial was in 1:10 dilution. However, the dry matter percentage was multiplied by 10 which resulted in 40%. The dry matter analysis of maltodextrin feed indicated that the maltodextrin feed had 96% dry matter. When the percentage dry matter of the maltodextrin feed was divided by dry matter percentage of the maltodextrin digesta (96/40), the result was 2.4. This was rounded to 2 and used as the constant for the calculations.

In the last *in vitro* trial, allicin and lysozyme were evaluated in culture using digesta generated from maltodoxtrin fed market-aged broiler digesta and *Salmonella enterica* serovar Heidelberg pure culture. It was demonstrated that lysozyme had an inhibitory effect on aerobic bacteria and *Salmonella enterica* serovar Heidelberg. There was a total volume of 12 ml aliquot in each test tube with 50, 100 or 150 mg of lysozyme or allicin. To evaluate the amount of lysozyme to add to the maltodextrin feed, the levels of lysozyme were divided by 12 ml (which was the total volume of aliquot in each tube): 50 mg/12, 100 mg/12, 150 mg/12 which equated 4.17 mg, 8.33 mg and 12.45 mg. These values were multiplied by 2, 2 being the value obtained from dividing dry matter percentage of maltodextrin by the dry matter percentage of the maltodextrin digesta sample. These resulted in 8.4, 16.6 and 25 mg, respectively and the values were rounded

to 8, 17 and 25 mg. However, the lysozyme levels were selected within the range of 0-25 mg. Every 1000 g maltodextrin feed was formulated with 0, 10 or 20 g of lysozyme.

4.7 CONCLUSION

The increased levels of lysozyme evaluated were effective in controlling aerobic bacteria numbers in culture generated from maltodextrin fed market-aged broiler digesta and *Salmonella enterica* serovar Heidelberg pure culture. Allicin did not have any inhibitory effect on aerobic bacteria numbers. Therefore, the lysozyme appears more promising to use in maltodextrin feed to control bacteria population than allicin.

CHAPTER 5: *IN VIVO* EFFECTS OF LYSOZYME-ENRICHED MALTODEXTRIN FEED ON BACTERIAL NUMBERS IN THE ILEAL CONTENT OF MARKET-AGED BROILERS

5.1 ABSTRACT

Poultry meats are often contaminated with pathogenic bacteria through digesta leakage during processing. It was anticipated that reducing bacteria load in the digesta of market-aged broilers prior to processing may reduce the impact of fecal contamination of carcasses at the processing plant. Lysozyme was incorporated in a maltodextrin feed intended for delivery during the normal pre-shipping feed withdrawal period. This was carried out to evaluate its effect on bacteria numbers in the ileal contents of market-aged broilers. Twenty 36 day-old broilers were randomly allocated to each of 16 pens. One of the following treatments was applied to each pen: feed withdrawal or maltodextrin feed with lysozyme added at 0, 10 or 20 g per 1000 g of feed. The birds were offered the feed treatments for 9 h. Feed consumption was determined then a minimum of 3 birds were randomly selected from each pen and euthanized. The ileal contents were removed and weighed. The digesta samples were analyzed for *Clostridium perfringens*, aerobic bacteria, Enterobacteriaceae, *E.coli* and coliform numbers using standard culturing techniques and next generation sequencing. The results from bacteria counts were transformed to $\log_{10}$ colony forming units (cfu) values and analyzed as a completely randomized design. The data from next generation sequencing was analyzed as a 3×5 factorial design using Proc Mixed of SAS. Lysozyme did not affect the amount of maltodextrin feed consumed by the birds and the weight of ileal contents for birds fed maltodextrin was not different than the birds on traditional feed withdrawal. *E. coli*/coliforms and Enterobacteriaceae plates had no signs of bacterial growth. The number of *Clostridium perfringens* and aerobic bacteria in the ileal contents of market-aged broilers was not different between treatments using the traditional culturing technique. The next generation sequencing was a useful alternative to traditional culture techniques as results revealed that bacilli were reduced and clostridia increased for 20 g of lysozyme per 1000 g of maltodextrin feed. Addition of lysozyme to a maltodextrin-based feed did not change overall numbers of bacteria but was effective in altering the participants in the bacteria community in ileal contents of market-aged broilers.

Key words: lysozyme, feed withdrawal, market-aged broilers, *Clostridium perfringens*, aerobic bacteria, next generation sequencing, lysozyme-enriched maltodextrin feed, Gram-negative and Gram-positive bacteria.

