STRATEGY TO DEVELOP ALTERNATIVE TO ANTIBIOTICS USING BACTERIAL SECOND MESSENGER 3’, 5’ CYCLIC DIGUANYLIC ACID AS AN IMMUNOSTIMULATOR IN BROILER CHICKEN

by

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Abstract

In this study the bacterial second messenger, 3', 5' cyclic diguanylic acid (c-di-GMP) was evaluated as a vaccine and therapeutic adjuvant in broiler. The humoral immune response to an oral infectious bursal disease virus (IBDV) vaccine administrated in conjunction with intramuscular injection (IM) or oral administration of 10 nmol or 100 nmol c-di-GMP in 192 broiler chickens. The antibody titers were determined in blood sera weekly in 8 birds /treatment. From d 14 to 35, an increase ($P < 0.05$) in the total immunoglobulin (IgA) titers was observed regardless of treatments. On d 35, birds receiving c-di-GMP by gavage showed higher serum IgA levels when compared the control birds ($P < 0.05$). To explore strategies to control *Clostridium perfringens* colonization in gut, the synergistic effect of c-di-GMP with penicillin G was investigated in a broiler challenge model. A mixture of *C. perfringens* type A strains from necrotic enteritis outbreaks were inoculated on d 14-16. Birds were treated with saline (control group) or 20 nmol of c-di-GMP by gavage or IM on d 24, all in conjunction with penicillin G (PG) in water for 5 d. Weekly samplings of ceca and ileum were performed on d 21 to 35 for *C. perfringens* and *Lactobacillus* enumeration. On d 35 of age, the IM treatment ($P < 0.05$) reduced *C. perfringens* in the ceca, suggesting possible synergistic activity between PG and c-di-GMP against *C. perfringens* in broiler ceca. The AFLP and the *cpa gene* prevalence results demonstrated that c-di-GMP per IM decreased the prevalence of *cpa* while restored the normal microflora on the day 35 after being challenged by high doses of *C. perfringens*. Thus c-di-GMP has a promise to improve poultry health by modulating mucosal immunity and reducing pathogens in the gut.

Preface

This thesis study was designed in consultation and supervision of my research supervisor Dr. Moussa Diarra. Planning of experiments, sample collection, performing laboratory test, and interpretation of results were mostly done by me. Sample Collection and laboratory tests were performed with the help of the laboratory technician Heidi Rempel and co-op students Tina and Tallie. Statistical analysis was conducted with the help of Dr. Diarra. I interpreted the results and wrote the various drafts of the manuscript while Drs. Moussa Diarra, Kim Cheng and Kevin Allen provided valuable suggestions for improvements.

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All experimental procedures performed in the above studies were approved by the local Institutional Animal Care Committee (Agassiz, BC, Canada) according to guidelines described by the Canadian Council on Animal Care (CCAC, 1993).

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List of Abbreviations

AAFC	Agriculture and Agri-Food Canada
AFLP	Amplified fragment length polymorphism
AGPs	Antimicrobial growth promoters
AMR	Antimicrobial resistance
APC	Antigen presenting cells
CD	Cluster of differentiation
C-di-GMP	3', 5' Cyclic Diguanylic Acid
CFIA	Canadian Food Inspection Agency
CFU	Colony-forming unit
CLFA	Clumping factor A
CMI	Cell-mediated immunity
CPA	Clostridium perfringens alpha toxin
CTL	Cytotoxic T-lymphocytes
DC	Dendritic cells
DGCs	Diguanylate cyclase
DNA	Deoxy- ribonucleic acid
EGTs	Environmental gene tags
EPS	Exopolysaccharide
ESBL	Extended- spectrum β-lactamases
EU	European Union

FBA	Fructose 1, 6-biphosphate aldolase
FCR	Feed conversion ratio
FOS	Fructo-oligosaccharide
GDP	Glyceraldehyde-3-phosphate dehydrogenase
GIT	Gastrointestinal tract
HI	Humoral immunity
HP	Hypothetical protein
IBD	Infectious bursal disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramascular injection
LMH	Leghorn male hepatoma cell lines
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MOS	Mannan-oligosaccharides
NCCLS	National committee for clinical laboratory standards
NE	Necrotic enteritis
NK	Natural killer cells
NO	Nitric oxide
NSP	Non-starch polysaccharides
OS	Oligosaccharides

PAMPS	Pathogen associated molecular pattern
PDE	Phosphodiesterase enzyme
PFOR	Pyrivate: ferradoxin oxidoreductase
PG	Penicillin G
PRR	Pathogen recognizing receptors
QS	Quorum sensing
SOP	Standard operating procedures
STING	Stimulator of IFN genes
TCR	T cell receptors
Th	T- helper
TLR	Toll-like receptors
TNF	Tumor necrosis factor
WHO	World health organization

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Dedication

This Thesis is dedicated to:

My Dear Parents, Mr. and Mrs. K.M. Abdul Khaliq

For always encouraging and believing in me

My Loving Husband, Mohammad Aizaz Burney

For all the love and support he gave during my studies

My Dearest Son, Musab Burney

I always enjoyed being with you, you made me cheer even during my tough times, you are a gift and blessing of God

Chapter 1: Literature Review

1.1 Introduction

Globally, increasing demand for poultry meat has resulted in increased broiler production. Data obtained during a 10-years span (1995-2005), shows 53% increase of the global production of chicken meat, which is estimated to be 38% in the United States of America (USA) (Scanes, 2007).

In 2012, the average Canadian chicken farms produced 526,000 kilograms of chicken meat (http://www.agr.gc.ca/poultry/glch_eng.htm , accessed November 1, 2013). Specifically, British Columbia (BC) contributes 15.5% to the nation's chicken production and ranks third following Ontario and Quebec ("FAST STATS 2011-Agriculture, Seafood and Agrifood," 2012).

Due to the increased demand for broiler meat, producers have adapted strategies to improve the feed conversion ratio (FCR) while increasing production of chicken meat. This involves the use of antimicrobial growth promoters (AGPs) in feed. For the last 50 years, chicken producers have been using antibiotics in feed for prophylactic purposes, to enhance growth, improve feed efficiency and reduce mortality (Dibner and Richards, 2005; Graham et al., 2007). The exact mechanisms through which AGPs improve bird's performance are still unclear; however studies have shown that their actions are mediated by their antibacterial effects (Butaye, 2003). Presumably, four mechanisms of actions of AGPs have been suggested: 1) inhibition of subclinical infections, 2) reduction of growth-suppressing toxins or metabolites 3) limit nutrient uptake by microbes and 4) thinning of the bird's intestinal wall to enhance the nutrient uptake.

These mechanisms would enhance growth of broilers and result in increased body weight (Gaskins et al., 2002; Butaye, 2003).

Despite the tremendous benefits of AGPs in poultry feed to control or inhibit diseases and increase production, concerns have been raised about the emergence and dissemination of antibiotic resistance in bacteria. Use of antibiotics at sub-therapeutic doses as feed supplements creates selective pressure which may contribute in promoting the development of antimicrobial resistance (AMR) genes in bacteria, and studies demonstrated that these AMR genes can be transmitted from animal to human microbiota (Butaye, 2003; Bywater, 2005; Dibner and Richards, 2005; Bonnet et al., 2009). Thus, increasing antimicrobial resistance in animals is a threat to human health which led to the ban, initially, to some antibiotics used as AGPs in 1999 by the European Union (EU) but later in 2006 a complete ban was enforced by the EU on the use of AGPs in animal feed (Castanon, 2007).

Consequently, there is an urgent need for the exploration of new strategies for alternatives to AGPs. Ideally, the best approach would be an alternative substance which possesses similar characteristics as an AGP but does not promote AMR. Immunomodulation in many contexts have proved to be a useful therapeutically, including prevention and treatment of infections, reduction of autoimmune and inflammatory responses and stimulate anti-tumor immunity in cancer patients (Hancock et al., 2012). Modulation of immunity by either suppression or enhancement according to its need has many advantages. First it targets the host rather than the pathogen and second, it induces little selective pressure for promotion of microbial resistance (Scherer and McLean, 2002). Moreover, protection provided against infections through vaccination over the decades have proved that stimulation of adaptive

immunity has been resilient in evolution of microbial resistance (Scherer and McLean, 2002; Hancock et al., 2012).

Furthermore, after the discovery and in-depth understanding of the mechanism through which the key pathogen recognition receptors (PRRs) act to stimulate innate and adaptive immunity leading to a rapid and effective clearance of pathogen there is a tremendous focus on utilizing these PRRs as therapeutic and vaccine adjuvants (Hamill et al., 2008; Hancock et al., 2012). Therefore this led to the fact that immunomodulation to control bacterial infections may be a promising approach for alternatives to antibiotics (Hancock et al., 2012). The 3', 5' cyclic diguanylic acid (c-di-GMP) a bacterial second messenger, which was reported as a direct stimulator of STING (stimulator of IFN gene) signaling pathway of innate immunity when administered in eukaryotes (Burdette et al., 2011). The c-di-GMP demonstrated its potential clinical application during *in vivo* and *ex vivo* studies as an immunomodulator, immune enhancer, vaccine adjuvant, and inhibition of infection and infectious disease (Karaolis and Rashid, 2005; Karaolis et al., 2007a; 2007b; Ebensen et al., 2007b; Hu et al., 2009; Yan et al., 2009).

This study aims to evaluate the utilization of c-di-GMP as a vaccine and therapeutic adjuvant in broiler chicken production. Precisely, focus of this study is to determine the usefulness of c-di-GMP as a vaccine adjuvant with Infectious bursal disease vaccine and therapeutic adjuvant with penicillin in the clearance of *Clostridium perfringens* in the chicken gut. The findings of this thesis may provide insights of the potential use of c-di-GMP during broiler production to protect and maintain good health of the chickens against these economically important infections in the absence of AGPs.

1.2 Broiler Chicken Production

1.2.1 Economic Importance

Chickens bred and raised specifically for meat are referred to as broilers. Broiler production in Canada is not only important to fulfill the food demand for Canadians, but is also a major export commodity, particularly to the US, Mexico, Japan, the Philippines, China and other countries (http://www.agr.gc.ca/poultry/glch_eng.htm , accessed November 1,2013). According to Agriculture and Agri-food Canada (AAFC) poultry market place report, in 2012 average chicken farms produced 526,000 kilograms of meat. Retail purchases in the same year were approximately 634 million kilograms, which represents 62% of Canada's total consumption. On the other hand Canada exported over 5.9 million chicks worth over $14.5 million to 13 countries. Additionally, the same report states that Canada exported worth more than 140.5 kilograms of meat worth $320.5 million of chicken meat and edible bi-products (fresh, chilled, frozen) to 61 countries (http://www.agr.gc.ca/poultry/glch_eng.htm , accessed November 1, 2013).

British Columbia contributes 15.5% of the nation's chicken production and ranks 3rd in the nation, by producing 158,379 kg chicken meat in the year 2011(http://www.agf.gov.bc.ca/stats/faststats/FastStats2011-lo.pdf, accessed August 29, 2013). Thus, chicken meat production is an important industry in Canada.

1.2.2 Systems of Broiler Production

Chicken farmers are responsible to provide basic bird requirements during their husbandry, including feed, shelter, water and treat health-related problems. There are two common practices

in rearing broilers in Canada; one employs conventional production practices and the other is organic practices. For conventional production, antimicrobial agents can be used for treatment and prevention of diseases. Moreover, these agents can also be used to improve performance such as enhancing growth and feed efficiency (Luangtongkum and Morishita, 2006). In contrast, organic production practices restrict antimicrobial agent usage and require feeding with only organically approved feed and supplements. Additionally, during organic farming the chickens have access to outside environment like sunlight and fresh air (El-Shibiny, 2005). However, organic farming legislations on strict prohibition of preventive medication led to occurrence of diseases, usually these diseases are controlled in conventional farming due to the use of antimicrobial agents (Van Overbeke et al., 2006).

1.2.3 Broiler Husbandry

Broiler farmers are responsible to provide basic needs of the poultry and the Canadian government has strict guidelines for it. The principal goal of the government for these regulations is to make sure that farmers raise the chickens in a healthy and humane way; secondly the next most important part is that they follow strict on farm food safety procedures. In Canada, chicken are raised following the feed and water supply, the housing and environment and the biosecurity guidelines as detailed below.

1.2.3.1 Feed and Water Supply

According to the BC chicken marketing board document on “Raising chicken in BC” (http://bcchicken.ca/index.php/bc-chicken-production/raising-chicken-in-bc/, viewed, May 9, 2013), chickens have to be provided with feed and fresh, clean water *ad libitum*. Feed should be properly formulated according to the nutriments requirement of broiler birds. The Canadian Food

Inspection Agency (CFIA) is responsible for the regulation of feed ingredients. It is illegal to use steroids and hormones in broiler feed in Canada. Moreover, water lines providing water to the birds should be cleaned and disinfected before every new flock and checked annually for any contamination.

1.2.3.2 Housing Environment

Chickens and their wastes contaminate the air with hazardous pollutants like ammonia, carbon dioxide, hydrogen Sulphite, methane nitrous oxide gases and dust (Kocaman et al., 2006). In Canada, poultry are raised in confined housing which results in exposure of birds and workers to airborne contaminants such as dust, endotoxins and ammonia for a long period (Senthilselvan et al., 2011). Studies have shown that inhalation of these air pollutants are associated with respiratory diseases in workers (Bakutis et al., 2004; Cambra-López et al., 2010; Senthilselvan et al., 2011). Poor quality air in the barn due to the presence of environmental physical and biological contaminants may not directly impact the chicken health; however their immune response can be compromised leading to increased bird's susceptibility to diseases (Kocaman et al., 2006). The overall impact of harass environment in poultry facilities include decreased chicken performance like reduction in feed consumption, feed efficiency, live weight gain, carcass quality and egg production (Kocaman et al., 2006; Nwagwu et al., 2012).

Air pollutant produced in poultry barn can be controlled by proper management techniques. Appropriate ventilation, i.e. make sure fresh air is present at all times, in the poultry houses is extremely important. Ventilation can control the humidity, temperature and emission of harmful gases in the barn (Kocaman et al., 2006). Dust particles from poultry houses consists of fecal material, urine, ammonia, carbon dioxide, feathers, feed and litter particles, pathogens,

endotoxins, and mycotoxins (Just et al., 2009). Good management strategies can control dust and potentially reduce air pollutants in the barns. Some measures to control air pollutants are regular and thorough cleaning of the barn, oil and water fogging in the barn, use of natural and artificial windbreaks, use of different filters, use of certain housing systems and equipment, precipitation and vegetative shelterbelts (Patterson, 2005).

1.2.3.3 Biosecurity

Biosecurity is the collection of procedures that farmers have to follow to prevent the transmission of infectious agents from the outside environment into the barn and vice versa. BC Poultry Biosecurity reference guide (Bill Cox, 2006), describes in detail about the mandatory standards to maintain good biosecurity in farm practice. Key features are:

1.2.3.3.1 Farm Access Standards

The first step to restrict the access of infectious agents in and out of the barn is to have secure barriers at all entry points to the controlled access zones. Secure barriers, closed and latched at all times except when authorized vehicles pass can impede the vehicles and discourage unauthorized foot traffic to enter the controlled access zone. These secure barriers should have appropriate biosecurity signage which clearly demonstrates that biosecurity is in effect and access is in control. Primary access zones should be constructed with hard surface or gravel to avoid accumulation of water. Stagnant water should be strictly avoided around poultry production as it may serve as a reservoir for pathogenic bacteria that may be transferred into the barn through vehicles, boots, equipment, etc. On the other hand, cleaning and decontamination of vehicles and personnel is important in minimizing the possible transfer of microorganisms and debris, and should be done in the primary access zone before entering the barn. In short, it is

mandatory that all accesses to the controlled access zone should be maintained to be free from any organic material or debris at all times as organic debris can act as an ideal environment for the growth of infectious agents and thereby transferred in and out of the barn through different modes.

1.2.3.3.2 Barn Access Standards

All access to the barn should be limited. The barn is considered as the restricted access zone. Locked barn doors and having appropriate approved signs clearly displayed" Restricted zone" is mandatory. The barn entrances are high risk pathogen transmission area and the last line of defense in preventing entry and exit of pathogenic organisms. Therefore it is imperative to have and maintain an anteroom at all primary access to the barn. The anteroom is a transition zone between outside and inside the barn having appropriate space and physically separated from outside area and inside area. Essentially, anteroom should be equipped with washing facilities and disinfecting utensils, permitting hand washing, cleaning and disinfection of boots, outwear and use of a head cover.

