PROTECTION AND STIMULATION OF INTESTINAL INNATE IMMUNITY USING MANNAN OLIGOSACCHARIDES

by

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A Thesis Submitted to the Faculty

of Graduate Studies

The University of Manitoba

In Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Animal Science

University of Manitoba

Winnipeg

ABSTRACT

Brady, Jessica Christine. M.Sc., The University of Manitoba, May 2011. Protection and Stimulation of the Intestinal Innate Immunity Using Mannan oligosaccharides. Major Professor; Juan Carlos Rodriguez-Lecompte.

Necrotic enteritis (NE) caused by *Clostridium perfringen*s is a re-emerging disease of economic importance in areas of the world where antibiotic growth promoters have been banned. Various alternatives to antibiotic growth promoters are being tested including prebiotics known as mannan oligosaccharides (MOS) which have been shown to mitigate the effects of NE and potentially boost the immune system, though the mechanism is not completely understood. The objective of this study is to test the effectiveness of MOS on balance of microbial populations, gut morphology and immune system ability; specifically: *C. perfringens* genetic populations, villi architecture and TLR2 and TLR4 activity.

This study focuses on organic broiler chickens fed MOS at 0, 2, 4, 6 and 8g/kg feed and challenged with an inoculation of *C. perfringens* isolated from an outbreak on a local organic farm. Toxinotype and 16S phylogenetic analysis of *C. perfringens* were reviewed as well as feed efficiency, gut morphology and gene expression of Toll Like Receptors 2 and TLR4 using qRT-PCR.

All field isolates were found to be Type A *C. perfringens*, as were most experimental isolates except for two isolates taken pre-innoculation, which were more likely attributed to contamination of the experiment room by cattle which were housed there previously. No association between pathogenecity and toxin genes cpb2 or netB could be established during this study. 16S analysis found all C. perfringens isolates to be highly related, though there seemed to be a change in populations post inoculation which did match the field isolate used for inoculation. Gut morphology readings including villi height, width and area, crypt depth and lymphocyte and goblet cell concentrations showed some significant effect though it was not in a common area of the intestine and was often due to the interaction between treatments and time. These results also failed to reproduce effects found by other authors. TLR2 and 4 were not found to be significantly different between treatments, though certain trends were noted. The lack of significant treatment effects as well as the low reproducibility of these outcomes leads to the conclusion that, though MOS may contribute to gut health and maturity based on these and conclusions found by other authors, its effect is hinging on other factors such as management and nutrition.

FOREWARD

This thesis is presented in a manuscript style, consisting of a literature review, two manuscripts and general discussion and conclusions. Manuscript 1 – Toxinotyping of necrotic enteritis-producing and commensal isolates of *Clostridium perfringens* from chickens fed organic diets has been published in Avian Pathology December 2010, Volume 39 pages 475-481. The full listing of authors for this publication is as follows: Jessica Brady, Juan D. Hernandez-Doria, Carlyle Bennett, Wilhelm Guenter, James D. House and Juan C. Rodriguez-Lecompte. Manuscript 2 – The effect of prebiotic supplemented organic feed on gut health and immunity in broilers challenged with necrotic enteritis producing isolates of *Clostridium perfringens* will not be submitted as is, though the information found within the text will be submitted as part of a larger publication at a later date.

ACKNOWLEDGMENTS

I would like to start by thanking my supervisor, Dr. Juan Carlos Rodriguez-Lecompte for his hands on support throughout my research. Much of what went into this was a result of his direct input and training, thus he assisted me in all facets of this work and for that I am ever grateful.

I would also like to thank my committee, Dr. James House, Dr. Karmin O and Dr. Keith Fowke for the support and suggestions throughout the process. Each member brought a unique background and perspective to the table which was paramount to the completetion of the final product.