5.2 INTRODUCTION

The poultry industry is fully aware of the association of bacteria from poultry products with foodborne illness. Some of the more prominent pathogens include *Salmonella* (Crump et al., 2002) and *Clostridium perfringens* (Sinosh et al., 2010). One of the most likely points of contamination of poultry meat is the processing plant (Berrang and Bailey, 2009). During processing digesta from the GIT can become a source of bacteria for contamination of the carcass (Chao et al., 2008). This is often referred to as fecal contamination.

Broilers are fed low doses of antibiotics to control pathogenic bacteria and improve growth performance (Apajalahti et al. 2004). However, long term use of antibiotics may lead to the emergence of antibiotic resistant bacteria (Butaye et al., 2003, Mayrhofer et al., 2004). Antibiotic resistant bacteria can be transferred to humans through the food chain (Van Looveren et al., 2001) when contaminated meats are consumed (Hurd et al., 2004). Due to these concerns, poultry researchers have investigated the use of alternatives to antibiotics from plant and animal sources (Abdalla, 2011, Kamysz, 2005). Alternatives to antimicrobials include prebiotics, probiotics, organic acids, animal and plant extracts (La Ragione et al., 2001). An example of an alternative antimicrobial is lysozyme from hen egg white (Mine et al., 2004). Lysozyme has been incorporated in broiler feed in a study by Liu et al. (2010) and was found to inhibit growth of *Clostridium perfringens* in chickens. They supplemented broiler feed with 40 mg of lysozyme per kg of feed and reported improved intestinal barrier function and growth performance of broilers compared to the control. The barrier function was evaluated by the indirect method of bacterial translocation to the liver and spleen.

In our study, lysozyme from hen egg white was evaluated in a maltodextrin feed offered to market-age broilers for a short time prior to shipping. Maltodextrin is a highly digestible feed supplement that was developed to feed poultry prior to slaughter to ensure the evacuation of the GIT and to maintain body weight of broilers (Farhat et al., 2002, Rathgeber et al., 2007).

Bacteria populations were evaluated using both NGS and traditional culturing method. The traditional culturing method of enumerating bacteria provides information on the number of bacteria (Zengler, 2009). The NGS effectively allows the evaluation of the broad group of bacterial diversity and the manipulation of the bacteria community (Metzker, 2010).

5.3 OBJECTIVE

The objective of this study was to evaluate the effect of lysozyme added to maltodextrin feed on bacterial numbers in the ileal contents of market-aged broilers prior to shipping.

5.4 HYPOTHESIS

The addition of lysozyme in maltodextrin-based feed for broilers for a short time will change bacterial numbers and type of bacteria in the ileum.

5.5 MATERIALS AND METHODS

5.5.1 Maltodextrin Feed Formulation

A maltodextrin feed was produced by modifying a formulation used by Farhat et al. (2002). The feed withdrawal supplement in this study consisted of 70% maltodextrin (Grain Processing Corporation, Muscatine, IA), 3% tallow, 2% corn germ (Corn Products International, Westchester, IL, USA), 2% mono and diglycerides (Univar, Quebec, BC,

Canada), 0.33% caramel color (Sethness Caramel Color, W. Touhy Avenue, Lincolnwood, IL, U.S.A) and 0.36% salt. To ensure that the feed supplement formed pellets, 22.31% finisher diet was added. Melted tallow was mixed with the finisher feed in a mixer (Hobart Mixer, Closter, NJ, USA) and added to the other ingredients. All ingredients were added to the mixer and mixed for 15 min. Three treatments were formulated to contain 0, 10 or 20 g of lysozyme (Neova Technologies, Abbotsford BC, Canada) per 1000 g of feed. The finisher feed component was reduced by the amount of lysozyme added. Rate of feed delivery of the pellet (California Pellet Mill Co. CPM, Crawfordsville, IN 47933, United States) equipped with a die with 3mm holes was adjusted to facilitate pellet formation and to control pellet temperature at exit from the pelleter die. The pellets were formed at 72±1°C.