1.2.3.3.3 Farm Management Standards

Farm management, includes pests control, protection of feed and water from contamination, cleaning and sanitizing farm equipment, manure management, on-farm biosecurity training, accessibility to standard operating procedures (SOP's) for on-farm biosecurity to all farm personnel and maintaining visitors log book. Successful incorporation of a biosecurity program depends on the farm management.

1.2.4 Antibiotics Used in Feed and Treatment of Infection

Antibiotics may be naturally occurring, semi-synthetic or synthetic compounds, and are frequently used in human and veterinary medicine to prevent and treat infections. Beside their use in treating diseases some antibiotics are also used in animal feed as growth promoters. National Committee for Clinical Laboratory Standards (NCCLS) have defined the use antibiotics in farm animals, as a therapy, Control, Prevention / prophylaxis, metaphylaxis, and growth promoter (Table 1.1) (National committee for Clinical laborotory Standards (NCCLS), 2002).

Antibiotics have been used in chicken production for the last 50 years as growth promoters in feed and water for disease prevention. AGPs were discovered in 1940, yet the exact mode of action in promoting growth of animals is not been known but has been suggested to be associated with antibacterial properties. The exact mechanism through which AGPs improve bird health and improved FCR is still unclear; however studies have shown that their action is mediated by their antibacterial effect (Butaye, 2003). Presumably, four proposals have been suggested to explain the mode of action of antibiotic growth promoters (1) inhibition of subclinical infections, (2) growth-suppressing toxins or metabolite production is reduced (3) reduced nutrient uptake by microbes (4) thinner intestinal wall of the AGPs fed animals enhances the nutrient uptake and use resulting in increased body weight (Gaskins et al., 2002; Butaye, 2003;).

In addition to this, it was also hypothesized that AGPs may increase body mass by impeding the production and excretion of cytokines by immune cells. As cytokines released during the immune response also stimulates the release of catabolic hormones, which may reduce muscle mass. Since AGPs in feed reduces the occurrence of gastrointestinal infection by their

microcidal action, as a result the immune response is not initiated and the cytokines and catabolic hormones are also not released thus there is an increase in muscle mass in the presence of AGPs (Thomke and Elwinger, 1998; Niewold, 2007).

For prophylaxis, antibiotics are added in feed at sub therapeutic levels. Many antibiotics have been used as AGP in broiler production, with the benefits relating to increased performance as measure by improved weight gain and decreased FCR ratio (Dibner and Richards, 2005). Thus, the usage of AGP is economically beneficial for poultry producers. However, the consequence of using antibiotics at a sub-therapeutic level results in development of AMR genes (Wegener, 2003).

The use of antimicrobials may result in the development, maintenance, and potential dissemination of AMR genes in food-producing animals (Butaye, 2003; Diarrassouba et al., 2007; Diarra et al., 2007b). In addition, antimicrobial residues and resistant bacteria may find their way into the environment from via fecal material and litter (Furtula et al., 2010; Merchant et al., 2012). Due to increasing concerns regarding the rapid dissemination of AMR and the preservation of therapeutic effectiveness of antibiotics for humans, Sweden banned on all AGPs in 1986 and Denmark banned avoparcin and virginiamycin in 1995. In 1998 the EU banned avoparcin use as AGPs in animal feed as a precautionary measure (Casewell et al., 2003; Graham et al., 2007). Later in January 2006, EU banned and phased out the ultimate use and marketing of AGPS (EC Regulation No. 1831/2003, http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2003:268:0029:0043:EN:PDF).

Interestingly, following this ban, substantially increased use of therapeutic antibiotics in animal production was reported (Casewell et al., 2003; Castanon, 2007). Phillips et al. (2004) in

his review has stated that the removal of AGPs from feed may contribute to the prevalence of gastrointestinal (GIT) problems, resulting in the increased therapeutic use of antibiotics. Furthermore, animal health deterioration symptoms like weight loss, increased diarrhea and mortality due to *Escherichia coli* and *Lawsonia intracellularis* in pigs and clostridial necrotic enteritis in broilers significantly increased (Casewell et al., 2003).

Thus, although there are valid concerns regarding the development and dissemination of AMR, the importance and benefits of AGPs in animal husbandry cannot be ignored. Therefore, alternatives to AGPs for use in poultry production are a priority.

1.2.5 Alternatives to Antibiotics in Broiler Production

The use of AGPs has become controversial; EU completed its process of removal of AGPs in 2006, but they are still used in other parts of the world. Therefore the World Health Organization (WHO) (1997) has recommended phasing out the use of AGPs and replacing it by alternative strategies (Bywater, 2005). Ideally, AGPs alternatives in poultry feed should have the same beneficial effects as the growth promoters. Consequently, numerous reports have been made evaluating possible AGP alternates, following are some alternatives used to promote growth in poultry.

1.2.5.1 Exogenous Enzymes

Restrictions in the use of AGPs led to the increased use of enzymes as a feed additive manifolds. Enzymes commonly used in poultry diets are carbohydrases and phytases. Non-starch polysaccharides (NSP) are complex group of components and include celluloses, pectin, oligosaccharides, arabinoxylans and β-glucans. They are mostly present in grains like wheat, rye, oat and barley and when fed to poultry they increase bulk and viscosity in the intestine, thereby

depresses digestion. The addition of appropriate enzyme(s) controls these effects and improves digestibility (Bedford, 2000; Huyghebaert, 2005).

Phytases are another type of enzymes added to feed to help chickens utilizing plant phytate phosphorus while reducing the addition of inorganic phosphorus to poultry feed and phosphorus pollution (Bedford, 2000).

Besides the benefits of adding enzymes to feed there are some limitations like: 1) not enough knowledge of substrate for specific enzymes; 2) enzymes cannot withstand high feed processing temperatures like pelleting (95°C), and therefore restricts the use of only thermo tolerant enzymes in feed; and 3) enzyme end-product activity is an important concern, because we need to be sure that the growth of the beneficial bacteria is encouraged and pathogens are excluded if we use it as AGPs alternative (Bedford, 2000).

1.2.5.2 Organic Acids

Organic acids are naturally found in plants and animal tissues, and are known for their *in vitro* and *in vivo* antimicrobial effects (Huyghebaert, 2005; Hernández et al., 2006). They have been used in poultry feed as antibiotic alternatives. Studies have shown that when organic acids are added in feed it decreases intraluminal concentrations of coliforms and other pathogens like *Campylobacter* and *Salmonella* associated with most gastrointestinal problems (Hernández et al., 2006).

Antimicrobial activity of organic acids has been associated with their pKa, which is the pH at which 50% of the acid is dissociated. The un-dissociated form of organic acids is capable of penetrating the semi permeable cell membrane of microorganisms and once in the cell, where the pH is almost 7, it dissociates and thereby suppresses cellular enzymes and nutrient transport

systems (Huyghebaert, 2005; Hernández et al., 2006). Some commonly used organic acids in animal production include formic, acetic, propionic, butyric, and lactic acid.

Furthermore, short chain fatty acid butyrate has been shown to down regulate the expression of genes involved in *Salmonella* spp. invasion and to have bactericidal and bacteriostatic effects against C*ampylobacter* spp. (VanImmerseel et al., 2006; Van Deun et al., 2008). Thus, use of organic acids may potentially control foodborne outbreaks caused by *Salmonella* and *Campylobacter* spp. which are transmitted mainly through consumption of contaminated eggs and other poultry products (Chaveerach et al., 2004; De Vylder et al., 2009).

Organic acids have been successful in maintaining a balanced intestinal flora by restricting pathogen colonization. However, an emerging problem is that acid sensitive food borne pathogens have been able to survive when exposed to low pH enhanced by organic acids through the stimulation of the acid tolerance response (Ricke, 2003).

1.2.5.3 Probiotics or Direct Fed Microbial (DFM)

In an effort to explore safe and effective alternatives to antibiotics, probiotics have been shown to be beneficial in improving gut health in broilers, while increasing the overall performance in poultry (Lutful Kabir, 2009). Probiotics are beneficial bacteria (natural inhabitant of intestine) supplemented in feed that are capable of improving intestinal health (Houshmand et al., 2011). It is considerably important to maintain gut normal microflora in order to obtain good performance and sustain chicken health, any imbalance may favor the growth of pathogens (Huyghebaert, 2005).

Probiotics include bacterial species belonging to *Aspergillus, Bacillus, Bifidobacterium, Candida, Enterococcus, Lactobacillus, Saccharomyces, and Streptococcus.* Beneficial effects

like improved bird performance, immunomodulation, modulation of intestinal microflora and inhibiting pathogen growth, hemato-biochemical parameters, improving sensory characteristics of dressed broiler meat and promoting microbiological meat quality of broilers were observed when these species of bacteria were used in broiler feed (Huyghebaert, 2005; Lutful Kabir, 2009).

The mechanism by which probiotics work includes competition for substrates, inhibition of pathogen growth by producing toxic compounds, competition for attachment sites and immune stimulation (Patterson and Burkholder, 2003; Huyghebaert, 2005; Lutful Kabir, 2009). Thus beneficial features of probiotics make them promising candidates as AGP alternatives.

1.2.5.4 Prebiotics

Prebiotics are non-digestible carbohydrates; mostly oligosaccharides (OS) which when added to animal feed stimulate the growth, activity or selection of beneficial gut microbiota (Huyghebaert, 2005; Callaway et al., 2008). Examples of commonly used prebiotics in poultry feed are fructo-oligosaccharide (FOS), mannan-oligosaccharide (MOS) and glucooligosaccharide (MacroGard) (Patterson and Burkholder, 2003; Huyghebaert, 2005).

Prebiotics, when fed to chickens, selectively stimulate the growth of only beneficial bacterial species such as *Bifidobacteria* and *Lactobacillus* in the intestine (Huyghebaert, 2005), or prevented colonization by pathogens like *Escherichia coli* and *Salmonella* spp. in the GIT by binding them (Patterson and Burkholder, 2003; Callaway et al., 2008; Gaggìa et al., 2010).

1.2.5.5 Phytobiotics

Phytobiotics, botanicals or phytogenics , are well known for their pharmacological effects like stimulation of feed intake, antibacterial, coccidiostatic, anthelmintic and immunstimulation

when added in animal feed (Grashorn, 2010). They comprise a wide range of compounds deriving from herbs, spices and plant extracts (mainly essential oils) (Grashorn, 2010). Due to the beneficial pharmacological activity, many botanicals have been studied as alternatives to AGPs in poultry feed, including black cumin, turmeric, cranberry, rosemary and oregano (Samarasinghe and Wenk, 2003; Guo et al., 2004; Nasir and Grashorn, 2006; Leusink et al., 2010; Mathlouthi et al., 2012).

Presumably the beneficial effects on farm animals are associated with enhanced feed intake and secretion of digestive enzymes, immune stimulation, anti-microbial, coccidiostatic, anthelmintic, anti-inflammatory and/or antioxidant properties (Wenk, 2003). These compounds are mostly secondary metabolites belonging to classes of isoprene derivatives, flavonoides and glucosinolates; most of these also possess antimicrobial and/or antioxidants activity in vivo and in food (Wenk, 2003; Huyghebaert, 2005)

1.3 Chicken Immunology

The immune system of chickens, like humans and other living organisms, consists of several sophisticated mechanisms to cope with a wide range of pathogens. The core elements of an immune system and their important functions are surveillance against pathogens, foreign proteins, parasites and cancerous cells, killing them after their recognition and most importantly, discriminate between self and non-self-cells. The immune system is divided in two categories depending to the types of responses: innate immune response and adaptive immune response.

1.3.1 Innate Immune Response

The innate immune system is the primary line of defense against infection; it is activated within seconds of exposure to any antigen, but there is no memory associated with this immune

system. For many years the innate immune system was considered non-specific, assuming that it has the same mode of action towards all pathogens. However, it is now clear that it produces a specific immune response, if not to certain pathogen but to a certain class of pathogens (Kaiser, 2010). Moreover, it directs the adaptive immune response in a direction to combat specific disease causing agents appropriately.

The innate immune system has exogenous and endogenous PRRs. These receptors require the pathogen associated molecular patterns (PAMPs) to initiate an effective response distinguishing between self and non-self-cells (Ozinsky et al., 2000). PAMPS possess the distinct invariant molecular structure in pathogens which helps the innate system to discriminate between self and non-self (Medzhitov and Jr, 1997). Examples of PAMPs are lipopolysaccharide (LPS), lipoteichoic acid, flagellin, and peptidoglycans or pathogen nucleic acid including single stranded RNA, double stranded RNA or CpG DNA (Kaiser, 2010). Initiation of the innate response requires macrophages to recognize PAMPS that discriminates between self and non-self-particles (Ozinsky et al., 2000). Macrophages are cells capable of detecting, phagocytizing and killing pathogenic microorganisms; therefore macrophages can be characterized as inflammatory (response of the body towards disease causing microorganisms) or cytocidal (killing of the pathogen) cells (Klasing, 1998). Recognition of pathogens by the immune system is mediated by receptors recognizing conserved motifs on pathogens not found in higher animals. For instance many bacterial components like, LPS, peptidoglycan, lipoteichoic acid, lipoarabinomannan(LAM), lipopeptides, and bacterial DNA can stimulate the innate immune response by binding to these receptors (Aderem and Ulevitch, 2000). Toll-like receptors (TLRs),

the basic signaling receptor of innate immunity, participate in the detection of foreign invading organisms inside the body.

The cells of the innate immunity after microbial recognition work in three ways:

1.3.1.1 Activation of Microcidal Mechanism

Toll like receptors (TLRs) belonging to the family of PRRs, recognize the cell surface components of the pathogen. Triggering of the TLRs through their specific PAMPS leads to the initiation of different signaling pathways (NF-κB, mitogen activated protein kinase, and the type I IFN), leading to the production of pro-inflammatory cytokines and chemokines. Subsequently, this results in the recruitment and activation of other cells in the innate and adaptive immunity (Kaiser, 2010).

Another important peptide contributing to the innate immune response are defensins; these are small anti-microbial peptides whose main function is to bind and form pores in microbial cell membranes. The second function of defensins is to chemoattract effector cells of the innate immunity system (Kaiser, 2010)

1.3.1.2 Release of Cytokines and Chemokines

Cytokines are important molecules for initiating and coordinating the process of eradicating pathogens in both innate and adaptive immunity, whereas chemokines regulate the movement of leukocytes (Swaggerty et al., 2009). Cytokines are produced by macrophages activated by bacteria cell surface components such as LPS on Gram negative or by viral products. They are also activated by T lymphocytes which connect cytokines to the adaptive immunity. Examples include tumor necrosis factor (TNF), interleukin (IL)-1, Type 1 interferon, IL-6, IL-10, IL-12 and chemokines (Abbas and Janeway, 2000; Kaiser, 2010).

1.3.1.3 Production of Molecules to Stimulate Adaptive Immunity

Macrophages kill and degrade microorganisms and the degraded components of the pathogens are presented to T-cells to establish protective immunity against the infectious organism (Aderem and Ulevitch, 2000).

Natural killer (NK) cells provide primary surveillance mechanisms against cancers and tumors (Fairbrother et al., 2004). They also create a bridge between innate and adaptive immune response (Semple and Freedman, 2010).

1.3.2 Adaptive Immune Response

Adaptive immunity is mediated exclusively by B and T cells. Its activation is slow, antigen specific, but generates antigenic memory (Semple and Freedman, 2010). In some cases, innate immunity is sufficient to combat the infection. However, adaptive immunity is required to clear the pathogens which are important for generating immunological memory either by primary infection or vaccination (Kaiser, 2010). The adaptive immune response is further divided into humoral immunity (HI) and cell-mediated immunity (CMI). Intracellular pathogens are cleared by CMI, whereas the extracellular ones are eliminated by HI.