For their technical support and training, I would like to thank Robert Stuski, Harry Muc, Alex Yitbarek, Mingyan Jing, Juan Hernandez, Angela Kroeker, Chris Link-Muirhead and Andre Hamel. As well I would like to thank all the graduate students for the help and support along the way.

A special thanks to my parents, Fran and Gord, as well as to Stewart, all of whom supported me through the best and worst of it and pushed me to follow the path I felt was best even if it included some detours.

Thanks as well to the many people who helped me along the way, I apologize that I was unable to name everyone individually.

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LIST OF ABBREVIATIONS

AGP	Antibiotic Growth Promoter
AMP	Antimicrobial Peptide
CD	Complex of Differentiation
CFU	Colony Forming Units
FDC	Follicular Dendritic Cell
Ig	Immunoglobulin
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MOS	Mannan oligosaccharide
MyD-88	Myeloid Differentiation factor-88
NE	Necrotic Enteritis
NF-κB	Nuclear Factor- κB
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
PRR	Pathogen Recognition Receptor
PAMP	Pathogen Associated Molecular Pattern
sIg	Secretory Immunoglobulin
TLR	Toll-like Receptor

GENERAL INTRODUCTION

The following studies were undertaken with the objective to determine the effect of mannan oligosaccharides on the gut health in organically raised broiler chickens challenged with *Clostridium perfringens* as it pertains to Toll like receptors 2 and 4, gut morphology and *C. perfringens* ecology.

As a major interface between the host and the outside world, the gut is constantly under pressure by antigens and must maintain itself to keep the animal healthy and in peak production (Brisbin et al 2008; Yegani and Korver 2008). To conserve balance in conventional broiler production systems, chickens most often receive feeds which have been supplemented with sub-therapeutic levels of antibiotics called antibiotic growth promoters (AGPs), used to boost animal performance through the depression of non-beneficial microflora, consequently preventing diseases (Bjerrum et al 2006; Sarson et al 2009). Fears of antibiotic resistant genes in bacteria moving through the food chain promoted a ban of AGPs in the European Union and increased the popularity of organic production systems which do not use any AGPs, in North America (Witte 2000; Oberholtzer 2006).

The absence of antibiotic growth promoters (AGPs) in the diets of chickens in countries where AGPs have been banned such as Denmark has significantly impacted animal agriculture, causing an increased incidence of necrotic enteritis (a re-emerging disease)

caused by the ubiquitous anaerobe *Clostridium perfringens*, impaired feed efficiency and doubled the use of therapeutic antibiotics causing economic consequences (Casewell et al 2003; Grave et al 2006).

Necrotic enteritis may be hard to diagnose and often the only sign is a mortality rate up to 1% in a flock (Hemboldt and Bryant 1971). In order to control diseases such as necrotic enteritis, researchers have begun to study new AGP alternatives. The alternatives, including prebiotics and probiotics, tested within poultry production may help to maintain gut health by promoting beneficial bacteria while pathogenic bacteria are descreased in relation (Yang 2009). Probiotics are live fed microbial cultures which are resistant to gastric enzyme digestion and therefore may survive and colonize the gut, improving the gut microbial population through competitive exclusion of intestinal receptors and creating a gut environment inhospitable to pathogenic bacteria through the production of metabolic products such lactic acid (Fuller 1989; Gibson and Roberfroid 1995; Netherwood 1999). Prebiotics are feed ingredients not digestible by the animal, creating a source of nutrients and energy for beneficial bacteria and boosting their populations, thereby causing secondary effects similar to probiotics (Gibson and Roberfroid 1995). Prebiotics such as mannan oligosaccharides may also have direct effects on pathogenic bacteria acting as a receptor of Pilli I structures present in some gram negative bacteria and enhancing the immune system interaction with carbohydrate receptors in some antigen presenting cells (Spring 2000; Shashidhara and Devegowda 2003).