5.5.2 Feeding and Allocation of Market-aged Broilers

Male, Ross 508×Ross 508 broilers were randomly allocated to 16 pens with 20 birds per pen at 37 days of age. Each of four rooms housed four pens of birds with each treatment group randomly assigned to a pen in each room. The treatments included conventional feed withdrawal and maltodextrin feed with 0, 10 or 20 g of lysozyme per 1000 g of maltodextrin feed. The feed treatments were applied after synchronizing the feeding behavior of the broilers by removing finisher feed for 4 h and followed by access to finisher feed for 2 h (Zuidhof et al., 2004). Feed synchronization was carried out to ensure minimized variation in the volume of GIT material. Once the feed supplement was introduced, the birds had access to the maltodextrin feed for 9 h. Bird feeding behavior was observed during the feeding trial. All animal use was approved by the Local Animal

Care and Use Committee (Nova Scotia Agricultural College) following the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

5.5.3 Sample Collection

After 9 h of access to maltodextrin feed, the remaining feed was weighed and recorded. Feed consumption was monitored by subtracting feed weight following consumption from the initial feed supplied. A minimum of three birds were selected at random from each pen. The birds were euthanized by cervical dislocation and ileal contents were harvested aseptically and pooled to produce two 30 g samples. The number of birds required to produce a 30 g sample of digesta was recorded. The digesta samples were stored on ice until further analysis.

5.5.4 Laboratory Analysis

5.5.4.1 Microbial Plating

From each of the 30 g digesta samples, 25±0.05 g was placed in a blender bag. Two hundred twenty five ml of BPW was used as a diluent prior to blending for 60 s to ensure homogeneity. Eight serial dilutions were performed. The first three dilutions were plated on *Clostridium perfringens* pour plates, the 4^{th} to the 6^{th} dilutions were plated on 3M aerobic count petrifilm count. The 7^{th} to 8^{th} dilutions were plated on Enterobacteriaceae and *E.coli*/coliform petrifilm plates.

The pour plates of *Clostridium perfringens* agar were incubated in a controlled atmosphere incubator (glove box) for 48 h at 35±1°C under anaerobic conditions and all black colonies with characteristics of *Clostridium perfringens* were enumerated as *Clostridium perfringens* (Bolder et al., 1999). The aerobic plates were incubated at a temperature of 35±1°C for 24 h and all colonies were enumerated as aerobic bacteria

(AOAC 940.36, 2005). Enterobacteriaceae plates were incubated for 24 h and red colonies with yellow zones, with or without gas production were enumerated as Enterobacteriaceae (AOAC 940.37, 2005). All red colonies were enumerated as coliforms after 24 h incubation at 35±1°C. The plates were reincubated for another 24 h at 35±1°C and all blue colonies with gas production were counted as *E. coli* (AOAC 989.10, 2005).

5.5.4.2 Extraction of DNA from Digesta for Bacteria Detection

One gram subsamples from each digesta sample were taken and stored at -80°C for DNA extraction. These samples were thawed at 20°C. AW1 (QIAGEN Mat.No. 1014790) and AW2 (QIAGEN Mat No. 1014592) BPW were prepared according to the manufacturer's instructions. Digesta was placed in 15 ml tubes and 10 ml of buffer ASL (QIAGEN Mat. No. 1014755) was added to the digesta sample and placed in a water bath (Fisher Scientific) at 95°C for 10 min. At 5 min, the samples were removed and vortexed for 1 min until a homogenous mixture was formed and returned to the water bath. The samples were centrifuged (Catalog No. C2400) at 3000 *x g* for 5 min. One and a half ml of the supernatant of each digesta samples was pipetted into a 2 ml microcentrifuge tube (Mandel Sorvail Legen RT, Mandel, Guelph, ON, Canada) and the pellets discarded. One tablet of inhibitEX was added to each sample and vortexed for 30 s (Scientific Industries Vortex Genie 2, Orville Drive, Bohemia, NY 11716, USA) until the tablet was completely suspended. The suspension was allowed to sit for 1 min to ensure that all inhibitors were adsorbed to the inhibitorEX matrix. The samples were then centrifuged (Spectrofuge Labnet spectrafuge 24D, Mayfield Avenue, Edison, NJ, 08837, USA) at 16,300 *x g* for 3 min to ensure that all inhibitors were bound to the inhibitEX matrix. The