One important initiation step in the adaptive immune response is the inter-linking of T cell receptors (TCR) with the cluster of differentiation (CD) 4/ CD8 molecule (as a group on αβ T cell), and antigen peptide bound to the major histocompatibility complex (pMHC) on antigen presenting cells (APC) (Liu et al., 2010a). The T-cells, which play important regulatory roles in the adaptive immunity, are the T helper (Th) cells classified by CD4+ surface antigens. In poultry, like humans, there are two types of Th cells: type-1 (Th1) which initiates CMI and type-

2 (Th2) involved in the stimulation of HI (Erf, 2004). The generation of Th-1 and Th-2 cells directs the immune response to a particular pathogen towards CMI or HI (Erf, 2004).

The role of major histocompatibility complex (MHC) is to bind antigen peptides which are formed as a result of exogenous or endogenous processes, with these being expressed on the APC surface. They are classified into MHC class I and II molecules. Class I MHC binds peptides present in the cytoplasm, they are present in almost all cells and are recognized by CD8-bearing T-cells generally cytotoxic T-lymphocytes (CTL). Class II MHC binds to peptides in intracellular vesicles taken up from extracellular space, they are primarily present on the APC and recognized by CD4 bearing T-lymphocytes (Davison and Kaspers, 2008). The stimulation of Th cells results in augmentation of their active form and their differentiation into effector and memory cells. The later cells are important for further protection of the organism against specific antigen while the effector cells secrete cytokines and express membrane-bound cell surface molecules that aid the production of other immune cells system (Erf, 2004). The Th1 and Th2 effectors are differentiated according to their cytokine production profile, which leads to the type of function they can initiate by stimulating specific cells of the immune system. For example, the Th1 cells generate cytokines such as interferon gamma (IFN-ɣ), tumor necrotizing factor alpha (TNF-α) and interleukin 2 (IL-2) which activates CMI while the Th2 produce cytokines like IL-4, IL-5, transforming growth factor-β and IL-10 which activates HI (Erf, 2004).

Professional APC (dendritic cells (DCs), macrophages, and B-cells) processes and presents exogenous antigens resulting in the activation of T-cells (de Jong et al., 2006). These authors reported that DCs are the only professional APCs involved in the activation of class II MHC restricted $CD4^+$ T-cells following FCɣR facilitated antigen capture. DCs recognize

pathogens through PRRs such as TLRs and C-type lectin receptors (CLRs), thereafter, they present these antigens to naive T-cells which results in their amplification and initiation of adaptive immune response. Their activation by DCs cause naive $CD4^+$ T cells differentiate into Th1 or Th2 depending on the type of pathogens (Wu and Kaiser, 2011).

Humoral immune response acts by producing antibodies, and the main lymphoid organ in chicken involved in this response is the bursa of Fabricius (BF). Antibodies produced by the activation of B-cells are more efficient in eliminating extracellular pathogens (Erf, 2004). There are three types of immunoglobulins (Ig) in chickens: IgM, IgG or IgY and IgA (Ratcliffe, 2006). IgM is the first antibody generated during primary antibody response. IgG in chickens are also referred to as IgY because phylogenetically it is similar to mammalian IgG and IgE and its function is mainly in the secondary immune response. Chicken IgA is found in secretions like serum and bile. Predominant Igs in chicken's external intestinal secretions are IgA and IgM. Secretory IgM are structurally pentameric molecules and are effective in eliminating microbes. However, secretory IgA prevents environmental antigen influx into internal body compartments, neutralizes viruses and microbial toxins, and prevents adherence and colonization of mucosal surfaces by pathogenic microbes (Lillehoj and Trout, 1996). Immunoglobulin G (IgY) may be found in the gut but presumably derived from the secretion or leached from the lymphatics during any permeability changes occurred in response to any infection.

1.4 Vaccines

Vaccines aim to protect the targets host by stimulating immunity against a specific infectious agent. Chickens are susceptible to infections because of their exposure to infectious agents during their rearing period. There are several viruses and bacteria endemic in poultry

producing areas (Table 1.2), which have the ability to cause recurring infections in commercial flocks and vaccination can protect birds from these infections. Table.1.2 shows different vaccines commonly used against various infections in commercial chicken productions.

1.4.1 Types of Vaccines

There are four types of vaccines: 1) live vaccines, 2) inactivated vaccines, 3) recombinant vaccines and 4) nucleic acid vaccines

1.4.1.1 Live Vaccine

These vaccines are live but attenuated bacteria, virus or parasites. The attenuated pathogens have weakened pathogenicity and do not cause significant infections upon administration. Conversely, they induce protection by multiplication in the host and activate the memory immune response which remember the specific pathogen upon second exposure and destroy in smaller time than first exposed. Live vaccines are the only vaccines which can be administered by drinking water or aerosol sprays.

1.4.1.2 Inactivated Vaccine

Inactivated or dead vaccines are prepared by growing the pathogenic agent and subsequently inactivating it chemically. The inactivated organisms are then administrated by injection and generally require an adjuvant to provide improved immune system stimulation.

1.4.1.3 Recombinant Vector Vaccine

These are prepared by genetic engineering; they introduce the nucleic acid of one organism by using attenuated bacteria or virus of interest into the host body employed by a delivery agent called vectors which thereby induce protective immune response against the specific antigen introduced.

1.4.1.4 Nucleic Acid Vaccine

These are new generation vaccines obtained using molecular biology techniques in which nucleic acid (DNA) of pathogen of interest is administrated into the host. DNA vaccines may provide both cellular and humoral protective responses, which are typically difficult to achieve with other vaccines. Specifically, it stimulates both antibody responses and cytotoxic T cell responses that could be effective against intracellular pathogens (Sharma and Khuller, 2001).

1.5 Infectious Bursal Disease Virus

Viral immunosuppressive infections have severely affected the economics of broiler production, often as a result of the chicks' increased susceptibility to secondary infections and sub-optimal response to vaccinations. Infectious bursal disease virus (IBDV), a bi-segmented, double stranded RNA virus that belongs to the family *Birnavirideae*, is one of the major immunosuppressive viruses affecting broilers. This virus cause infectious bursal disease which is an acute, highly contagious disease of young chicks (Käufer and Weiss, 1980). There are two known serotypes of IBDV. The pathogenic strains are grouped in serotype-1 while non-pathogenic strains form the serotype-2 class. Infection caused by IBDV serotype-1 is cytolytic and directly leads to immunosuppressant effects through depletion of IgM+ B-lymphocyte precursors. On the contrary, serotype-2 strain neither causes disease nor induces apoptosis (Rodríguez-Lecompte et al., 2005).

Infectious bursal disease (IBD; Gumboro disease) is highly lethal in chickens. This disease commonly affects young chickens 3-6 weeks of age. IBDV infections effect both HI and CMI (Sharma et al., 1989). The effect on humoral immunity is due to the depletion of immunoglobulins though other mechanisms like suppression of macrophages and T-cells may

also take place due to the virus (Sharma et al., 2000). Furthermore, T-cells which were not normal residents of bursa were observed at elevated levels in the early phase of the disease (i.e. 1 day post infection) (Kim et al., 2000). Previously it was considered that humoral immunity contributed to IBDV recovery. However, Rautenschlein et al. (2002a; b) demonstrated that T-cells were important for the protection against viral infections, specifically IBDV.

Immunization is the primary method used to control IBD in chickens. Young chicks are protected by maternal antibodies but when the antibody levels decrease, vaccination is required. Currently, protection against the disease is obtained by live and inactivated IBDV vaccines (Sharma et al., 1989). However there are concerns about live attenuated vaccines i.e. their extensive use could be the reason for the increased virulence of the pathogen due to mutation. On the contrary, recombinant vaccines have demonstrated success in protecting against IBDV infections, particularly when compared to live attenuated and inactivated vaccines (Mahgoub, 2012).

DNA vaccines mimic natural viral infections by delivering protective antigens. The encoded antigens are produced in their native structure and presented in the context of MHC class I and II. Moreover, they induce a balanced immune response and multiple component vaccines can be administered in a single dose (Mahgoub, 2012).

1.6 Poultry Gut Microbiology

The poultry gut natural microbiota plays an important role in poultry health. Any imbalance may result in poor performance and/or increased morbidity/mortality. The gut colonization occurs soon after the chicks hatch, however their composition changes over time

depending on their diet, age, use of antibiotics and/or probiotics until a normal microflora is established (Lu et al., 2003; Brisbin et al., 2008).

1.6.1 Importance of Poultry Gut Microflora

The gut microbiota plays a vital role in digestion, intestinal morphology, and immune system stimulation (Ahir et al., 2012). Most importantly, the commensal bacterial population communicates with the gut-associated immune system when in contact with any microbe (antigen) resulting in the modulation of host immune system (Haghighi et al., 2006). Thus, intestinal microbiota also contributes to host defense by activating its innate and adaptive immune system (Brisbin et al., 2008).

1.6.2 Bacterial Community

A diversified microbial population is found in the GIT of chickens with a maximum population in the cecum (Amit-Romach et al., 2004). Monitoring the chicken microflora is very important since many human pathogens reside in the gut and can potentially be transferred into the food chain through consumption of contaminated food (Amit-Romach et al., 2004). Conventionally, the only way to detect a microbial population was through traditional culturing on selective media followed by a number of biochemical tests to identify and characterize the organism. These methods are laborious, time consuming, and therefore not suitable for ascertaining the gut microbial ecology where complex and diverse species are expected (Apajalahti et al., 2004). Recently, molecular methods using 16S ribosomal DNA (rDNA) have been successful in investigating poultry gut microbiota complexity (Amit-Romach et al., 2004; Ahir et al., 2012).

A culture-independent flow cytometry method has shown that the microbial population of day old chicks is 10^8 and 10^{10} colony-forming units (CFU)/per gram digesta in the ileum and cecum, respectively. This population gradually increased over time, reaching 10^{11} /gram of ceca digesta and 10^9CFU/ gram of ileum digesta and remained stable until day 30 (Apajalahti et al., 2004).

In a study using 16S rDNA gene sequences method, bacterial diversity in ileum and ceca on chickens fed with vegetarian-corn based- diet devoid of any feed additives was determined. Results illustrate that ileum consists of approximately 70% of the bacterial species belong to *Lactobacillus*, 11% *Clostridiaceae*, 6.5% *Streptococcus* and *Enterococcus.* Conversely, *Clostridiaceae* species were abundant in the cecum constituting about 65%, *Fusobacterium* 14% ranking second, with others species detected including *Lactobacillus* 8% and *Bacteriodes* only 5% (Lu et al., 2003).

Ultimately, the gut microflora is influenced by the diet, age, antibiotic administration, and infections, thereby any alteration in the gut microbial population may be beneficial or detrimental to host's health, growth and maturation (Lu et al., 2003). For example, it has been recently demonstrated by metagenomic sequencing approaches that salinomycin-feeding has a profound impact on the dynamics of the chicken ceca microbiome (Fung et al., 2013). The salinomycin fed group had an increased abundance of the Elusimicrobia, and a decreased abundance of Chloroflexi, cyanobacteria, and Synergistetes. There was also increased abundance of *Clostridium perfringens* in the salinomycin fed ceca. By contrast, *Campylobacter jejuni*, *Salmonella* spp., and *Escherichia E. coli* were not detected in salinomycin fed group. The abundance of *Bifidiobacterium* spp. and *Lactobacillus* spp. increased significantly in the

salinomycin fed-birds. A functional analysis of environmental gene tag (EGTs) revealed that abundance in the cell wall and capsule, iron acquisition, and motility categories increased in salinomycin treatment while an increase of β-lactamase family and a decrease of multidrug efflux pump EGTs were detected in the salinomycin treated-birds (Fung et al., 2013).

1.6.3 *Clostridium Perfringens*

C. perfringens is an anaerobic, Gram positive, spore forming bacterium, ubiquitously found in soil, water, sewage and in the intestinal tract of animals and humans (Brynestad and Granum, 2002; Gurjar et al., 2008). This bacterium is the etiological agent of a human foodborne disease. It causes Type A diarrhea which is mild and more common in industrialized world, and a second more serious Type C human necrotic enteritis (Brynestad and Granum, 2002). In addition, *C. perfringens* causes necrotic enteritis (NE) in poultry, an enteric disease of significant economic importance in poultry production (Parish, 1961; McDevitt et al., 2006).

1.6.3.1 Disease and Pathogenesis

Normal microflora of chicken contains *C. perfringens* counts from 0-10^5 CFU/gram of the intestinal content. However, birds with NE have concentrations of *C. perfringens* between 10^6- 10^8 CFU/gram of intestinal content (McDevitt et al., 2006; Timbermont et al., 2011). There is some evidence suggesting that *C. perfringens* number in chicken gut vary in relation to husbandry practices (Yost et al., 2011). Increased *C. perfringens* counts do not guarantee the onset of NE, though. Not all *C. perfringens* strains are capable of inducing NE. In particular, specific virulence factors are required.

C. perfringens produces a number of extracellular enzymes and toxins. They are classified into subtypes A, B, C, D, and E based on their capability to produce four major toxins

alpha (α), Beta (β), epsilon (ε) and iota (ι) (Table 1.3) (Gurjar et al., 2008). Das et al, (2008) investigated the cause of NE in six broiler birds that died from NE, for the specific virulent factors. They observed that alpha toxin gene, *cpa*, in all isolated strains, confirming *C. perfringens* Type A (CPA) as the NE-inducing strain (Das et al., 2008).

More recently, another toxin, NetB, was identified in NE-inducing strains of *C. perfringens* (Cooper and Songer, 2009). The amino acid sequence of NetB has 38% homology to the beta toxin protein and 31% homology to the *Staphylococcus aureus* alpha toxin. The role of NetB toxin in NE was confirmed when both purified and recombinant NetB toxin induced cytotoxicity in chicken leghorn male hepatoma cell lines (LMH), causing cell lysis whilst *netB* mutant did not induce NE (Keyburn et al., 2008).

Subsequently, another study on broilers to further confirm the role of NetB in NE was performed. In this study, they treated chickens with laboratory grown cultures of *C. perfringens* field cases of NE mixed with feed (3 parts culture to 4 parts feed) and examined rates of NE. Results showed that *netB* gene positive strains were able to induce NE, interestingly, the disease was also observed in birds infected with *netB* negative strains. Based on this, it appears that other factors may contribute to NE (Cooper and Songer, 2010).

1.6.3.2 Epidemiology

Necrotic enteritis in broilers, which was previously controlled and treated by the prophylactic use of antibiotics in feed, has re-emerged because of the recent ban on this practice, causing significant economic losses for broiler producers. Due to insufficient data on accurate number of NE incidences, estimates vary from 1-40% of broiler flocks being affected in North America and EU (McDevitt et al., 2006). In broilers NE can be sub-clinical or severe, resulting

in decreased weight gain, poor feed conversion and high rates of mortality (Cooper and Songer, 2010).

Most importantly, consumption of *C. perfringens* contaminated meat and meat products are the main source of introducing *C. perfringens* in the food chain (Brynestad and Granum, 2002). The prevalence of NE in chickens is dependent to the colonization status the chickens by *C. perfringens* themselves. Small numbers of *C. perfringens,* is present in the gut of normal chickens, and are shed in the environment during hatching and/ or during subclinical NE, and infect other healthy birds (Cooper and Songer, 2009).

1.6.3.3 Prevention and Treatment

Despite significant progress in management, the control and eradication of *C. perfringens* infections in broiler chickens remain a major challenge. Due to the widespread and presence of *C. perfringens* in the environment, it is imperative to prevent its growth and colonization in the intestine and cause NE disease. The overgrowth and NE can be prevented through prophylactic use of antimicrobial (virginiamycin, bacitracin and lincomycin) in feed. However the incidence of *C. perfringens*-associated NE in poultry has increased in countries where antibiotic growth promoters used has been banned as mentioned above. Factor such as protein rich diet (fish meal), cereals containing NSP (wheat, barley, etc.) or coccidiosis may results in the increase of the *C. perfringens* number and the onset of NE. Thereby, occurrence of NE can be substantially controlled by reducing the exposure of their risk factors (McDevitt et al., 2006; Cooper and Songer, 2009). For the treatment of *C. perfringens* infection in broiler production, bacitracin (200-400 mg/gal. for 5-7 days), penicillin (1,500,000 u/gal. for 5 days),

and lincomycin (64 mg/gal. for 7 days) have been often used in the drinking water (http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/201200.htm).