In order to test the effectiveness of mannan oligosaccharides this study will look into their effect on overall gut health defined as the balance of the microbial population, gut

morphology and mucosal immune system responses (Choct 2009). Previously it has been hypothesized that mannan oligosaccharides are able to directly affect *C. perfringens* populations (Spring et al 2000). In Manuscript 1 and 2 it was expected that there would be a clear control of this microbe post challenge (Brady et al 2010). Gut morphology as it relates to villi architecture effects nutrient absorption and pathogen migration through the intestine and is expected to be improved based on past results of similar studies (Savage et al 1997; Stokes et al 2001; Yang et al 2007). The effect of mannan oligosaccharides on lymphocyte proliferation in germinal centres post infection has not been studied previously, it is expected that a higher level of lymphocytes will be noted regardless of infection due to earlier immune maturation and that number will remain stable post challenge as *C. perfringens* numbers will be mitigated in response to MOS. Quantification of toll like receptors 2 and 4 allows for deduction of up regulating of the innate immune system as well as general bacterial populations as TLR 2 and 4 are able to recognize patterns displayed by gram positive and gram negative bacteria, respectively (Higuchi et al 2008; Keestra and Van Putten 2008).

LITERATURE REVIEW

1. Introduction

Necrotic enteritis is a serious disease affecting chickens and has become more commonplace due to the exclusion of antibiotic growth promoters from the daily diet. Prebiotics and probiotics are being tested as alternative preventative measures in order to control necrotic enteritis. Although prebiotic mannan oligosaccharides (MOS) have been used to induce immune stimulation in chickens, the actual mechanism has not yet been ascertained. Varying results have been shown as to the effects of MOS on gut morphology in avian species. It is thought that MOS has the ability to boost the immune system based on associated pathogen control and immune stimulation. Therefore, this study will focus on the effects of MOS on innate immune receptors toll like receptor 2 and 4, which recognize portions of bacterial cell wall of gram positive and gram negative bacteria.

2. Immune System

Chickens have undergone unique evolutionary pressures which have caused their immune system to differ from other animals as well as other avian species (Downing et al 2010). Much like in mammals, the chicken immune system can be academically broken down

into two branches: the innate system which is the first line of defense, reacting quickly and nonspecifically and the adaptive immune system which is slower to respond but mounts a stronger, more specific attack (Hoffmann et al 1999).

These branches of the immune system break down further into cellular and humoral responses, the former is made up of a variety of cells which act in both inductor and effector mechanisms:, the latter, involves cell products, where the mechanisms are undergone by proteins and molecules such as cytokines, antimicrobial peptides and antibodies (Beutler 2004).

2.1 Chicken Intestinal Innate Immunity

In order for the gut to remain a working part of the animal system it must be able to maintain homeostasis and respond appropriately to foreign antigens (Chirkin et al 1988). The innate immune system in the gut is especially important as it will define the immediate response to antigens ingested with food, causing oral tolerance to be defined at a very young age (Klipper et al 2001). Unique properties including cell surface barriers and tight junctions have been built into the intestinal innate immune system to ready it for foreign particles which are part of the necessary nutrients being ingested (Fugihashi et al 1999).

2.2 Gut Health

Gut health can be described in a variety of ways including microbial population, gut morphology and immune system ability (Brisvin et al 2008; Choct 2009). The microbial

population can be constituted of two major groups: beneficial and pathogenic;, beneficial bacteria live in harmony with the host animal and offer positive effects such as the production of organic acids which reduce viability in disease causing pathogenic populations (Tse and Chadee 1991; Revolledo et al 2006). Gut health is important part of complete health, homeostasis and production in an avian system (Brisbin et al 2008; Yegani and Korver 2008). Birds will only produce to maximum ability when they are at their best health status as this allows them to meet their full genetic potential without the need to divert energy towards processes such as immune reactions (Klasing 2007; Yegani and Korver 2008).