pellets were removed and 200 µl of the supernatant was pipetted off to a microcentrifuge tube and 10 µl of RNase was added to each of the samples. All the samples were allowed to sit for 10 min and centrifuged (Catalog No. C2400) for 3 min at 16,300 *x g*. Fifteen µl of proteinase K (QIAGEN Mat. 1014023) was pipetted into each of the samples. Following that, 200 µl of buffer AL (QIAGEN Mat.1014600) was added to each sample and inverted for 15 s. The samples were incubated at 70°C for 10 min. Then, the samples were centrifuged (Catalog No. C2400) briefly for 10 s at 16,300 *x g* to remove all traces of sample on the lid. Two hundred µl of ethanol (96-100%) was added to the lysate and inverted. New QIAamp spin column lids were leveled and placed in a 2 ml collection tube. Then 630 µl was carefully pipetted to the QIAamp spin column without moistening the rim. The caps were closed and centrifuged (Spectrofuge Labnet spectrafuge 24D, Mayfield Avenue, Edison, NJ, 08837, USA) at 16,300 *x g* for 1 min. Five hundred µl of buffer AW1 (QIAGEN Mat. 1014790) was pipetted into the QIAamp spin column. The samples were then centrifuged at 16,300 *x g* for 1 min. The filtrates were poured off and 500 µl of buffer AW2 (QIAGEN Mat. 1014592) was pipetted into QIAamp spin column, centrifuged at 16,300 *x g* for 3 min and the filtrate was discarded. The QIAamp spin column was transferred into a new labeled 1.5 ml microcentrifuge tube. Then 150 µl AE was pipetted to the QIAamp membrane and centrifuged (Catalog No. C2400) at 16,300 *x g* for 1 min to elute DNA. The QIAamp spin columns were discarded and the eluted DNA samples were stored at -4°C. The quantity of DNA was determined using a spectrophotometer at 260 nm. Buffer ASL was modified to 10 ml, the method was based on manufacturer's instructions (QIAGEN, Toronto, ON).

5.5.4.3 Electrophoresis

Fifty ml of tris-acetic acid EDTA (IX-TAE) electrophoresis buffer was placed into a measuring cylinder, 1.5% of the 50 ml (0.75 g) of agarose (AMRESCO Lot #1139B065, Cochran Road Solon, OH 44139-0098, USA) gel was weighed and added to the IX-TAE. The solution was microwaved (Kenmore, Model No. 88230) until agarose was completely dissolved. The solution was allowed to sit in the hood (Mott fume hood monitor FH 218-1) for 10 min to cool down. Two µl of ethidium bromide was added and swirled gently to obtain a homogenous solution. The solution was poured into an electrophoretic gel casting tray and allowed to sit for 20 min to form the gel. Before loading, the polymerase chain reaction samples were centrifuged briefly in a Sevant (Speed Vac SC110, Farmingdale, NY, USA) to collect sample at bottom of the tube. The PCR product was centrifuged in savant (Speed Vac SC110, Farmingdale, NY, USA) for 20 s. The gel casting tray (Owl, Knightsbridge Rd, Danville, IN 46122, USA) was placed in the electrophoresis tank and IX-TAE electrophoresis buffer was added to fill line indicated on the tank, to cover the gel. The comb was removed; 5 µl of the PCR product was mixed on the loading gel dye and loaded into the gels. A gene ruler of 1000 bp DNA (Fermentas No.SMO321) ladder (5 µl) was pipetted into one of the wells. The gel was run for 30 min at 80 volts, after which it was visualized with a UV transilluminator (Syngene Genius, Pegasus Court, Suite M, Frederick MD 21704, USA) at 320 nm. This procedure was used to determine whether the correct PCR product was produced.

5.5.4.4 Amplification of the Deoxyribonucleic Acid (DNA)

After checking for the quality of the DNA in each sample through imaging, it was realized that the concentrations of DNA in each sample were very low. However, the DNA was amplified by using polymerase chain reaction (PCR). The sample was vacuumed for 45 min using Savant (Speed Vac SC110, Farmingdale, NY, USA) to concentrate the sample. This enabled the DNA to be concentrated. To amplify the DNA in the samples, a PCR product master mix with 600 bp of DNA was prepared and 20 µl of the master mix was added to 5 µl of each sample. These samples were centrifuged with Sevant (speed Vac SC 110, Farmingdale, NY, USA) for 30s at 16300 *x g*. Following that, the PCR was run in a Dyad Disciple (Peltier Thermal Cycler Bio RAD, Lincoln Centre Drive, Foster City, California 94404, USA.) for 105 min. The manufacturer's instructions were followed (QIAGEN, Toronto, ON). The samples were sent to the Research and Testing Laboratory LLC (Lubbock, Texas, U.S.A) for next generation sequencing analysis. The 454 next generation sequencing technique (Roche 454 sequencer) was used. The sequencing was performed using a universal primer (preparatory primer used by the next generation sequencing group, Lubbock, Texas, U.S.A) on a ¼ slide with 5,000 reads per plate.