In humans and other mammals probiotic bacteria have been shown to reduce intestinal colonization by *C. perfringens* (Van Immerseel et al., 2004; Dahiya et al., 2006). In chicken, lactobacilli also have shown to reduce the gross lesion of NE and the mortality rate from 60% to 30% in an experimental trial when administrated on day one (Hofacre et al., 1998, 2003). Although promising results using lactobacilli were observed in chickens, the mechanism of action is still not clear and more work needs to be done to confirm its efficacy (Dahiya et al., 2006).

Presently, vaccination against *C. perfringens* maybe the best approach to prevent and control the infection. *C. perfringens* alpha toxin (CPA) was considered as the necrotizing factor responsible for causing NE (Das et al., 2008). Consequently, immunization against the CPA toxoid and boosting with active toxin has provided greatest degree of protection against NE, however other proteins such as glyceraldehydes-3-phosphate dehydrogenase (GDP), pyruvate: ferredoxin oxidoreductase (PFOR), fructose 1,6-biphosphate aldolase (FBA) and a hypothetical protein (HP), might also be good candidate for the use in vaccine in controlling and preventing NE in chickens (Dahiya et al., 2006; Kulkarni et al., 2007; Cooper and Songer, 2009).

1.6.4 *Lactobacillus*

Lactobacilli are Gram positive, non-spore forming bacteria. They are strictly fermentative, anaerobic and acidophilic and possess complex nutrient requirements (Tannock, 2004). Lactobacilli are the member of the lactic acid bacteria and are frequently used in food production. Specifically they are used for preparing foods that require lactic acid fermentation

such as yogurt, cheese, pickles, sauerkraut, bread and fermented meats like Salami (Tannock, 2004).

Most importantly, in chickens *Lactobacillus* are normal inhabitants of the gut, where they play a role as probiotics, competitive exclusion agents or delivery vector (Stephenson et al., 2010). For instance *L. salivarius* has been observed to reduce colonization of *Salmonella enterica* serovar Enteritidis (*S. enteritidis*) in chickens (Zhou et al., 2007). Lactobacilli as probiotics have shown promising immune modulatory effects, and have been useful in prevention and treatment of many diseases. For instance *L. salivarius* 3d strain has been shown to reduce the number of *S. enteritidis* and *C. perfringens* in a group of chickens treated with *Lactobacillus* (Kizerwetter-Swida and Binek, 2009). Studies using different strains of *Lactobacillus* demonstrated positive effects on humoral and cellular immunity of chickens depending on type of *Lactobacillus* strain used, age of the bird, dose of lactobacilli administered (Koenen et al., 2004; Lutful Kabir, 2009).

To date, it appears that *Lactobacillus* may be potentially used as a nutritive feed supplement for the growth promotion, modulation of intestinal microflora and restriction of pathogen colonization, immunomodulation and improving meat quality in poultry (Lutful Kabir, 2009).

Normal gut microbial composition of chickens consists of 70% of *Lactobacillus* spp. in the ileum and 8% in cecum which is important for their wellbeing (Lu et al., 2003). When antimicrobial agents, such as bacitracin, virginiamycin and salinomycin were used in chicken feed, drastic reductions in *Lactobacillus* spp. in the ileum microflora have been observed (Feng et al., 2010). Moreover, another study also supports the fact that the prophylactic use of

antibiotics in poultry feed significantly reduces the *Lactobacillus* and *C. perfringens* counts in the ileum (Knarreborg and Simon, 2002).

1.7 3', 5' Cyclic Diguanylic Acid (C-di-GMP)

1.7.1 Discovery

The c-di-GMP is a bacterial intracellular signaling molecule and was first discovered in 1987 in the laboratory of Moshe Benziman at the Department of Biological Chemistry of the Hebrew University of Jerusalem, Israel (Wolfe, Alan and Visick, 2010). Discovery of c-di-GMP was a result of investigation of complex plant cell wall biogenesis. Studies on cellulose biosynthesis in plants was a difficult task, therefore *Acetobacterium xylinum*, currently known as *Gluconecetobacter xylinus,* was used as an experimental model because the cellulose produced by this bacteria is similar in its crystalline unit structure and average microfibrillar width with many plant cell and algal sources (Ross et al., 1991).

Subsequently, as a result of a series of studies to reveal the stepwise process of cellulose biosynthesis using *A. xylinum* as a model, c-di-GMP was discovered as a direct activator of the enzyme cellulose synthase involved in the biosynthesis of cellulose (Ross et al., 1991; Delmer and Amor, 1995; Wolfe, Alan and Visick, 2010)

1.7.2 Role of C-di-GMP in Bacteria

C-di-GMP, is a cytoplasmic bacterial second messenger molecule, found in most of the human pathogens. This molecule depends on environmental stimuli, which reaches the target effector receptors inside the cell through the cell membrane as a part of signaling transduction cascade (Jenal and Malone, 2006). Metabolism of c-di-GMP is regulated by the opposing activities of diguanylate cyclase (DGCs) and phosphodiesterase (PDEs) enzymes. DGCs

containing domain GGDEF synthesizes c-di-GMP by two molecules of guanosine-triphosphate (GTP), and it is hydrolyzed by phosphodiesterases(PDEs) enzyme containing EAL domain into guanosine-monophosphate (GMP) (Jenal, 2004; Jenal and Malone, 2006).

C-di-GMP plays a role in a number of cellular processes. Increased concentration of c-di-GMP, results in exopolysaccharide (EPS) formation and cell-to-cell adhesion during biofilm production (Jenal and Malone, 2006; Cotter and Stibitz, 2007; Borlee et al., 2010; Srivastava et al., 2011). However, low concentration of c-di-GMP, are found in cells that move by flagella or retracting pili (Jenal and Malone, 2006).

Numerous studies on different bacteria have investigated the role of c-di GMP in bacteria. *Vibrio. cholerae* inside the host is in a motile, virulent state and in a sessile biofilm form in the aquatic environment. The two important signaling systems enabling this transition are c-di-GMP and quorum sensing (QS) (Srivastava et al., 2011). Moreover, c-di-GMP also regulates virulence expression (Cotter and Stibitz, 2007). Biofilm formation in *V. cholerae* protects bacteria from environmental stress due to acid in the stomach and enables the organism to reach the host intestine unaffected and cause disease (Zhu and Mekalanos, 2003).

Biofilm formation does not directly contribute to pathogenesis; it depends on biology and lifestyle of the bacteria, thus not all bacteria forming biofilm cause disease in humans (Tamayo et al., 2007). However, c-di-GMP has shown its role in regulating virulence gene expression in *V. cholerea, Salmonella enterica* serovar *typhimurium,* and *Pseudomonas aerugenosa,* in these examples increased concentration of c-di-GMP inhibits the expression of virulence factors (Hisert et al., 2005; Jenal and Malone, 2006; Cotter and Stibitz, 2007).

1.7.3 Role of C-di-GMP as an Immunomodulator in Infection Control

Besides the intracellular functions of c-di-GMP in bacteria, numerous studies reported the role of this molecule in the context of other biological system, particularly in the prevention and treatment of diseases (Karaolis et al., 2007a). Many of these studies suggest that this molecule may exert biological effects through the modulation of host immunity.

1.7.3.1 Effect of C-di-GMP on Host Innate Immunity

Innate immunity is the first line of defense against invading antigens; it consists of PRR which recognizes conserved microbial molecules (i.e. PAMP) for initiation. Exogenous c-di-GMP has been reported to initiate immune response in the host and thereby might potentially be used to minimize and control diseases (Karaolis et al., 2005, 2007b; Römling and Amikam, 2006). A transmembrane protein called STING (stimulator of IFN genes), has been identified as a direct sensor for the candidate c-di-GMP in the host, for the activation of innate immunity (Burdette et al., 2011). Therefore, c-di-GMP acts as a PAMP which is recognized by the host immune system and initiates an immune response.

Klebsiella pneumoniae is a highly pathogenic Gram negative bacterium and the etiological agent of both community-acquired or nosocomial pneumonia. Interestingly, Karaolis et al. (2007b) reported in mice that local intranasal or systemic subcutaneous treatment with c-di-GMP prior to intra-tracheal challenge with *K. pneumoniae* resulted in the enhanced stimulation of innate immunity. This stimulation was characterized by recruitment of neutrophils, NK cells, αβ T cells and B cells. Stimulation of immunity by c-di-GMP resulted in improved survival of mice by significantly reducing the count of the *K. pneumonia* in lungs and blood. Moreover, lung macrophages from the pre-treated mice treated with *K. pneumonia* expressed elevated levels of

inducible nitric oxide synthase and nitric oxide (NO) ex-vivo as compared to control mice. Thus, this suggests that c-di-GMP enhances innate immunity of the host by contributing to reductions in bacterial invasion (Karaolis et al., 2007b).

1.7.3.2 Effect of C-di-GMP on Host Adaptive Immunity

Adaptive immunity is initiated following innate immunity, and mediated by clonally distributed by B and T lymphocytes. These lymphocytes are required to clear pathogens from the host, and lead to the generation of immunological memory. Similarly, for animal and human health, vaccination directly acts by stimulating an adaptive immune response. Dendritic cells are professional APCs capable of activating naive T cells, thereby initiating adaptive immunity (Akira et al., 2001).

C-di-GMP has been shown as an activator of DC and initiator of adaptive immunity (Wolfe, Alan and Visick, 2010). Specifically, incubation of human immature DCs with c-di-GMP resulted in increased expression of CD80/CD86 and the maturation marker CD83. Moreover, increased MHC class II, cytokines and chemokines such as IL-12, IFN-γ, IL-8, MCP-1, IFN-γ-inducible protein-10 and RANTES, and altered expression of chemokine receptors CCR1, CCR7 and CXCR4 was observed. Thus, it appears that c-di-GMP has a T-cell stimulatory activity (Karaolis et al., 2007a).

In mice, c-di-GMP as a mucosal adjuvant was noted to produce a significant increase (512-fold) in anti-β-GAL IgG titers compared to controls. It also led to significantly increased release of β-GAL-specific IgA in the lungs (Ebensen et al., 2007b). Intranasal pre-treatment with c-di-GMP, or intraperitoneal co-administration of c-di-GMP with the pneumolysin toxoid prior to pneumococcal challenge resulted in significant reduction of bacterial numbers in the lungs and

blood, and significantly increased antigen-specific antibody titers survival of mice when compared to control groups (Ogunniyi *et al.,* 2008). Thus, c-di-GMP may play a central role in minimizing and/or control infectious diseases by modulating both humoral and cellular host immunity.

1.7.3.3 As a Vaccine Adjuvant

Although the use of vaccines is currently considered the best strategy for disease control and prevention, some vaccines lack adequate immunogenicity to be clinically useful. To overcome limitations in immune stimulation, a vaccine adjuvant can be used to enhance the host immune response to the target antigen (Perrie et al., 2008). Due to its immunostimulatry and immunomodulatory effects, C-di-GMP has been explored for its role as a potential vaccine adjuvant by many scientists. In 2006, Karaolis et al. used c-di-GMP for the first time as an adjuvant and immune enhancer. In their study, they intramuscularly vaccinated mice with c-di-GMP (as an adjuvant) and co-injected with *S. aureus* clumping factor A (CLfA) antigen (Ag). This resulted in significantly higher ($p< 0.001$) anti-ClfA IgG antibody titers in serum compared with injections of ClfA individually (Karaolis et al., 2007a).

Increasing the efficiency of mucosal immunity has been shown to play an important role in host resistance to mucosal pathogens. Results from Zhao et al. (2011) showed that the administration of 50 µg (72 nmol) of c-di-GMP 18 h prior to infection provided protection against *Acinetobacter baumannii* in an intranasal mouse model. Moreover, subcutaneous co-administration c-di-GMP with β-galactosidase (β-Gal) in mice resulted in significantly elevated levels of IgG serum titers compared to controls. Also, strong cellular immune responses were observed, being characterized by stimulation of a balanced Th1/Th2 response compared to mice

treated by β-Gal alone. These results suggest that c-di-GMP represents a promising vaccine adjuvant (Ebensen et al., 2007a).

Previous pre-clinical studies have highlighted the role of c-di-GMP as a mucosal vaccine adjuvant. Madhun et al. (2011) conducted a study focusing on a pandemic, highly fatal avian influenza virus, H5N1, which can be transmitted from birds to humans. They investigated the humoral and cellular immune response of following intra-nasal and intra-muscular administered plant derived influenza H5N1 virus antigen individually and in combination with c-di-GMP as an adjuvant. Results show that intranasal administration of vaccine using c-di-GMP as adjuvant showed highest levels of serum Ig A and Th1 cytokine when compared to control groups. Thus c-di-GMP as a vaccine adjuvant was successful in stimulating both CMI and HI (Madhun et al., 2011).

1.7.4 Application of C-di-GMP in Animal Production

Because of its immunomodulatory and successful role as a vaccine adjuvant, c-di-GMP has been investigated for its utility in animal production. Mastitis is an important and expensive to treat disease in dairy cows, therefore considering the promising results obtained by the above study on mastitis mouse model, thus c-di-GMP could potentially be used in dairy cows to treat and control of mastitis (Brouillette et al., 2005; Karaolis et al., 2005). A mastitis mouse model study reported that intramammary injections of c-di-GMP significantly reduced dose dependent teat colonization by *S. aureus*. Precisely, intramammary injection of 5 and 50 nmol of c-di-GMP induced *S. aureus* number reduction of 0.79 ($P > 0.05$) and 1.44 ($P < 0.01$) logs cfu/ gram of gland whereas, 200 nmol induced the highest clearance (4 logs reduction; $P < 0.001$) when compared to control group. Thus, the results clearly indicate the role of messenger-di-GMP in

reducing the colonization of pathogens in a dose dependent manner and can potentially be used in the disease prevention, treatment and control of mastitis (Brouillette et al., 2005). Optimistically, Ster et al. (2010) have initiated an ex-vivo study to determine the effect of c-di-GMP on bovine immune cells and a field study to investigate the adjuvant properties for immunizing calves (Ster et al., 2010).

Table: 1.1 Uses of antibiotics in farm animals

Antibiotics Used as	Definition
Therapy	When animals or group of animals have clear signs of any disease
Control	when the morbidity and/ or mortality have exceeded baseline levels normally observed
Prevention/ Prophylaxis	when antibiotics are administered to healthy flocks considered to be at risk of a disease but before the onset and prior to diagnosis
Metaphylaxis	When small proportion of animals are sick and all treated by the antibiotic
Growth Promoter	used as feed additives to promote improved growth performance

Table: 1.2 Commonly used poultry vaccines and their infectious agents

Vaccine	Species vaccinated	Infectious Agent
Marek's disease	Chickens	Marek's virus
Newcastle disease	Chickens, turkeys	Newcastle virus
Infectious bronchitis	Chickens	Infectious bronchitis virus
Infectious bursal disease	Chickens	Infectious bursal disease virus
Infectious laryngotracheitis	Chickens	laryngotracheitis virus
Fowl pox	Chickens	Fowl pox virus
Avian encephalomyelitis	Chickens	Avian encephalomyelitis virus
Reovirus Infection	Chickens	Reo virus
Cholera	Chickens, turkeys	Bacterium: *Pasteurella multocida*
Coccidiosis	Chickens, turkeys	Parasite: *Eimeria* spp.
Salmonella	Chickens, turkeys	Bacterium: *Salmonella* spp.