2.2.1. Gut Morphology. Gut morphology is important for both digestive and immune reasons (Choct 2009). Villus architecture affects nutrient absorption as well as movement of the lumen contents including potentially harmful bacteria reducing potential for attachment and infection (Stokes et al 2001). Longer villi and crypt depth are an indication of gut maturity and functional ability (Yang et al 2007). Simultaneous short villi and deep crypts have been associated with toxin effects in the intestine as well as diarrhea in newly weaned piglets (Nabuurs et al 1993). This effect has been shown to cause more secretor and fewer absorbtive cells, which is beneficial for fighting off disease but problematic for digestion and therefore production (Nabuurs et al 1993; Choct 2009). The mucosal barrier is made up of the protective mucus layer as well as the epithelial cells which lay below (Stokes et al 2001). Mucus is produced from goblet cells which originate in the intestinal crypt and it aids in lubrication and transportation as well as protection (Gaskins 2001). Though large crypts are often associated with gut maturity,

extremely deep crypts may also have the opposite effect as they may lead to rapid tissue turnover, not allowing cells to mature fully (Choct 2009).

2.2.2 Physical Intestinal Barriers. The mucosal barrier includes the epithelial cells and mucus layer which prevent entry of pathogens that may cause disease upon entry (Stokes et al 2001). There are two layers of physical barriers in the intestine; the mucus layer and the tight junctions of the epithelia (Stokes et al 2001). Mucus is both a physical and chemical barrier produced by goblet cells in the intestine; it blocks pathogens from directly contacting the epithelia of the intestine and causing infection (Forder et al 2007). While the inner layer of mucus remains bacteria free, the outer layer of mucus may be colonized by bacteria. Pathogens which are trapped in or set on top of the mucus may be carried away during peristalsis (Tse and Chadee 1991; Tlaskalova-Hogenova et al 2005). The mucus also helps to distribute secretory Immunoglobulin A (sIgA), which is known to capture pathogens, increase mucus secretion and can decrease inflammation reactions, throughout the intestinal tract (Wieland et al 2004; Bauer et al 2006). Trefoil factors, which are peptides found in mucus rich areas, aid in endothelial repair during inflammation (Kjellev 2009).

Tight junctions formed between the epithelial cells and intraepithelial lymphocytes (IELs) are an important part of maintaining surface integrity and barring microbes from gaining entry and causing infection (Nochi 2006; Stokes et al 2001). Molecules such as occludin are produced by IELs to keep endothelial cells close together only allowing substances access to the tissue below through small pores in the tight junction (Chediak et al 2003). As substances increase in size it becomes less likely they will pass through

these pores via paracellular transport making it difficult for pathogens to enter under normal conditions (Chediak et al 2003). There is potential for these barriers to separate, causing a 'leaky' barrier between cells and opening up the opportunity for pathogen entry thus it is important that these attachments remain strong and intact (Madara and Stafford 1989).

2.2.3. Gut Epithelium. Enterocytes line the intestine and have microvilli, which create a brush border that helps to increase surface area and transport particles. This brush border also helps to breakdown nutrients such as starches through the use of digestive enzymes (Uni et al 1998; Sklan 2005). The intestine uses multiple types of immune mechanisms to determine what is, and is not, a threat in order to maintain homeostasis in the intestine. The most basic method of determining this is through Pattern Recognition Receptors (PRR), such as toll-like receptors, PRRs are able to recognize molecular patterns specific to pathogens known as Pathogen Associated Molecular Patterns (PAMP) (Rackhouf-Nahoum et al 2004). These PAMPs are made up of patterns which cannot be created by the host, for example: double stranded RNA found in viruses is recognized by a PRR known as Toll-like receptor 3 (Alexopoulou et al 2001; Janeway and Medzhitov 2002). Self-non self determination is modulated by Major Histocompatibility Complexes (MHC) which are able to present foreign proteins to T-cells, in chickens the MHC found in its most basic evolutionary form, with different haplotypes defining susceptibility to particular viral pathogens (Kaufman and Salomonsen 1997).