5.6 STATISTICAL ANALYSIS

5.6.1 Statistics for Response Variables

The data were analyzed as a completely randomized design. There were four treatments and four replicates. All bacteria counts were transformed into $\log_{10}$ colony forming units (cfu) values before subjecting them to Analysis of Variance (ANOVA) using Proc Mixed of SAS model (SAS Institute Inc. 2003). The model for the statistics

was $\gamma_{ij} = \mu + \tau_i + \varepsilon_{ij}$. γ_{ij} was for the response variable: *Clostridium perfringens,* aerobic bacteria, digesta weight or feed consumption, μ for the overall mean, τ_i for the treatment effect of the feeding treatments (i=1,2,3,4), ε_{ij} for the error term. Significant differences among treatments were separated using the mean separation test of Tukey-Kramer ($P \leq 0.05$).

5.6.2 Statistics Used for next generation sequencing

The results from the next generation sequencing were analyzed using a denoising program to obtain the classification summary spread sheet. Following that, the sequences were trimmed to keep them with a minimum length of 200 bp. The data was aligned against the silva 16S backbone. This 16S backbone is software used for NGS sequenced read cleaning, assembling and explanation of transcriptome, read mapping and single nucleotide polymorphism selection (Blanca et al., 2011). The data were screened to ensure that chimeric sequences were removed as it was tested against the silva gold database (Quince et al., 2011). One of the NGS data points was found to be an outlier. The outlier was removed and the rest of the data were subsampled to obtain the bacteria classifications. The classifications were then run with silva database and the percentages of same sequences in the sample were calculated. The files were merged by selecting those classifications where the taxa had ≥0.5% representation in at least one sample. The data files were merged into the final file (Quince et al., 2011). The data were run as 3×5 factorial design using Proc Mixed of SAS (SAS Institute Inc. 2003). The model was $\gamma_{ijk} = \mu + \tau_i + \beta_j + \tau\beta_{ij} + \varepsilon_{ijk}$. γ_{ijk} was for the bacteria groups, μ was for the overall mean, τ_i was for lysozyme levels (i= 1,2,3). β_j was for bacteria groups (Gram-negative

and Gram-positive) and j= 1,2,3,4,5. $\tau\beta_{ij}$ was for the interaction effects of lysozyme levels and bacteria groups, ε_{ijk} was for the error term. Significant differences among treatments were separated using the mean separation test of Tukey-Kramer (P≤0.05).

5.7 RESULTS AND DISCUSSION

5.7.1 Feed Consumption

There were no differences (P>0.05) (Table 5.1) observed for feed consumption for all maltodextrin treatments (0, 10 and 20 g of lysozyme). Therefore, lysozyme levels in the diet had no influence on feed intake. Most birds did not feed on the maltodextrin until after 5 h of feed synchronization. The delay in feed consumption could be that the birds were not familiar with the feed supplement. Farhat et al. (2002) ensured that the

Table 5.1 Feed consumption of maltodextrin feed with or without lysozyme for broilers.

Feed treatments	Lysozyme levels (g.kg feed^{-1})	Feed consumption (g.bird^{-1})
Maltodextrin-based diet	0	30.11±3.06†
	10	28.89±3.06
	20	30.28±3.06

Anova	P-value
Feed treatments	0.94

† Mean ± Standard error of the mean.

birds consumed maltodextrin feed by initially exposing the birds to a 50:50 mixture of maltodextrin feed and commercial feed for 4h. This was followed by a 75:25 mixture of maltodextrin feed to commercial feed for another 4 h before 100% maltodextrin was offered for 9 h. Farhat et al. (2002) failed to report the feed consumptions for the commercial feed but reported 31 g maltodextrin feed consumption per bird for 9 h. Similar feed consumption per bird was observed in this research. A recent research by Northcutt et al. (2003a) indicated that birds offered maltodextrin consumed less feed than

broilers continuing to feed on finisher diet. Although, the authors did not report the feed consumption for both feeds, research on broilers feed consumption indicated that five 30-42 day old broilers consumed 1500 g of feed in 23 h (Corzo et al., 2003). This indicates that each bird consumed approximately 13 $g.h^{-1}$. However, in this study, feed consumption per bird was 29-30 g for 9 h. This confirms the observation made by Northcutt et al. (2003a) that birds consumed less maltodextrin feed than finisher feed.

5.7.2 Digesta Weight

The amount of digesta in the ileum of the treated birds was not different ($P>0.05$) from the feed withdrawal birds (Table 5.2). These results were different from

Table 5.2 Digesta weight in the ileum of 36 day old broilers after 9 h of feeding maltodextrin or feed withdrawal

Feed treatments	**Lysozyme levels ($g.kg^{-1}$ of feed)**	**Digesta weight ($g.bird^{-1}$)**
Maltodextrin-based feed	0	9.00±1.15†
	10	11.07±1.15
	20	11.86±1.15
Feed withdrawal	-	10.62±1.23

Anova	**P-value**
Feed treatments	0.36

† Mean ± Standard error of the mean.