Table: 1.3 *Clostridium perfringens* major toxin types and their genes

Toxins	Type A	Type B	Type C	Type D	Type E	Genes	**Pathogenesis** (Gurjar et al., 2008)
Alpha-toxin	+	+	+	+	+	*plc*	cell lysis by hydrolysis of membrane phospholipids in cells
Beta-toxin	-	+	+	-	-	*cpb1* *cpb2*	causes mucosal necrosis
Epsilon-toxin	-	+	-	+	-	*etx*	Responsible for lethal enterotoxaemia In livestock
Iota-toxin	-	-	-	-	+	*iap* *ibp*	Increase vascular permeability & Dermonecrosis and lethal in mice
Enterotoxin	+	+	+	+	+	*cpe*	Interacts with epithelial cell tight protein causing diarrhea and intestinal cramping

Chapter 2: Effect of Co-administration of 3´, 5´-Cyclic Diguanylic Acid with Infectious Bursal Disease Virus Vaccine on Serum Antibody Levels in Broilers

2.1 Introduction

Viral immunosuppressive infections have severely affected the economics of broiler production, often as a result of increased chick susceptibility to secondary infections and sub-optimal response to vaccination programs for Newcastle disease, Marek's disease and infectious bronchitis (Kibenge et al., 1988). Infectious bursal disease virus (IBDV, Gumboro disease) is one of the major immunosuppressive viruses affecting broilers. This virus, belonging to the *Birnaviridae* family, induces a highly contagious disease in 3 to 6 week-old chickens and continues to be one of the major causes of economic losses in poultry farming worldwide (Bumstead et al., 1993). The general mode of infection is oral; the virus enters the gut and subsequently, spreads to different organs. In addition to poor vaccination response, secondary bacterial, viral and protozoan infections, the causes of economic devastations associated with IBDV, also include poor growth performance of infected birds. The targets of the virus are the immature B cells from the bursa of Fabricius, the primary organ involved in the development of the chicken's immune system (Hirai and Calnek, 1979; Sharma et al., 2000). The consequence of IBDV infection is the depletion of immunoglobulin production by the B-lymphocytes in response to a variety of vaccines or pathogenic microorganisms (Berg, 2008). The inability of current IBDV vaccinations to effectively prevent and limit the spread of IBDV necessitates the development of more effective, alternate interventions. Correspondingly, studies have been conducted to enhance the immunity of chickens to fight against IBDV and other infections.

Recent studies have found that β-Glucan treatment can significantly stimulate immunity and improve bird growth (Rajapakse et al., 2010; Tang et al., 2011). Also, results from nutritional interventions suggested that tryptophan and arginine modulate systemic immune responses against IBDV (Emadi et al., 2011).

The compound 3’, 5’-Cyclic diguanylic acid (c-di-GMP) is an intracellular signalling molecule that is present in various bacterial species but no evidence has been shown for its presence in eukaryotes. The cellular levels of c-di-GMP are determined by the opposing activities of diguanylate cyclases and phosphodiesterases, which can control virulence through modulation of motility, cell adhesion and biofilm formation (Jenal and Malone, 2006; Römling and Amikam, 2006). As such, exogenous c-di-GMP was reported to inhibit intercellular adhesive interactions between *S. aureus* cells and to reduce biofilm formation (Brouillette et al., 2005; Karaolis et al., 2005). Moreover, prophylactic treatment of mice with exogenous c-di-GMP prior to experimental *S. aureus* infection provided a protective effect and a 10,000-fold reduction of bacterial counts in tissues. Also, intramuscular vaccination of mice with c-di-GMP as an adjuvant for a purified *S. aureus* ClfA antigen produced significantly higher anti-ClfA IgG antibody titers in serum compared with injections of ClfA alone (Karaolis et al., 2007a). These immunomodulatory activities and vaccine adjuvant effects of c-di GMP have also been reported by (Ogunniyi et al., 2008). These authors showed that intranasal pre-treatment with c-di-GMP, or intraperitoneal co-administration of c-di-GMP with the pneumolysin toxoid prior to pneumococcal challenge resulted in significant reduction of the bacterial number in the lungs and blood as well as a significant increase in antigen-specific antibody titers and the increased survival of mice when compared to control groups (Ogunniyi et al., 2008). Increasing the

efficiency of mucosal immunity has been shown to play an important role in the resistance to mucosal pathogens. In mice, c-di-GMP as a mucosal adjuvant, produced a significant increase (512-fold) in anti-β-GAL IgG titers compared to controls and significantly stimulated release of β-GAL-specific IgA in the lungs (Ebensen et al., 2007b). Results from Zhao et al. (2011) reported that the administration of 50 μg (72 nmol) of c-di-GMP 18 h prior to infection provided protection against *Acinetobacter baumannii* in a mouse model of intranasal infection. These observations suggest that c-di-GMP needs to be investigated for its remarkable properties as an immunomodulator.

The objective of the present study was to investigate the capacity of c-di-GMP to increase humoral responses of broiler chickens in an IBDV vaccination model. Total immunoglobulin IgA, IgG and IgM, as well as anti-virus antibodies titers to a number of relevant viruses was determined.

2.2 Materials and Methods

2.2.1 Chicken Housing and Treatment

One hundred-ninety-two male day-old broiler chicks (Western Hatchery Abbotsford, B.C) were randomly placed in 24 cages (8 chicks /cage). Before placement, all chicks were visually examined for health and inferior chicks were excluded from the trial. Each cage was equipped with a drinker and a feeder providing free access (*ad libitum)* to feed and water. Heat was provided through gas-fired brooders and airflow was provided by negative pressure. The temperature was initially set at 32°C and then was progressively reduced by 1.7°C each week to reach 23°C at 35 days of age. Chicks were exposed to light for 24 h for the first day, 23 h for the second and third days, and then 18 h thereafter as previously described (Leusink et al., 2010).

The composition of the diets used in this study is presented in Table 2.1. The starter, grower and finisher diets were formulated in accordance with the broiler diet used in Western Canada using wheat, barley and corn as the principal cereals and soybean and canola meals as protein concentrates to meet the National Research Council (NRC) nutrient requirements for broiler chickens (NRC, 1994). On days 7 and 14, two birds were removed from each cage and the remaining 96 birds (4/cage) were vaccinated by oral administration with 1 ml of Bursal Disease Vaccine (S-706) as recommended by the manufacturer (Canadian Poultry Consultants, Ltd. Abbotsford, BC, Canada). On the same day, the vaccinated birds were assigned to six treatment groups: Group I and group II were administrated with saline orally (gavage) or by intramuscular (IM) injection respectively. These two groups did not receive c-di GMP; Group III and IV received 10 nmol c-di-GMP by gavage or IM respectively; Group V and VI received 100 nmol of c-di-GMP by gavage or IM respectively. All experimental procedures performed in this study were approved by the local Institutional Animal Care Committee (Agassiz, BC, Canada) according to guidelines described by the Canadian Council on Animal Care (CCAC, 1993).

2.2.2 Blood Collection and Measurement of Antibodies

From days 7 to 35, two birds per cage (2 birds per treatment group) were randomly selected for blood sample collection. Prior to centrifugation, blood samples were allowed to clot at room temperature. The serums were then transferred to sterile eppendorf tubes and stored at -20°C. Total serum Ig concentrations were determined using a commercial chicken IgA, IgG and IgM ELISA quantification kits (Bethyl Laboratories Inc, Montgomery, TX, USA), according to the manufacturer's instructions. Antibodies against IBDV, infectious bronchitis disease virus,

Newcastle disease virus and Reovirus in serum were also determined at the Animal Health Center of BC Ministry of Agriculture and Lands (Abbotsford, BC).

2.2.3 Statistical Analysis

Statistical analyses were conducted according to a randomized complete block design using the GLM procedure of (Statistical Analysis System, 2002) with treatment groups as sources of variation and the individual cages as experimental units (four cages per treatment group). Data from serum samples were log transformed before analysis. Mode of administration and bird ages (days) were included as sources of variation. The least significance difference was used to separate treatment means whenever the F value was significant. The 0.05 *P*-value was used to declare significance.

2.3 Results and Discussion

The various reported immunomodulatory activities and vaccine adjuvant effects of c-di GMP (Karaolis et al., 2007a; Ogunniyi et al., 2008; Hu et al., 2009) suggest that c-di-GMP may represent a new pathogen-associated molecular pattern recognized by the immune system of animals. Based on these observations, I evaluated in the present study the potential of c-di-GMP as an immunostimulant and a novel adjuvant for commonly used vaccines in broiler chickens.

2.3.1 General Immunoglobulin

The effect of 10 nmol (6.9 µg) and 100 nmol (69 µg) c-di-GMP administrated orally or IM on the humoral immunity of broilers from days 14 to 35 was evaluated. Data from this study showed that oral or IM administration of 10 or 100 nmol c-di-GMP did not induce any adverse effect in broiler chickens. Regardless of treatments, significant increase ($P < 0.05$) in the immunoglobulins IgA and IgM concentrations were observed over time (*i.e.* day 7 to 35)

whereas, the serum immunoglobulin IgG titers remained relatively stable during this period (Figure 2.1 A, B and C). No significant treatment effect was noted for IgG and IgM titers on any of the sampling days after administration of 10 or 100 nmol of the test compound. On day 35, a significant treatment effect ($P < 0.05$) on IgA titers was observed in birds receiving 10 and 100 nmol of c-di-GMP by gavage. These birds showed the highest IgA antibody titers when compared to birds of the control group. Thus, it can be concluded that, regardless of treatment, IgA and IgM titers significantly increased with age, but IgA titers can be significantly increased by treatment with 10 and 100 nmol of c-di-GMP using gavage.

As stated above, stimulation of secretory IgA production in the lung by c-di-GMP has been reported (Ebensen et al., 2011). Mucosal IgA plays an important role in mucosal immunity which is part of the first line of defense against bacteria and viruses (Fagarasan and Honjo, 2003; Suzuki et al., 2007). In serum, IgA may function as a second-line of defense by eliminating pathogens that have breached the mucosal surface (Snoeck et al., 2006). In my study, intestinal Ig concentrations were not determined; however, if a significant increase of serum IgA concentration translated into an increase of the intestinal IgA concentration after oral administration of c-di-GMP, this would confirm the potential of c-di-GMP as mucosal adjuvant for broilers.

2.3.2 Anti-Virus Antibodies

A well-developed immune system and optimal immune response are important for the welfare and growth performance of chickens. The ability of high productive meat-type chickens to build sufficient immune responses to infections during the rearing period is of concern (Koenen et al., 2002). Prophylactic measures such as vaccination and antimicrobial feed-

supplementation have been used to reduce infectious diseases. Despite these good management practices, including vaccination against major diseases such as IBD, infectious bronchitis disease, Newcastle disease and avian Reovirus infections, these viruses continue to be important health issues for broilers and a major constraint for the poultry industry worldwide (Hoerr, 2010). The increase in virulence and/or emerging new field virus variants is contributing to the failure of the live-attenuated viral vaccines that are presently being used. Thus, the development of new vaccination strategies using immune stimulatory adjuvants for the poultry industry would help to develop better control of major pathogenic poultry viruses. The recent interest in c-di-GMP has been stimulated by its reported immune-stimulatory effects. Therefore, in the present study, I also evaluated the effects of a co-administration of c-di-GMP with the IBDV vaccine on the humoral response of broiler chickens.

After vaccination on day 14, the antibody levels against IBDV decreased from days 7 to 35, and thus no significant response of birds to this vaccine was observed. It is possible that maternal IBDV antibodies, which were high on day 14, may have played a role in the neutralizing antigen, thereby limiting antibody titers (Berg, 2008). However, on day 35, birds receiving c-di-GMP (10 or 100 nmol) orally (gavage) or IM apparently had higher IBDV antibodies titers (Figure 2.2A) than the control groups, although this was not statistically significant. Thus I plan to evaluate the effect of a higher dose than 100 nmol of c-di-GMP since, in mice, significant increases in titers of IgG1, IgG2a, IgG2b and IgG3 were observed compared to the control group in immunization studies using a dose of 200 nmol of c-di-GMP (Karaolis et al., 2007a; Hu et al., 2009). It would also be interesting to evaluate the effect of c-di-GMP on the cellular immunity of broilers since some cell-mediated immune-related cytokine could play

crucial roles in driving cellular immune responses during IBDV infection (Liu et al., 2010b). Before placement, the birds were initially vaccinated on day 0 against bronchitis at the hatchery. However, I observed low antibody titers on day 21 after administration of c-di-GMP in all treatment groups (Figure 2.2). On day 28 (except for birds treated with 100 nmol c-di-GMP) and day 35, apparent increased antibody titers (not statistically significant) were observed in comparison to control groups. Birds receiving 100 nmol c-di-GMP orally showed the highest antibody titers on day 35. The antibody titers against Newcastle virus also decreased when the birds aged ($P < 0.05$). On day 35, birds receiving c-di-GMP both by oral and IM administrations showed higher antibody titers than birds receiving saline (Figure 2.2C). On day 35, birds receiving 100 nmol c-di-GMP orally showed higher serum titers against Reovirus (figure 2.2D). Although this study did not find significant humoral response effects associated with two different doses of c-di-GMP on antivirus antibody levels in broilers, future work on the beneficial effects of this molecule in broilers using larger bird numbers in field, and evaluations at higher dosages, such as 200 nmol, could lead to the development of new vaccination strategies against major poultry pathogenic viruses such IBDV.

2.4 Conclusion

In this study my results showed no clear linear (dose dependence increase or decrease) effects on the humoral response in broilers. One of the explanations for this lack of clear effects could be due to the use of low doses of c-di-GMP. However, the observed increases in serum IgA at day 35 in birds orally administered with c-di-GMP suggest potential mucosal immune-stimulatory effects. In general the immunostimulatory effects of c-di-GMP were shortcoming in some aspects and optimal utilization of this molecule remains to be defined. Future work

evaluating increased concentrations of c-di-GMP administration or ovo inoculation at the embryonic stage may promote improved humoral antibody responses, potentially reducing the morbidity and mortality in chickens.

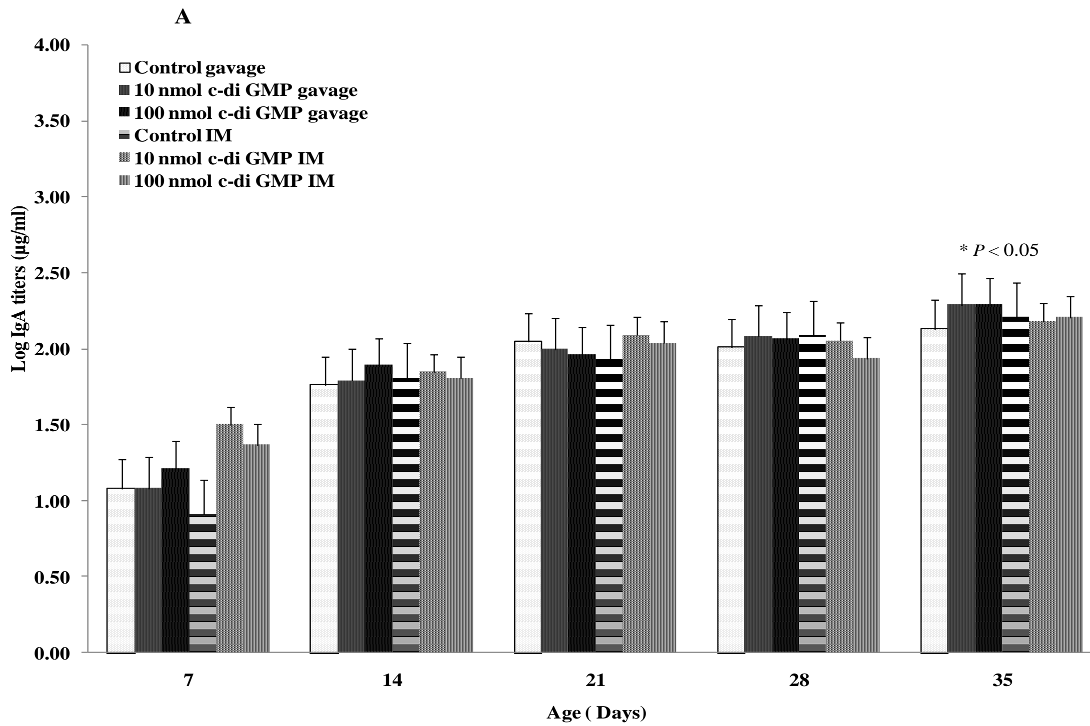
Table: 2.1 Composition of the diet that was used in the study

Ingredients	% Inclusion in Diet		
	Starter	**Grower**	**Finisher**
Wheat	34.96	35.03	40.79
Soya	23	0	1.54
Barley	10	0	0
Canola	11	24	18
Canola Oil	5.6	5	5
Corn	8	25	26
Corn Gluten	2.3	6	4
Limestone	1.6	1.3	1.2
Dicalcium phosphate[1]	1.6	1.5	1.4
Vitamin-mineral mix[2]	1.0	1.0	1.0
Lysine	0.4	0.71	0.6
Iodized Salt	0.31	0.32	0.32