Intraepithelial lymphocytes (IEL) are largely made up of T-cells, but also include natural killer cells (NK cells), which are able to recognize and destroy infected epithelial cells

(Göbel et al 2001). Only a small proportion of the T-cells (~16% at week 15) that make up the IEL population are the common variety containing T-cell Receptor (TCR) 2. Depending on age, 42-78% of the TCR2 T-cell type, are Cluster of Differentiation (CD8+) cytotoxic T-cells, while, only a small amount are attributed to CD4+ helper T-cells. The greatest populations of T-cells that make up the IEL are those that display TCR1 γδ polypeptide chains that are only found amongst epithelial cells of the mucosa and skin and are strongly cytolytic (Lillehog and Chung 1992). Intraepitheial lymphocytes manage immune responses by producing both pro-inflammatory and anti-inflammatory factors such as cytokines and chemokines (Fujihashi et al 1999). IELs play an important role in recognizing and reacting to pathogens which have managed to pass the mucus layer and infect epithelial cells as they may control further movement of the pathogens deeper into the tissue by destroying infected epithelial cells (Groh et al 1998; Göbel et al 2001).

Enterocytes are able to secrete antimicrobial peptides (AMPs), which are extracellular immune effector proteins found in a wide variety of species that defend the epithelium and help to control the microbial population of the intestine through the disruption of the negatively charged phospholipids of microbial membranes (Lynn et al 2004; Buetler 2004; Zasloff 2002; Lynn et al 2004; Tlaskalova-Hogenova et al 2005). It seems that there are many different isotypes of AMPs due to evolutionary changes that may allow for a species to have a specialized group based on the microbial challenges specific to its environment (Cormican et al 2009). Vertebrates have AMPs known as defensins and cathelicidins; in chickens β-defensins have evolved into a unique group called gallinacins

which are better suited to fight pathogens that afflict chickens specifically (Lynn et al 2004).

2.2.4. Peyers Patches and Cecal Tonsils. Peyers Patches and Cecal Tonsils are highly organized lymphoid tissues containing dense areas of immune cells such as microfold (M) cells, dendritic cells, B and T-cells (Nochi and Kiyono 2006). Before hatch, many peyer's patches may occur in the intestine, however, these numbers tend to decrease as the chicken grows older. One area found about 5-10 cm proximal to the ileocecal junction consistently houses Peyer's patches (Befus et al 1980). Peyer's patches may be found in the lamina propria consisting largely of B-cell aggregates separated by T-cells (Befus et al 1980). Cecal tonsils are found at the transition from the ceca to the rectum, made up of T-cell and B-cells capsules separated by connective tissue (Casteleyn et al 2010). Cecal tonsils mature quickly as germinal centers have generally formed within 2 weeks post hatch (Jeurissen et al 1989). Both of these are sites of secondary immune maturation and depend M-cells to transport and present antigens to the immune cells below, causing an adaptive immune reaction (Casteleyn et al 2010).

2.3 Toll like Receptors

Toll Like Receptors are a component of the innate immune system called Pathogen Recognition Receptors (PRR) which recognize generalized molecular patterns of pathogens called PAMPs causing a chain reaction which stimulates the immune system to destroy the pathogen (Creagh and O'Neil 2006). PAMPs are highly conserved prokaryotic indicators found in a variety of pathogens (Aderem and Ulevitch 2000). PRR of PAMPs help the immune system to distinguishing pathogens into types while allowing

the innate immune system to mount an early and quick response (Ozinsky 2000). The PAMP recognized by a specific TLR is known as its 'ligand', however many TLRs have been shown to recognize multiple ligands, sometimes in conjunction with other TLRs of the same family (Akira et al 2001; Cormican et al 2009).