Farhat et al. (2002) study where they observed less digesta volume in the maltodextrin fed birds as compared to the feed withdrawal birds. Although, the authors did not show figures, it was indicated that after keeping the birds on the maltodextrin feed for 9 h, followed by a 3 h waiting time prior to slaughter, the birds GIT were emptier than birds withdrawn from feed for 12 h. The purpose of the feed withdrawal period is to ensure that the birds emptied their GIT prior to slaughter (Farhat et al., 2002; Rathgeber et al., 2007, Northcutt et al., 2003a). Farhat et al. (2002) found maltodextrin feed more useful for GIT

emptying than withdrawing feed from birds. However, providing maltodextrin feed to market-aged broilers did not result in further emptying of the GIT of broilers in the current study.

5.7.3 *Clostridium perfringens*

The lysozyme-enriched maltodextrin feed did not influence the number of *Clostridium perfringens* per gram of digesta in the ileum of market-aged broilers (P>0.05) (Table 5.3). Although, lysozyme did not affect *Clostridium perfringens* numbers, current research has shown that lysozyme is effective on *Clostridium*

Table 5.3 The *in vivo* effect of maltodextrin feed with or without lysozyme on *Clostridium perfringens* in ileal contents of market-aged broilers.

Feed treatments	**Lysozyme (g.kg feed^{-1})**	***Clostridium perfringens* log$_{10}$ (cfu. g^{-1})**
Maltodextrin-based feed	0	2.21±0.48†
	10	2.56±0.35
	20	2.43±0.35
Feed withdrawal	-	2.50±0.35

Anova	**P-value**
Feed treatments	0.92

† Mean ± Standard error of the mean.
Cfu-colony forming units.

perfringens. An *in vitro* assay by Zhang et al. (2006) on *Clostridium perfringens* type A and its α-toxin production revealed that lysozyme at 100 μg.ml^{-1} inhibited α-toxin production and that 156 μg.ml^{-1} completely inhibited the growth of *Clostridium perfringens* type A in a nutrient broth. At these levels it caused massive damage to the cell wall of *Clostridium perfringens.* The massive damage caused on the bacterial cell wall was due to the *N*-acetylmuramoylhydrolase enzymatic activity of lysozyme which led to the lysis of the peptidoglycan layer of the cell wall of *Clostridium perfringens* (Masschalck and Michiels, 2003). This indicates that, lysozyme is effective on

Clostridium perfringens even at low levels. Liu et al. (2010) fed a diet with lysozyme at 40 $mg.kg^{-1}$ to broilers for 28 days and found that lysozyme reduced *Clostridium perfringens* in the ileum of broilers. The time exposure for the lysozyme to act on *Clostridium perfringens* may have been too short in this study. The market-aged broilers used in this study were fed for approximately 9 h, which may not be enough for the lysozyme to have an inhibitory effect on *Clostridium perfringens.*

5.7.4 Aerobic Bacteria

There were no differences (P>0.05) in the aerobic bacteria numbers among treatments (Table 5.4). The maltodextrin feed (control) did not affect overall bacterial numbers in the ileum of market-aged broilers. Others have investigated the effects of maltodextrin on bacterial numbers in the upper GIT and in carcass rinses. Rathgeber et al. (2007) found no differences in aerobic bacteria in the crop after subjecting turkeys to the maltodextrin feed for 5 h. Northcutt et al. (2003a) found no difference for numbers of *Campylobacter*, *E. coli* and coliforms in whole carcass rinses in buffered peptone water after subjecting the birds to 8 h of maltodextrin feed followed by 0, 4, 8 and 12 h of feed withdrawal. It could be that the carcasses were not contaminated with the digesta from the market-aged broilers used in their study. The addition of lysozyme to maltodextrin

Table 5.4 The *in vivo* effect of maltodextrin feed with or without lysozyme on the aerobic bacteria numbers in the ileum of market-aged broilers.

Feed treatments	**Lysozyme (g.kg feed^{-1})**	**Aerobes (cfu.g^{-1})**
Maltodextrin-based feed	0	6.36±0.38†
	10	5.71±0.34
	20	5.83±0.34
Feed withdrawal	-	6.38±0.34

Anova	**P-value**
Feed treatments	0.24

† Mean ± Standard error of the mean.