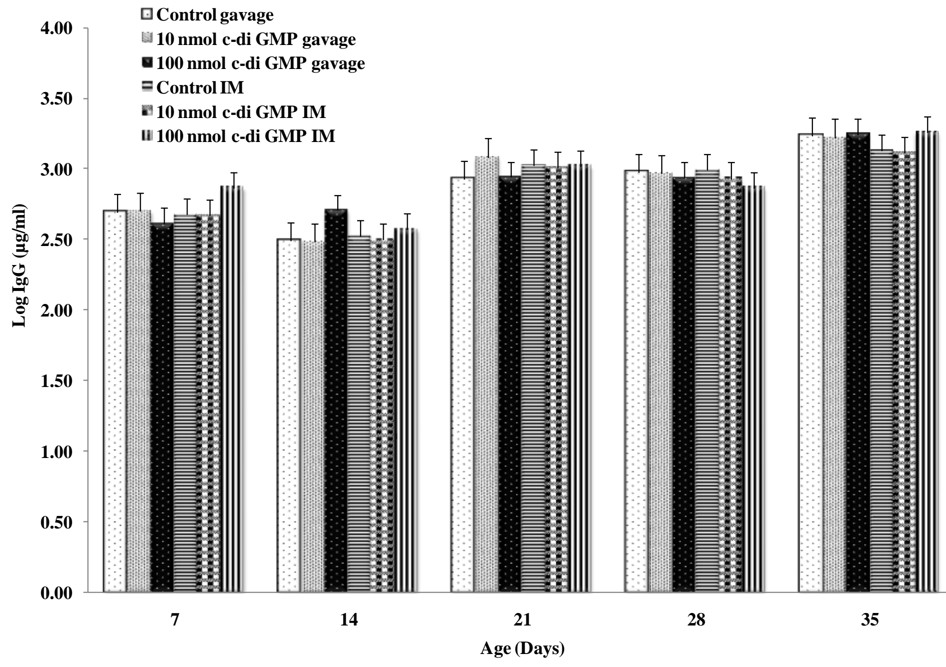
Ingredients	% Inclusion in Diet		
	Starter	**Grower**	**Finisher**
Methionine	0.18	0.09	0.1
Avizyme[3]	0.05	0.05	0.05
Total	100	100	100

[1]A mixture of mono- and dicalcium phosphate containing 18% calcium and 21% phosphate.
[2]Supplied per kilogram of diet: vitamin A, 9,000 IU; cholecalciferol, 1,500 IU; vitamin E, 10 IU; vitamin K, 0.5 mg; vitamin B12, 0.007 mg; thiamine, 0.4 mg; riboflavin, 6 mg; folic acid, 1 mg; biotin, 0.15 mg; niacin, 135 mg; pyridoxine, 4 mg; choline chloride, 1,000 mg; dl-methionine, 1,184 mg; ethoxyquin, 125 mg; NaCl, 2 g; manganese sulfate, 60 mg; copper sulfate, 5 mg; selenium (sodium selenium), 0.1 mg; iodine, 0.35 mg; and zinc sulfate, 50 mg.
[3]Multi-Enzyme System for Wheat-Based Poultry Feed (Halchemix Canada Inc., Toronto, ON, Canada) containing 5,000 U/g of xylanase and 1,600 U/g of protease

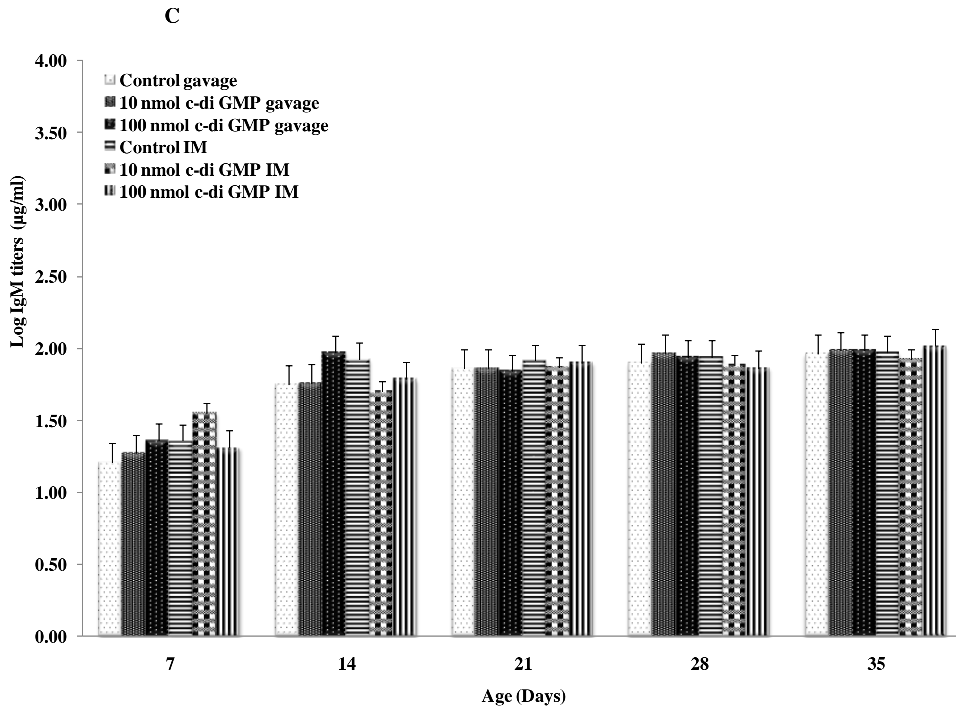
Figure: 2.1: General antibody profiles in a broiler immunization model with IBDV vaccine on day 14 using 100 nmol or 10 nmol c-di GMP administrated orally (gavage) or intramuscularly (IM). Total IgA (2.1A), IgG (2.1 B) and IgM (2.1 C) titers in chicken serum.



B

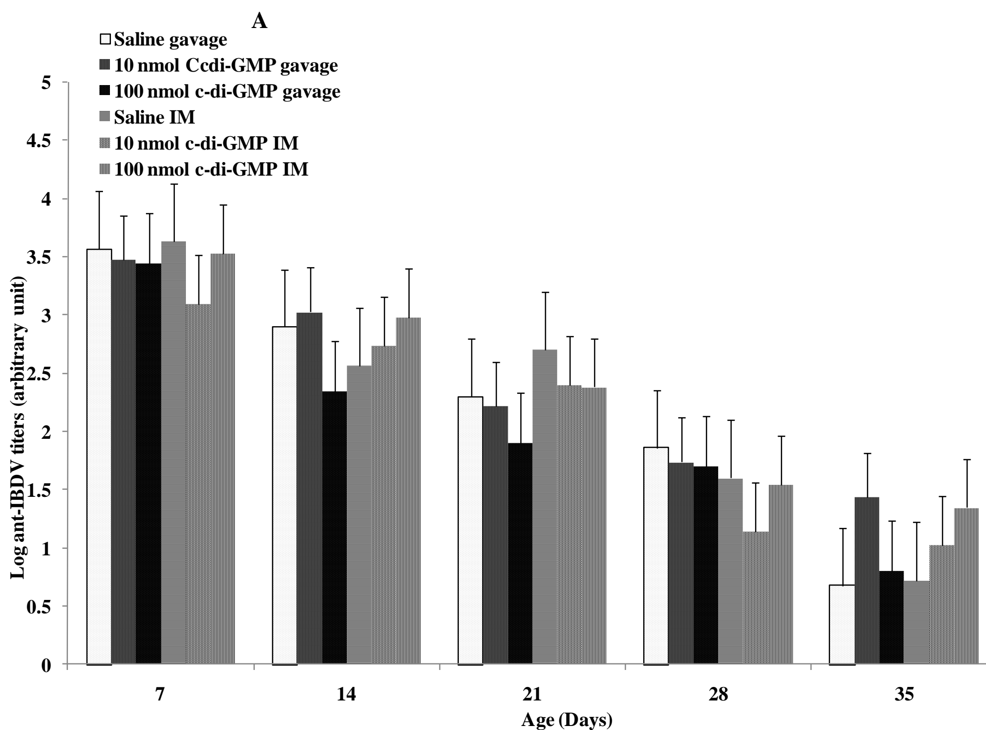


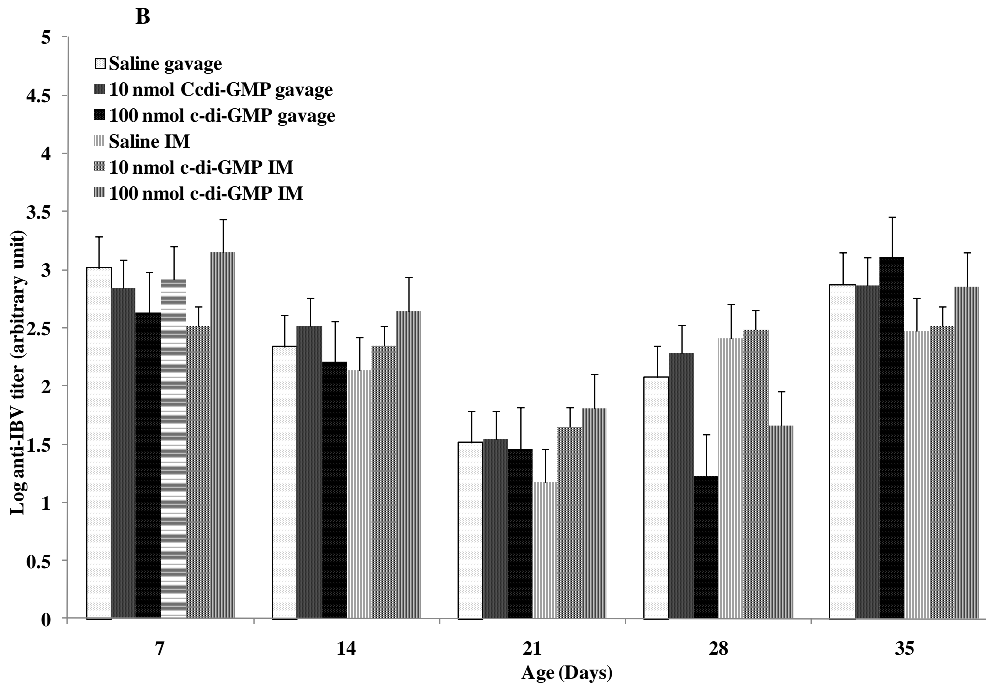
C

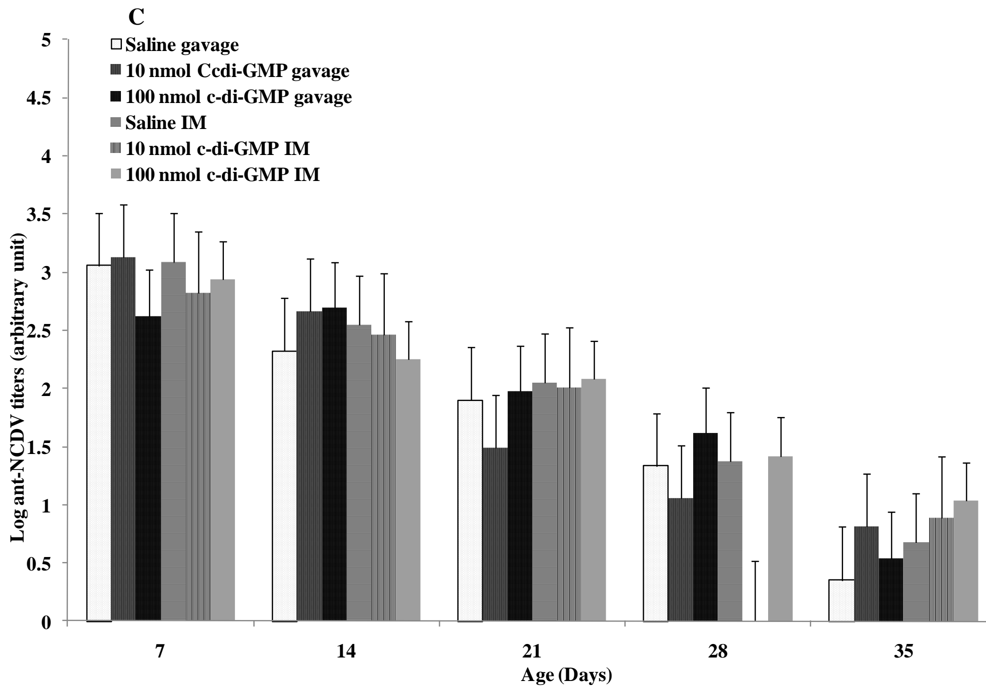


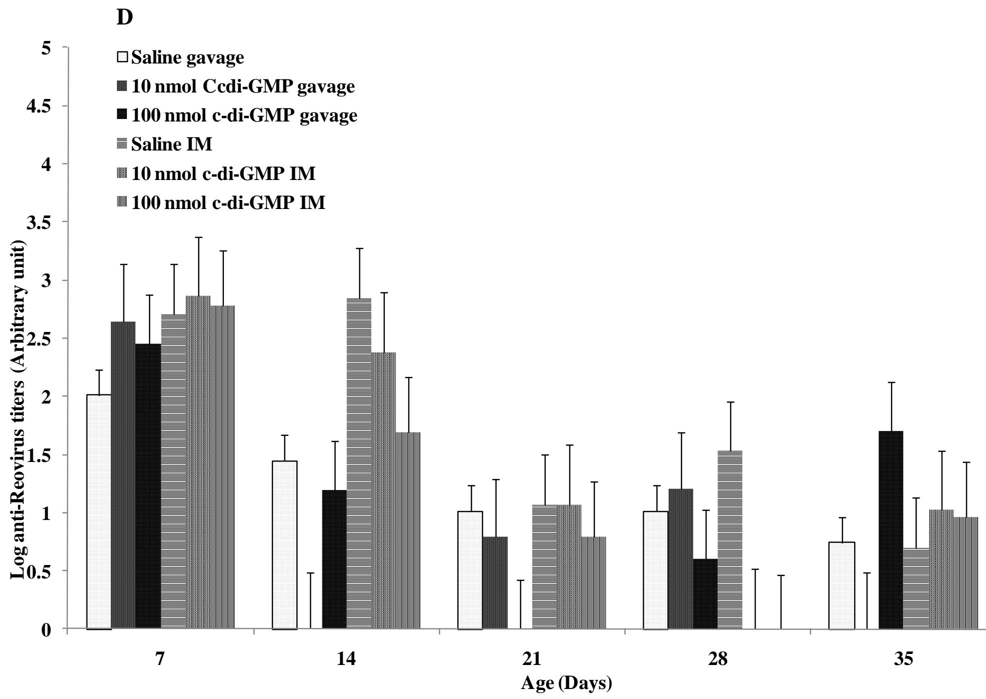
***Significant differences between treatments were observed in the IgA titers at the day 35 (P< 0.05). Data represent means ± SEM of 4 replicates / treatment (n=4 pens; 2chickens/pen at sampling day) arranged in a completely randomized block design**

Figure: 2.2: Antivirus antibody titers in serum after broiler immunization with IBDV vaccine on day 14 using 100 or 10 nmole C-di-GMP administered orally (gavage) or intramuscularly (IM) (Figure 2.2 A) titers of anti-IBDV, (Figure 2.2 B) titers of anti-Infectious Bronchitis Virus, (Figure 2.2 C) titers of anti-Newcastle Disease Virus and (Figure 2.2 D) anti- ReoVirus .




B
Saline gavage
10 nmol Ccdi-GMP gavage
100 nmol c-di-GMP gavage
Saline IM
10 nmol c-di-GMP IM
100 nmol c-di-GMP IM
Log anti-IBV titer (arbitrary unit)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
7
14
21
28
35
Age (Days)


C
Saline gavage
10 nmol Ccdi-GMP gavage
100 nmol c-di-GMP gavage
Saline IM
10 nmol c-di-GMP IM
100 nmol c-di-GMP IM
Log ant-NCDV titers (arbitrary unit)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
7
14
21
28
35
Age (Days)



Data represents mean ± SEM of 4 replicates/ treatment (n=4 pens; 2 Chickens/ pen at sampling day) arranged in a completely randomized block design.