2.3.1 Chicken Toll-like Receptors. Chicken Toll-like receptors (chTLR) have been found using other animal TLRs as models to isolate and clone (Yilmaz et al 2005). Though many of the mechanisms and ligands of chTLR are similar to mammalian counterparts, the receptor itself is often unique having a varied structure, duplicate genes or are exclusive to the avian genome (Temperley et al 2008). Chicken TLRs are found throughout the intestine (Iqbal et al 2005). In mammals, TLRs tend to be expressed in a polarized fashion at the apical and basolateral surfaces, this expression helps to maintain homeostasis as TLRs associated with tolerance tend to be on the apical surface, decreasing unnecessary inflammation (Schauber et al 2004; Lee et al 2008). In order to maintain gut homeostasis, macrophages in the intestine are less sensitive to TLRs and instead are largely phagocytic, reducing the need for inflammatory responses to minor aberrations from the normal gut mucosa (Smythies et al 2005).

TLR2 is known to be activated by a wide variety of ligands associated with gram positive bacteria such as *C. perfringens* possibly due to having multiple binding sites (Meng et al 2003). TLR2 recognizes peptidoglycan and lipoteichoic acid which make up portions of the gram-positive bacterial cell wall (Higuchi et al 2008). Duplicate genes have been found of chicken TLR 2 (chTLR2) and are referred to as TLR2t1 and TLR2t2 (Fukui et al 2001). Duplicates of TLR1 were originally referred to as TLR1t1 and TLR1t2 but more

recently it has been proposed that they be called TLR1LA and TLR1LB, respectively (Temperley et al 2008). Chicken TLR2t1 in conjunction with TLR1LB as well as TLR2t2 and TLR1LA were able to recognize a triacylated and diacylated lipopeptide, while chTLR2t1and TLR1LA was able to only recognize triacylated lipopeptides to a lesser extent, the TLR2 receptors are unable to mount a full recognition response without the help of TLR1(Higuchi et al 2008). In chickens, TLR4 is best known for its ability to recognize gram negative cell wall related lipopolysacchirides (LPS), which can cause septic shock, though chickens appear to be far less sensitive to these effects (Keestra and von Putten 2008). TLR4 is only able to recognise LPS when it has been bound by LPS-binding protein (LBP) which can then be recognised by CD14 on monocytes and heterophils, these CD14 molecules are found close to TLR4 allowing for receptor stimulation by bringing LPS within proximity (Jiang et al 2000).

Unique toll like receptors include TLR15 and TLR21 (known as TLR9 in humans) whose ligands are bacterial heat stable compounds and CpG DNA, respectively (Brownlie et al 2009; Nerren et al 2010). Table 1 describes known chicken toll like receptors and their ligands.

TABLE 1. Known chicken Toll Like Receptors and their associated ligands

TLR	Ligands	Reference
TLR1LA	Acts as huTLR1/6/10 (lipoproteins)	Keestra et al 2007
TLR1LB	Lipopeptides in conjunction with TLR2t1	de Zoete et al 2010
TLR2t1	PG, LPA	Fukui et al 2001
TLR2t2	Lipoproteins	Fukui et al 2001
TLR3	dsRNA	Alexopoulou et al 2001
TLR4	LPS	Keestra et al 2008
TLR5	Flagella	Iqbal et al 2005
TLR7	Chloroquine agonists	Philbin et al 2005
TLR15	Gram-/+ heat stable components	Nerren et al 2010
TLR21	CpG DNA	Brownlie et al 2009

2.3.2 Signalling. TLR signalling pathways are initiated when recognition causes dimerization forming independent homodimers or heterodimers such as with the TLR2 and TLR1 signalling complex (Higgs et al 2006). Myleoid Differentiation factor 88 (MyD88) is a receptor protein commonly seen in TLR cascades which allows up regulation of immune related genes such as those that produce cytokines, though MyD88 independent cascades also exist (Medzhitov et al 1998; Akira et al 2001; Li and Verma 2002; Keestra and Putten 2008). In chickens several forms of MyD88 have been discovered including MyD88-1, 2 and 3 (Wheaton et al 2007). MyD88-1 is the most similar to mammalian types though it is unable to initiate the death-associated protein kinase, MyD88-1 and -3 both showed a stronger ability to promote Nuclear Factor–κB

(NF-κB), a protein complex that regulates the immune system, than MyD88-2 did though it is considered to be a major player in signalling (Qui et al 2008).