Cfu-colony forming units.

feed did not have any inhibitory effect on aerobic bacteria (P>0.05). This is the first report on the effect of lysozyme delivered for a short period in a maltodextrin feed on aerobic bacteria. There have been many investigations on the inhibitory effect of lysozyme against Gram-negative and Gram-positive bacteria, as well as foodborne pathogens and food spoilage bacteria (Hughey and Johnson, 1987, Gill and Holley, 2000, Nattress et al., 2001). The authors reported that lysozyme reduced Gram-negative and Gram-positive bacterial numbers. An inhibitory effect of lysozyme on aerobic bacteria was not observed in this study with traditional culturing methods. It is possible that there were some substances present in the feed and the digesta that hindered the antimicrobial activity of the lysozyme.

5.7.5 Next Generation Sequencing (454 Sequencing)

This is the first report to describe the intestinal bacterial profile from broilers fed lysozyme-enriched maltodextrin using NGS. Gram-negative and Gram-positive bacteria were identified in the GIT of the market-aged broilers to represent approximately ≤0.7% and ≤99.2% respectively. Gram-positive bacteria were reduced by 20 g of lysozyme per kg of maltodextrin feed but did not affect the Gram-negative bacteria (Table 5.5). This confirms the results from Cunningham et al. (1991) and Liu et al. (2010) that lysozyme is more effective against Gram-positive than Gram-negative bacteria. Among the class of Gram-positive bacteria group, Actinobacteria and Firmicutes were identified. The sequencing results showed that Firmicutes bacteria that exist in the GIT of broilers used in this trial had two subgroups of bacteria, these include bacilli and clostridia (Table 5.5). Bacilli were found to have the highest population followed by the clostridia group. The

clostridia found in the digesta were non-harmful clostridia species. It is possible that the use of the universal primer in this study was not sensitive enough to detect the small numbers of those clostridia species that are of clinical importance such as *Clostridium perfringens* (Bjerrum et al., 2006). Clostridia levels were reduced in maltodextrin digesta more so than lysozyme-enriched maltodextrin feed, however, it increased with an increase in lysozyme levels. Perhaps the maltodextrin feed itself influenced the clostridia species.

Bacilli decreased with an increase in lysozyme levels which demonstrated the antimicrobial activity of lysozyme. Similarly, lysozyme was indicated to have bactericidal effect on four spoilage lactic acid bacteria (*Lactobacillus kunkeei, Lactobacillus brevis, Pediococcus parvulus, and Pediococcus damnosus*) at a concentration of 125 and 250 $mg.l^{-1}$ in grape juice at 20±0.5°C (Gao et al., 2002). This indicates that lysozyme is effective against Gram-positive bacteria. The proportion of

Table 5.5 Next generation sequencing information and diversity of bacteria in the digesta of market-aged broilers.

Bacteria class		**Level of lysozyme**		
Gram sensitivity	**Subgroups**	**0g**	**10g**	**20g**
Actinobacteria (+)		$0.13±9.71^{c}$	$0.00±9.71^{c}$	$0.43±9.71^{c†}$
Firmicutes (+)		$99.47±9.71^{a}$	$99.70±9.71^{a}$	$93.10±9.71^{a}$
	Bacilli	$98.43±9.71^{a}$	$98.17±9.71^{a}$	$58.37±9.71^{b}$
	Clostridia	$0.57±9.71^{c}$	$1.53±9.71^{c}$	$34.87±9.71^{b}$
Proteobacteria (-)		$0.37±9.71^{c}$	$0.23±9.71^{c}$	$4.57±9.71^{c}$

Anova	**P-value**
Group (Bacteria class)	<0.00
Treatment	0.95
Group×treatment	0.04

Means with the same superscript are not significantly different (P≥0.05).
† Mean ± Standard error of the mean.
Data for bacteria are expressed in percentages (%).

clostridia increased with increase in lysozyme levels relative to the bacilli. Research has indicated that lysozyme was effective on *Clostridium perfringens* when delivered through broiler soy bean-based diet or in a micro-broth. Liu et al. (2010) reported that lysozyme inhibited *Clostridium perfringens* in the ileum of older birds challenged with *Clostridium perfringens*. Similarly, Zhang et al. (2006) found that lysozyme had inhibitory effect on *Clostridium perfringens* in a micro-broth. It is possible that some bacteria may be sensitive to higher levels of lysozyme.

Lysozyme-enriched maltodextrin shows promise in reducing Gram-positive bacteria but as to whether its effects will be extended to the Gram-negative bacteria should be investigated with high levels of lysozyme.