Chapter 3: Effect of 3´, 5´-Cyclic Diguanylic Acid in a Broiler *Clostridium Perfringens* Infection Model

3.1 Introduction

Worldwide enteric diseases in poultry lead to enormous economic losses annually. Correspondingly, efficient control of these diseases may potentially save poultry producers millions of dollars. An enteric pathogen of particular concern in poultry is *Clostridium perfringens* Type A, the causative agent of necrotic enteritis (NE**,** characterized by necrotic lesions in the small intestine). This ubiquitous pathogen is a low G+C anaerobic spore-forming bacterium naturally found in soil and sewage and in the normal bacterial flora of the gastrointestinal tract (GIT) of many animals and humans (Collier et al., 2003).

Normally, many avian species have less than 10^4 colony forming units (CFU) of non-pathogenic *C. perfringens* in their normal gut flora. However, increase in *C. perfringens* counts in the GIT due to any predisposing factors supporting their propagation would result in the development of NE (McDevitt et al., 2006). Indeed, massive colonization (> 10^4 CFU) of *C. perfringens* in the gut could lead to acute clinical conditions resulting in mortality rates from 10 to 40% in the affected flock (McDevitt et al., 2006). Moreover, subclinical infections could lead to production losses associated with reduced weight gain and increased feed conversion ratio**.** Intestinal damage caused by NE gives bacteria access to the bile duct and blood stream, consequently damaging the birds' other organs (Timbermont et al., 2011). Due to the zoonotic character, poultry meat contaminated with *C. perfringens* type A, poultry meat contaminated

with is pathogen can therefore be a source of food poisoning in humans, causing a major public health concern (Brynestad and Granum, 2002; Van Immerseel et al., 2004).

Antimicrobial compounds including penicillin are used to treat NE caused by *C. perfringens*. Although treatment may be effective, a more proactive practice focusing on NE prevention would be the use of ionophores (salinomycin) or other antimicrobial agents like avilamycin, virginiamycin, bacitracin and avoparcin to effectively minimize the growth of *C. perfringens* (Kocher, 2003). Moreover, the use of such agents also minimizes the rate of mortality and morbidity due to subclinical and clinical diseases, and is of obvious economic benefit to producers (Van Immerseel et al., 2009). The use of antimicrobials can result in the development, maintenance, and potential dissemination of antimicrobial resistance (AMR) genes in food-producing animals (Butaye, 2003; Diarra et al., 2007b). In addition, antimicrobial residues and resistant bacteria may find their way into the environment from fecal materials and litter (Furtula et al. 2010; Merchant et al., 2012). Increasing public health concerns due to spread of AMR and the concomitant reduction in therapeutic efficacy and potential for treatment failures have led to the ban of antimicrobial growth promoters (AGPs) in European countries (Graham et al., 2007). These authors reported that due to minor economic benefit, the use of growth promoting antibiotics in poultry production should be reconsidered.

Correspondingly, in an effort to improve interventions for controlling NE in poultry, I examined the effect of 3’, 5’-Cyclic diguanylic acid (c-di-GMP), an immuno-stimulatory intracellular bacterial signalling molecule, against *C. perfringens* in broiler chickens. It has been shown that exogenous c-di-GMP treatment significantly reduced *Staphylococcus aureus* infections in an *in vitro* model as well as in mice (Karaolis et al., 2007a). In addition, c-di-GMP

treatment prior to *Klebsiella pneumoniae* challenge significantly reduced bacterial pneumonia and stimulated innate immunity response in a mouse model (Karaolis et al., 2007b). In a previous study, the study showed a significant increase in immunoglobulin A (IgA) concentration in the serum of treated birds when compared to control birds, confirming c-di-GMP's role as a mucosal adjuvant (Fatima et al., 2011).

The objective of my present study is to evaluate the *in vivo* effects of c-di-GMP against *C. perfringens* type A in a broiler chicken challenge model. I hypothesized that the universal bacterial second messenger molecules c-di-GMP when administrated with penicillin G could increase *C. perfringens* clearance in chicken gut.

3.2 Materials and Methods

3.2.1 Chicken Housing

A total of 108 male day-old Ross 308 broiler chicks (Western Hatchery Abbotsford, BC) were randomly placed in 18 cages (6-chicks/cage) after visual inspection for health to remove inferior chicks from the trial. Each cage was equipped with a drinker and a feeder providing feed and water *ad libitum.* Heat was provided through gas-fired brooders and airflow was provided by negative pressure. The temperature was initially set at 32°C and was then gradually reduced by 1.7°C each week to reach 23°C at 35 days of age. Chicks were exposed to light for 24 h on the 1st day, 23 h on the 2nd and 3rd day, and 18 h thereafter (Leusink et al., 2010). The composition of the diets used in the study was similar to my previous study (Fatima et al., 2011). Briefly, the starter, grower and finisher diets were formulated with wheat, barley and corn as the principal cereals and soybean and canola meals as protein concentrates to meet the National Research Council nutrient requirements for broiler chickens (NRC, 1994). All experimental procedures

performed in this study were approved by the Animal Care Committee of the Pacific Agri-Food Research Center (Agassiz, BC, Canada) according to guidelines described by the Canadian Council on Animal Care (CCAC, 1993).

3.2.2 Experimental Challenge and Treatment

Four *C. perfringens* type A strains (ABB004-1534, ABB008-3964, ABB009-3791 and ABB007-3648) isolated from NE outbreak cases, were used in the study. These strains were initially stored in thioglycolate supplemented with 15% glycerol at -80°C until used. The challenge doses were prepared fresh on each challenge day by cultivation into freshly pre-reduced cooked meat broth (CMB, Oxoid, Nepean, ON, Canada) anaerobically for 18 to 24 h at 37°C. Cells were then harvested from the supernatant by centrifugation (4500 x g at 4°C for 10 min) and the pellets were re-suspended in 10 volumes of physiological saline to have approximately 8-9 Log_{10} cfu/ml (Siragusa et al., 2008). The doses were verified by platting onto Brucella Agar supplemented with hemin and vitamin K and incubating overnight anaerobically at 37°C. All chicks were inoculated in the crop by gavage on days 14, 15, and 16 with 9 log_{10} CFU of a mixture of the four *C. perfringens* strains. At 24 days of age, they were then randomly divided into three treatment groups: 1) control untreated, 2) gavage with 20 nmol c-di-GMP and 3) intramuscularly injected (IM) with 20 nmol c-di-GMP. On the same day, all three groups received penicillin G (300 000 UI/l) in drinking water for five days (until day 28) to measure synergistic effects of c-di-GMP and penicillin against colonization by *C. perfringens*.

3.2.3 Sample Collection and Microbial Counts

On days 21, 28 and 35, two birds from each cage (12/treatment) were euthanized by cervical dislocation, and the ceca and ileum were removed. Samples (pooled samples from 2 birds of the

same pen constituting 1 sample) were then 10-fold diluted for bacteriological analysis. A total of 108 samples (54 intestines (ileum) 18/sampling day: 6/treatment and 54 ceca - 18/sampling day: 6/treatment) were analyzed. *C. perfringens* was enumerated according to Knarreborg et al. (2002). Briefly, samples were spread on tryptose sulfite agar (Oxoid) supplemented with cycloserine (SR088E, Oxoid) and incubated anaerobically for 24 h at 37°C. *Lactobacillus* spp. populations were quantified using Lactobacilli MRS Agar (Oxoid) according to the manufacturer's methods.

At 21, 28, and 35 days of age, the intestine of sacrificed birds (2 per pen) were examined for evidence of NE. The intestines were longitudinally opened and intestinal mucosa were scored on a scale of 0 to 3 for NE lesions for the upper gut, mid gut, lower gut, and ceca according to the method of Collier et al. (2003).

3.2.4 Detection of Virulence and Antibiotic Resistance Genes

The presence of alpha-toxin (*cpa*), beta-toxin (*cpb*), epsilon-toxin (*etx*), iota-toxin (*iA*), enterotoxin (*cpe*) and beta2-toxin (*cpb2*) was investigated by PCR in tested *C. perfringens* strains and subsequently in extracted cecal DNA from challenged birds at day 21, 28 and 35 of age using specific primers (Das et al., 2008). PCR amplifications were performed using AccuStart Taq PCR Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and 0.5 µM concentration of each primer. PCR reactions involved 30 cycles of denaturation (94°C for 1 min.), annealing (55°C for 1 min.), and extension (72°C for 1 min). The β-lactamase $bla_{\text{CMY-2}}$, bla_{SHV} and bla_{TEM} genes were screened using primers as previously described (Merchant et al., 2012). PCR products were run on a 1.5% Tris-acetate-EDTA buffer (TAE) agarose electrophoresis gel

stained with ethidium bromide (1 μl/10 ml) and amplicon sizes were referenced to a 1 kbp gene ruler (Fermentas, Burlington, Canada).

3.2.5 Culture Independent Method for Analysis of the Cecal Microflora

At each sampling day (21, 28, 35), six ceca samples from each treatment group (c-di GMP gavage, c-di-GMP IM and control) were pooled for the microbiota analysis. DNA of these nine cecal samples was thus extracted using QIAamp® DNA Stool Mini Kit (Qiagen, Mississauga, ON, Canada) and stored at -80°C until used. Microbial flora variation in these samples was determined by the amplified fragment length polymorphism (AFLP). Extracted ceca DNA was first amplified using FAM labelled 16S rRNA gene primer (Forward -5´CCT ACG GGA GGC AGC AG 3´) (Reverse- 5´ CCG TCA ATT CCT TTG AGT TT 3´) by PCR and sent to the University of British Columbia Nucleic Acid Protein Service Unit (Vancouver, BC, Canada) to perform the AFLP test. The images were analyzed using Pearson's product-moment correlation coefficient to construct dendrograms (BioNumerics Analysis software version 5.10, Applied Maths, Ghent, Belgium).

3.2.6 Statistical Analysis

Bacterial counts were log transformed and analyzed according to a randomized complete block design using the repeated statement of SAS (Statistical Analysis System, 2000) considering the cage as the experimental unit. The association test of Cochran-Mantel-Haenszel was used to determine the relationship between the frequency of screened genes and the treatment using the FREQ procedures. The least significance difference was used to separate treatment means whenever the F value was significant. The P-value (0.05) was used to determine significance.

3.3 Results and Discussion

Partial control of NE can be achieved by using growth-promoting antibiotics that alter the microbial composition of the GIT and increase availability of nutrients to the host (Butaye et al., 2003; Van Immerseel et al., 2004). However, beside their beneficial effects, it seems possible that antibiotics may have a negative correlation with the health and well-being of the animal (Brisbin et al., 2008). The impact of antimicrobials used in chicken immunity is poorly understood and the uses of agents that enhance the immunological responses to infection need to be developed for chicken production. The immune stimulatory effects of c-di-GMP against infections have previously been reported (Karaolis et al., 2007a). Indeed, treatment with c-di-GMP in mouse infection models have shown significant reductions in *K. pneumoniae*, *S. aureus* and *Streptococcus pneumoniae* counts, resulting in increased survival and protective effects (Karaolis et al., 2007ab; Hu et al., 2009). Furthermore, the intranasal administration of antigen with c-di GMP significantly stimulated humoral and cellular immune responses at systemic and mucosal levels, showing as mentioned above, the beneficial effect of c-di-GMP (Ebensen et al., 2007b).

3.3.1 Clostridium Challenge Model

The present study investigated the effect of c-di GMP against *C. perfringens* in a broiler chicken challenge model. Based on gross observations from day 0 to 35, the health of the birds treated with c-di-GMP in the trial was good, with no mortalities due to the *Clostridium* challenge observed. However, a single mortality was observed in a cage of control birds due to undetermined physiological conditions. Despite administration of penicillin G in drinking water, some c-di-GMP untreated (control) birds also showed some sickness symptoms such as reduced

weight gain, ruffled feathers and movement difficulties (data not shown). Intestinal mucosae examinations were initiated after challenge at 21 days of age. Despite administration of high doses of *C. perfringens*, the birds did not develop obvious coccidiosis (no *Eimeria* used in this model) or intestinal NE lesions during the study period, confirming that the *C. perfringens* infection model used was not a NE model as previously reported (Siragusa et al., 2008). In birds examined on days 21, 28 and 35, small foci of necrosis of about 1 mm in diameter (lesions scoring 1 in the upper intestine were observed in a few birds. Several factors may have been associated with the inability to induce experimental NE using this model, with the most likely being linked to the virulence characteristics of *C. perfringens* strains used in this challenge (Lee et al., 2011).

The production of alpha-toxin is thought to play a major role in the virulence of *C. perfringens*. In the present study, the alpha-toxin gene (*cpa)* was detected in all four *C. perfringens* strains used for challenge while *cpb2* gene was additionally detected in only one strain (ABB007-3648). None of the other screened genes (*etx, iA. cpe, cpb)* were detected in challenge strains. Furthermore, screening DNA extracted from cecal samples by PCR amplification revealed the presence of the *cpa* gene in 13.9% and 44.4% of samples on days 21 and 35, respectively. This gene was detected in 30% of the cecal samples from birds treated with intramuscularly administration of c-di-GMP at both 21 and 35 days while on the same days the prevalence of *cpa* increased from 10 to 60% in control birds and those receiving c-di-GMP by gavage (Figure 3.1). The *cpb2* gene was detected in one of the challenge *C. perfringens* isolates; however, only one cecal sample from c-di-GMP intramuscularly administrated birds was positive for this gene. Detection of these genes was not correlated with NE in the experimental birds

suggesting that other factors could be involved in the induction of disease. Conditions that promote excessive growth of *C. perfringens* in the chicken intestine and lead to toxin production causing mucosal lesions are less understood. It has been reported that the alpha-toxin may not be required for the development of NE as birds challenged with *C. perfringens* strains lacking this toxin were shown to develop NE (VanImmerseel et al., 2009; Lee et al., 2011; Timbermont et al., 2011). The mechanism behind the negative effect of c-di-GMP on bacterial virulence factors seems to be complex; however, the importance of this molecule in bacterial regulation of virulence factors including motility and toxin production has been shown (Bordeleau et al., 2011; Srivastava et al., 2011). Thus, the apparent effect of c-di-GMP administrated intramuscularly on the prevalence of *Clostridium* alpha-toxin observed in the present study requires additional investigation.

3.3.2 Bacterial Enumeration

For the treatment of *C. perfringens* in broiler production, penicillin has been used, but few studies actually report the efficacy of this clinically important antibiotic (Gadbois et al., 2008). Furthermore, the current global concern over the use of important antibiotics such as penicillin has already resulted in the loss of many very useful products due to the development of resistance with no new replacements on the horizon. In the present study, it was hypothesized that the universal bacterial second messenger molecules c-di-GMP when administrated with penicillin G could increase *C. perfringens* clearance in chicken gut. Detection of bacterial diversity in broiler chickens by 16S rRNA gene sequencing revealed that nearly 70% of ileum sequences represent *Lactobacillus* spp., while the ceca population was abundant in *Clostridiaceae*-related sequences (Lu et al., 2003). In the present study, at day 21 (i.e. four days

after challenge) and before the administration of c-di-GMP, *Clostridium* numbers were higher in the ceca than in the ileum by about 2-Log, confirming the ceca as the ecological niche for this bacterium. Administration of c-di-GMP on day 24 followed by penicillin G treatment led to significant ($P < 0.05$) decrease in *Clostridium* numbers in both the ileum and ceca as observed on day 28. On the same sampling day, ceca from birds treated with 20 nmol c-di-GMP by gavage as well as both ceca and ileum from birds treated with 20 nmol c-di-GMP IM showed the lowest *C. perfringens* number per gram of sample, but no significant treatment effects ($P > 0.05$) were noted. However, on day 35 of age, IM administration of 20 nmol of c-di-GMP significantly ($P < 0.05$) reduced the *C. perfringens* numbers in the ceca (Figure 3.2A) suggesting a possible synergistic activity between penicillin G and c-di-GMP against *C. perfringens* in broiler ceca. Similarly to that used in the present study, a *C. perfringens* colonization model similar in two-week old chickens showed that administration of lupulone, a hop plant (*Humulus lupulus*) bitter acid, decreased intestinal levels of inoculated pathogenic clostridia (Siragusa et al., 2008). The decrease of *C. perfringens* number in the ceca could lead to the decrease of *C. perfringens* translocation to the upper intestine. The observed decrease of *Clostridium* numbers also correlated with the decrease of the *cpa* gene prevalence mentioned above. Four strains were used (all from NE cases) to simulate the natural *C. perfringens* population in the gut. Since *C. perfringens* counts were positively associated with the severity of NE-specific lesions (Srivastava et al., 2011), the findings indicate that strategies using c-di-GMP combined with penicillin G could be developed to decrease *C. perfringens* number in broiler gut.