2.3.4. Effector Mechanisms. TLRs display a variety of effector mechanisms which create a tailored response from both the innate and adaptive immune system (Creagh and O`Neill 2006). TLRs are found on a number of different cells (i.e. macrophages, heterophils) and will congregate on cells for which they directly affect (Iqbal et al 2005).

The adaptive immune system is dependent on NF-κB produced by TLR activation in many ways; it has been shown to be a key aspect in dendritic cell and T-cell development and function (Zheng et al 2003; Lopez et al 2003). Defects in the NF-κB system affects late stage B-cell maturation, proliferation and isotype switching (Snapper et al 1996).

In the innate system NF-κB has been shown to up regulate the production of nitric oxide, a potent killer produced by macrophages, and delays heterophil apoptosis, allowing heterophils to work longer in areas of inflammation (Xie et al 1994; Ward et al 1999).

TLR and heterophil interactions have varying responses, though TLR2 and 4 are the most likely to cause oxidative burst reactions and under TLR activation heterophils can produce inflammatory mediators help to induce further immune response (Farnell et al 2003; Kogut et al 2005). TLRs are also able to induce the expression of antimicrobial peptides (AMPs) to protect mucosal surfaces (Birchler et al 2001). TLRs may also produce kinases which upregulate the production of cytokines, substances that act as the messaging and control system of immunity, swaying immune reactions depending on cytokine type (Martin et al 2005). Stimulation of TLR2 is important to gut homeostasis as it can help maintain the integrity of cellular tight junctions (Cario et al 2007).

3. *Clostridium perfringens* in chickens

Environmental and dietary effects are contributing factors to growth of anerobic, gram positive *C. perfringens* in the chicken intestine (Kocher 2003; Williams 2005). In chickens the presence of intestinal damage produced by Coccidia, and the use of high amounts of wheat in the diet are considered predisposing factors that increase *C. perfringens* proliferation and the incidence of Necrotic Enteritis (NE), through the production of extra-cellular toxins that degrade host tissues (Al-Sheikhly and Al-Saieg 1980; Annett et al 2002).

The threat of antibiotic resistance in *C. perfringens* can be associated with not only the pathogen itself but also the possibility of the transfer of resistance genes to commensal bacteria (Teuber 1999). Though antibiotic resistance has been found in both types of production the levels appear higher in conventional systems and multi-resistant strains of bacteria from chickens have been noted as early as 1966 (Smith 1966; Garcia-Migura et al 2005). Unfortunately, the absence of antibiotic growth promotants (AGPs) in the diets of chickens in countries where AGPs have been banned has significantly impacted animal agriculture, causing an increased incidence of NE (a reemerging disease), impaired feed efficiency and doubled the use of therapeutic antibiotics (Casewell et al 2003; Grave et al 2006).

3.1 Toxinotype

C. perfringens types A, B, C, D and E are currently recognized as the major pathogenic contributors and are associated with the production of one or more of six different major lethal toxins (Granum 1990; Songer 1996). Types A and C are the most commonly linked with disease observed in avian species (Tschirdewahn et al 1991; Cooper and Songer 2006). Avian *C. perfringens* produce many extracellular toxins, however toxins α (cpa), β (cpb), β-2 (cpb2), enterotoxins (cpe) and NetB are most significant (Engström et al 2003; Martin and Smyth 2009).