In general pathogenic bacteria such as *Salmonella, Clostridium perfringens*, *Campylobacter*, *Escherichia coli* and *Shigella* were not found in the sequencing results in this research.

5.8 CONCLUSION

In this current study, enriching maltodextrin feed with lysozyme had no effect on feed consumption and ileal weight contents. The different lysozyme levels did not affect *Clostridium perfringens* and aerobic bacterial numbers in ileal contents using the traditional culturing method but was found to reduce Gram-positive bacteria (bacilli) when NGS was used. NGS is an ideal tool for determining bacterial population in complex metrices such as digesta. No bacterial growth was visible on the *E. coli*/coliform and Enterobacteriaceae plates.

CHAPTER 6: CONCLUSIONS

6.1 RELATIONSHIP AMONG COMPONENTS OF THE STUDY

This project was carried out to develop an *in vitro* method to evaluate allicin and lysozyme in the GIT contents from market-aged broilers. To determine the levels of alternative antimicrobials (allicin from garlic and EDTA modified lysozyme from hen egg whites) for maltodextrin feed. The addition of allicin inhibited Enterobacteriaceae numbers in the maltodextrin digesta at 5 and 12.5 mg *in vitro*. The lysozyme had an inhibitory effect on *Clostridium perfringens* at 5 and 12.5 mg *in vitro* but not on the other bacteria evaluated.

Excessive bacterial growth and a lack of detectable response of bacteria to allicin and lysozyme in this *in vitro* models resulted in additional trials conducted with overnight broth cultures of *Salmonella* and bacteria from digesta of maltodextrin fed broilers. It was indicated that lysozyme affected aerobic bacteria while allicin did not.

Lysozyme affected bacterial population when it was fed to market-aged broilers in the maltodextrin feed. The sequencing results indicated that, lysozyme had an effect on Gram-positive bacteria (bacilli) and clostridia but not on Gram-negative bacteria. It may be concluded that lysozyme delivered in maltodextrin feed is an effective strategy for reducing some Gram-positive bacteria in the ileum of market-aged broilers. Comparison of the *in vitro* and *in vivo* method indicated that lysozyme inhibited bacteria when fed to broilers through maltodextrin feed (*in vivo*). This may be attributed to the differences in bacterial isolation techniques.

The NGS indicated a decrease in bacilli species and an increase in proteobacteria species as lysozyme levels increased, whereas the traditional culturing method was not

consistent in providing results on antimicrobials effects on the bacteria numbers. In the first trial, the *E.coli*/coliform and the Enterobacteriaceae plates were overgrown while *E.coli*/coliform and Enterobacteriaceae had no presumptive identifications on its plates when used in the *in vivo* trial. NGS had surprisingly few types of bacteria identified indicating that maltodextrin alone may significantly affect the type of bacteria found in the digesta of chickens.

6.2 IMPORTANCE OF THE STUDY ON BROILER INDUSTRY

The current research shows that maltodextrin feed replacement is an important feeding management practice prior to slaughter that will enable farmers to reduce market-aged broilers weight loss prior to processing. This may increase meat yield and maximize their production profit.

Additionally, the incorporation of alternative antimicrobials will help to reduce certain type of bacteria available for contamination from ileal contents during processing. Reduced bacteria may help extend the shelf life of their meat products as well as ensuring the health of their consumers.

6.3 FUTURE DIRECTION

Feeding the maltodextrin supplement decreased aerobic bacteria as time progressed. It is recommended that further research should be carried out where market-aged broilers will be fed with maltodextrin feed for a longer period than 9h. This may help to control other bacteria in the GIT.

The same *in vitro* trials should be carried out where higher levels of lysozyme or other alternative antimicrobials will be used. These antimicrobials will be incorporated in maltodextrin digesta harvested from different sections of the GIT (crop, ileum, duodenum

and the cecum) of market-aged broilers, to evaluate the variation in bacterial load at different sections after the pre-shipping preparation treatments. Therefore, it is recommended that lysozyme should be evaluated but not allicin. Lysozyme showed an inhibitory effect on aerobic bacteria, *Salmonella* in micro-broth system and *Clostridium perfringens* in the ileum.

The NGS results indicated that bacilli (Gram-positive bacteria) species were reduced as lysozyme levels increased. Further studies should be conducted where high levels of lysozyme will be incorporated in a maltodextrin feed for market-aged broilers and determine if the higher levels will enable the lysozyme to extend its inhibitory effects on Gram-negative bacteria.

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