Lactobacillus spp. are normal inhabitants of the gastrointestinal tract of the chicken, and some strains of *Lactobacillus* spp. are used as probiotic bacteria due to their health benefits

which include possible immune stimulation (Peña et al., 2005; Haghighi et al., 2006). Since some antimicrobial agents were shown to be associated with the reduction of *Lactobacillus* spp. frequency in broiler gut (Torok et al., 2011), I investigated the effect of c-di-GMP treatments on *Lactobacillus* numbers. Regardless of the treatments applied, *Lactobacillus* numbers were higher in the ceca than in the intestine ($P < 0.05$) at all sampling days (Figure 3.2B). Data from this study suggest that administration of 20 nmol of c-di GMP does not wipe out the natural microbial flora of the chicken, which is common during antibiotic treatments. Whether this high *Lactobacillus* population in ceca translates to the reduced colonization by pathogenic bacteria was not investigated.

3.3.3 Antibiotic Resistance Genes

The application of culture-independent approaches, such as PCR and DNA-microarray to study antibiotic resistance has uncovered a vast diversity of antibiotic resistance genes in bacteria. Using DNA microarrays, several antibiotic resistance genes have been described, including the extended-spectrum β-lactamases (ESBL) $bla_{\text{CMY-2}}$, bla_{SHV} and bla_{TEM}. These genes confer resistance to β-lactam antibiotics such as ceftiofur, ceftriaxone and ampicillin in Gram negative bacteria isolated from broiler gut samples (Bonnet et al., 2009). Since penicillin G was used in this study, I developed a PCR method to detect $bla_{\text{CMY-2}}$, bla_{SHV} and bla_{TEM} genes directly from gut DNA extracts. From day 21 to 35, no significant treatment effect was detected for the prevalence of the respective screened *bla* genes in 54 ceca samples. The $bla_{\text{CMY-2}}$ gene was detected in all screened cecal samples from all treatment groups except in birds from one pen of the control group at day 35. On the same day of age, the bla_{TEM} gene was detected in only one pen of the treatment group receiving c-di-GMP IM whereas bla_{SHV} was found in a pen of the

control group that was also negative to *bla*$_{CMY-2}$. The sources of these ESBL (*bla*$_{CMY-2}$, *bla*$_{SHV}$ and *bla*$_{TEM}$) genes, which were known to be carried by Gram negative bacteria such as *Salmonella* spp. and *E. coli* (Diarrassouba et al., 2007; Merchant et al., 2012), were not determined in this study. However, this study confirmed the presence of such genes in the ceca of broiler chickens independent from the used β-lactam antibiotics. The prevalence of such genes is of concern because they can inactivate extended-spectrum cephalosporins, such as ceftriaxone, that are commonly used in the treatment of invasive *Salmonella* infections. Furthermore, resistance genes to other antibiotics such as tetracycline and amikacin can be co-located on ESBL clarifying plasmids (Hamilton et al., 2012). My findings suggest that additional research is needed in order to determine the origins of ESBL genes in broiler and to evaluate the *in vivo* effect of c-di-GMP on the overall diversity of antibiotic resistance machinery in the poultry gut microbiome.

3.3.4 Gut Microflora

The identity of approximately 90% of bacteria in the chicken gastrointestinal tract is unknown (Apajalahti et al., 2004) and previous research studies have been conducted to evaluate the potential effects of alternative feeding practices on the dynamic of some of the chicken gut microflora members (Leusink et al., 2010). Additional work needs to be done for establishment of the nature of shifts in the chicken gut microflora in response to different feed regimes including new alternative approaches that are being used. Recent developments in molecular microbiology and computation analysis can generate information about bacterial population genetics and species evolution that has not been accessible in the past (Hamady et al., 2010). In this study, I used AFLP to gain insight on the dynamic of the cecal microfloral shift from days

21 to 35 following a *C. perfringens* challenge and c-di-GMP administration (Figure 3.3). The generated dendrograms from nine cecal samples (1 pooled sample from each treatment/sampling day) showed that at 70% similarity, cecal microflora from 35-day old birds gavaged with c-di-GMP clustered distinctly from the cecal microflora of other group. At 80% similarity, microflora of the 21 day-old birds treated IM with c-di-GMP also were distinct. The remaining microfloral clustered together or alone at varying degrees of similarity. For example, microflora from 28-day old birds treated with ci-di-GMP by gavage or IM were similar at 96%. Predominantly, results showed 92% similarity between 21-day old control birds' ceca and 35-day old IM treated c-di-GMP. This indicates that c-di-GMP IM treatment might be effective in restoring or maintaining a normal host microflora following *C. perfringens* challenge. Limited information exists about the broiler chicken gut microflora after a *C. perfringens* infection. However, suppression of some *Lactobacillus* species had been reported after *C. perfringens* infection (Feng et al., 2010). It would be interesting to identify the specific bacterial population present in the cecal samples to estimate the shift of microbial composition induced by my treatments.

3.4 Conclusion

In conclusion, it appears that c-di-GMP can modulate *C. perfringens* colonization in the host ceca without altering the normal microbiota. Additionally, c-di-GMP did not alter the commensal bacterial community of the intestine, which is known to stimulate the host immune response, and did not act as an antimicrobial drug in selecting resistant genes such as ESBL genes. It will be interesting to further study variations in the bacterial community in response to c-di-GMP, and the incidence, movement, spread and persistence of bacterial strains representing major threats to humans, birds and the environment.

Figure: 3.1: Prevalence of the *C. perfringens* alpha toxin gene (*cpa)* in the cecal DNA samples at various sampling days (21, 28 and 35) for the three treatment groups (c-di GMP gavage treated, c-di-GMP IM treated and control). Statistical differences in the *cpa* frequency were observed at day 21 and 35 (*P* < 0.05).

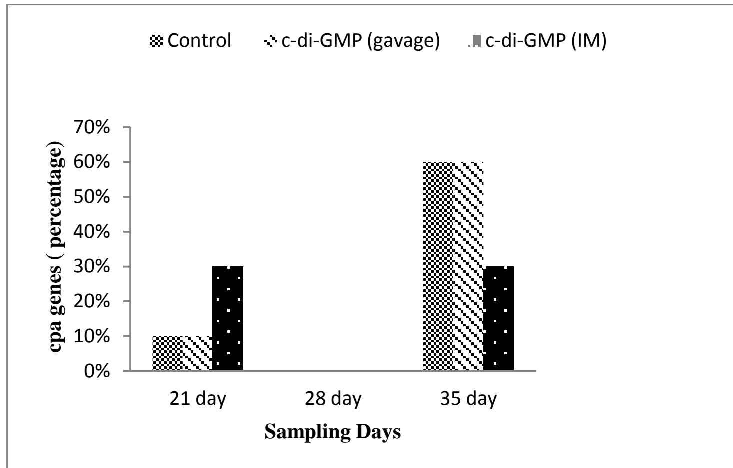
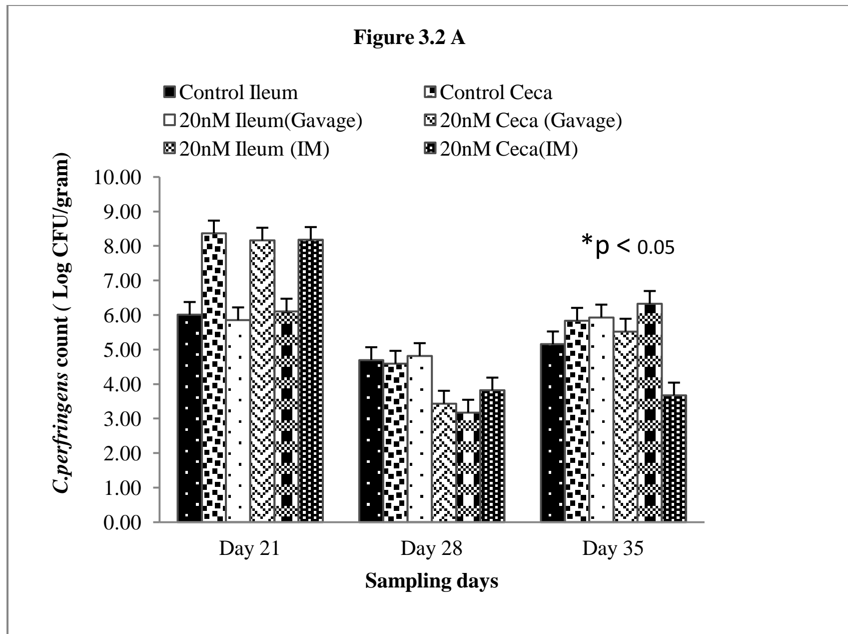
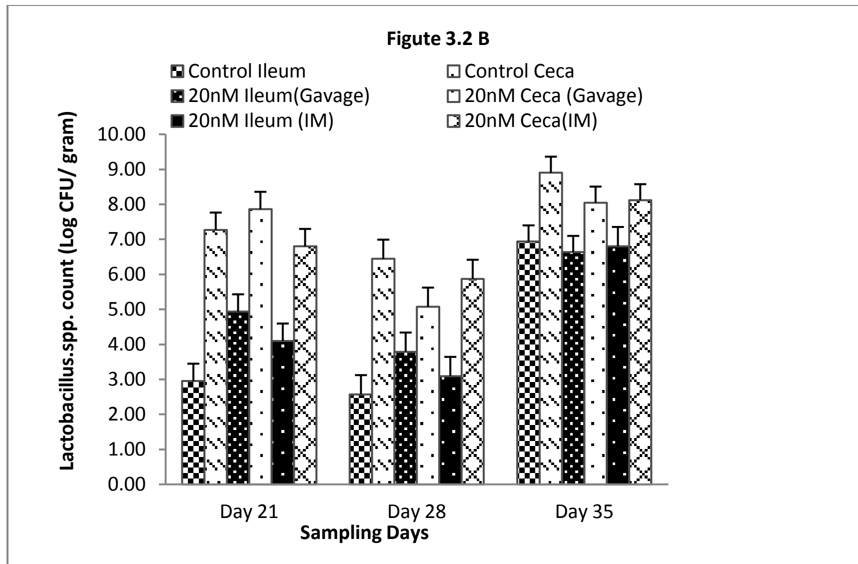


Figure: 3.2: Mean log cfu of *C. perfringens* (A) or Lactobacillus spp (B) per gram of ceca and ileum contents at various sampling days for three treatment groups (c-di GMP gavage treated, c-di-GMP IM treated and control.

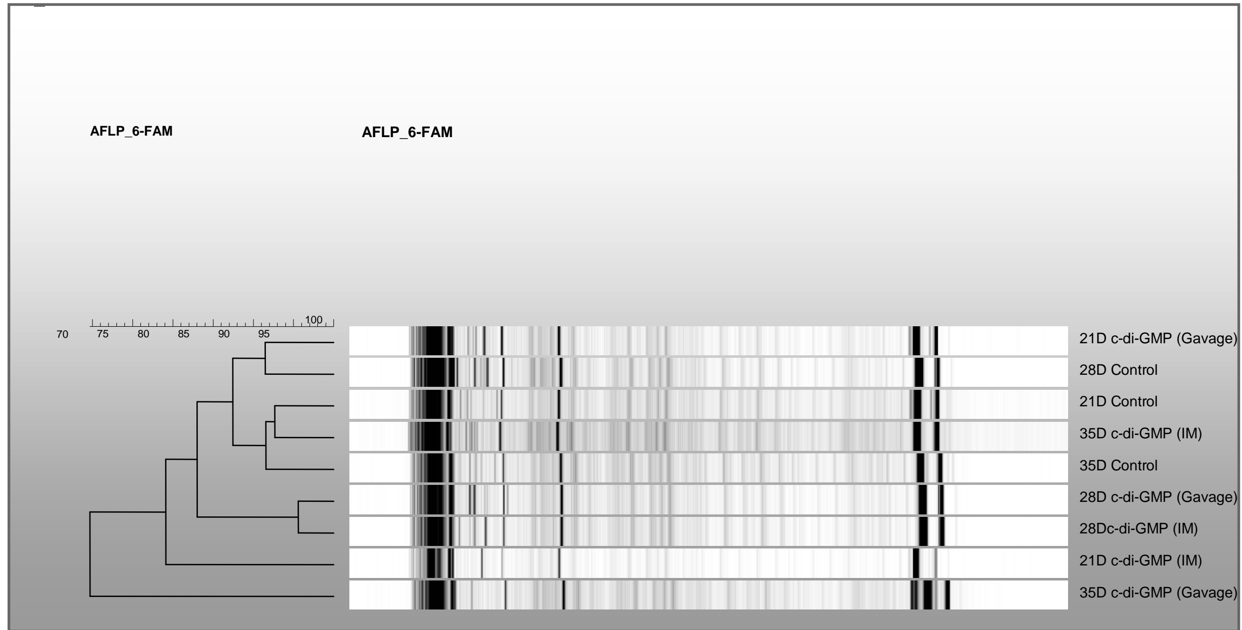


* Values are statistically different ($P < 0.05$).



At each sampling day, the concentrations of *Lactobacillus* were higher in the cecal samples than in the ileum samples ($P < 0.05$).

Figure: 3.3: Dendrogram of AFLP DNA fingerprinting showing the microbial population of ceca on sampling days 21, 28 and 35 for the three treatment groups (i.e., control, and c-di- GMP administered by gavage or IM).



Chapter 4: Conclusion and Future Research Prospects

Despite the tremendous benefits of AGPs in feed, concerns have been raised over time that use of AGPs in feed is the contributing factor of the increasing problem of AMR in human medicine. Therefore WHO has recommended restricted use of AGPs and replaced with alternatives. Consequently, this study was to evaluate the role of c-di-GMP as an alternative to antibiotic in broiler chickens. In this thesis the effect of c-di-GMP in chickens was studied for the first time, first as a vaccine adjuvant in combination of IBDV vaccine and secondly, in the prevention and control of NE. Both diseases are of persistent problems of economic significance for broiler producers.

The first study (Chapter 2) results of c-di-GMP as a vaccine adjuvant generally show no clear evidence of stimulation of humoral immunity. However, significant increases in serum IgA levels on the day 35, last day of sampling, orally administered, represents potential promise as a mucosal immune enhancer. To confirm the role of c-di-GMP as a mucosal immune enhancer in broiler chickens, an in depth evaluation of immuno-competent cells such as CD4/CD8, Th1/ Th2 profile, DCs, macrophages, maybe required. Moreover, increased doses of c-di-GMP could have resulted in significantly enhanced mucosal immune system.

In the second study (Chapter 3), my candidate c-di-GMP in combination with penicillin G significantly reduced the colonization of *C. perfringens* in ceca on day 35 when compared to controls following IM administration. This represented that c-di-GMP in combination with penicillin G reduced the colonization of *C. perfringens* in the ceca of broiler chicken, challenged by high doses of pathogenic *C. perfringens*. Additionally, c-di-GMP did not alter the microbiome population of the intestine thus the effect might be antipathogenic.

Brouillette et al. (2005) in his study have determined a dose dependent effect on *S. aureus* colonization in a mouse model, results show linear reduction of *S. aureus* colonization with increasing doses of c-di-GMP. Thus dose of c-di-GMP has a critical role in the reduction of pathogen colonization. Thereby, more insight on the effect of c-di-GMP on the chicken gut may be revealed if the effect was evaluated with different doses of c-di-GMP. Additionally, if a study is done using other bacterial species in chicken it could provide additional information. This could provide details whether c-di-GMP could potentially reduce gut colonization from other pathogens like *Salmonella, Campylobacter, E. coli.* In addition to this, specifically in the case of NE, newly discovered toxin NetB evaluation in my study could have given detail analysis of the disease too.

In view of the above experimental results, I can conclude that c-di-GMP has a promise as an antibiotic alternative in broiler production. Further in depth studies with different doses and evaluation of effects of c-di-GMP on different immunocompetent cells may provide insight on the use of c-di-GMP as an alternative to antibiotic growth promoters in animal production.

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