In order to characterize the C. perfringens toxin type, PCR is used (Daube et al 1994). Multiplex PCR techniques allow for multiple toxins to be tested at one time, as a time saving procedure. The toxin genes cpa, cpb, cpIo and etx are used to define type (Daube et al 1994; Petit et al 1999). Type A is associated with cpa, Type B with cpb, cpa and etx, Type C with cpb and cpa, Type D with cpa and etx and Type E is associated with cpIo and cpa. Other toxins commonly typed include cpb2 and cpe which are important as a potential virulence factor and food poisoning agent, respectively (McClane 1996; Gibert et al 1997). Recently, NetB has become a toxin of interest it has shown virulence potential in some cases (Keyburn et al 2008).

The chromosomally located *cpa* gene is associated with alpha toxin, a 42.5 kDa phospholipase C enzyme, originally thought to be the main factor associated with pathogenesis of the disease however recent work is indicating alpha toxin is not an essential virulence factor in NE in chickens (Canard and Cole 1989; Titball et al 1989; Titball et al 1999; Keyburn et al 2006). Alpha toxin causes membrane damage through

hydrolysis of phosphatidylcholine and sphingomyelin leading to cell death (Taguchi and Ikezawa 1976).

β toxin is produced by cpb and is present in various types of *C. perfringens* including type C found in poultry (Smedly et al 2004). The 35 kDa toxin leads to hypoxic tissue necrosis in severe cases due to its pore forming nature (Hunter et al 1993; Nagahama 2003).

Toxin genes cpb2 and netB are associated with *β-2* and NetB toxins and are found in a variety of bacterial types and have been indicated as possible factors of pathogenesis though incidence of these genes varies (Bueschel et al 2003; Keyburn et al 2006). These two toxins produce proteins of varied sizes, Beta-2 creates a protein of 27 kDa where as NetB is associated with a 33 kDa toxin protein (Gibert 1997; Keyburn et al 2003). Like β-toxin, β-2 and NetB toxins are thought to increase permeability as they appear to be pore forming in nature causing ionic imbalance followed by cell lysis (Shatursky et al 2000; Keyburn et al 2008; Ojcius and Young 1991).

Toxin gene cpe can be either chromosomal or plasmid bound and produces a 35 kDa toxin known as enterotoxin and has been linked to certain diarrheas, causing cell death by changing membrane permeability (Cornillot et al 1995). Human food poisoning is correlated with chromosomal bacteria whereas plasmid borne version is often seen in animal diseases (Cornillot et al 1995; Collie and McClane 1998). In plasmid cpe there is the potential to loose or gain this gene, thereby effecting pathogenicity (Collie and McClane 1998).

Studies of chickens from conventional systems found that Type A and C isolates are the most common in sick birds, many of which were also found to have the *cpb2* gene,

though toxin expression was detected in only half of the birds. (Crespo et al 2007). Interestingly, *C. perfringens* was also found in 10-15% of healthy birds, demonstrating that it is a ubiquitous bacterium and simple presence in the gut is not sufficient to induce pathogenesis (Shane et al 1984; Gholamiandekhordi et al 2006).

3.2 Clinical Necrotic Enteritis

The signs of clinical necrotic enteritis in live birds are common to those associated with many different avian diseases, making accurate identification and treatment very difficult, including ruffled feathers, decreased feed intake and diarrhoea (Hemboldt and Bryant 1971). A jump in mortality rate up to 1% is considered the main sign of necrotic enteritis and usually does not include other signs of illness (Hemboldt and Bryant 1971).

Upon necropsy of clinically infected animals, the turkish towel effect is the best defined lesion associated with necrotic enteritis, appearing as a pseudomembrane over the mucosal surface of the jejunum (Broussard 1986). This is caused by the sloughing of cells and disconnection of the mucosa from the intestine due to extensive lesions caused by *C. perfringens* toxins (Hemboldt and Bryant 1971).

3.3 Subclinical Necrotic Enteritis

In recent years, subclinical necrotic enteritis has become the more apparent form of the disease (Van Immerseel et al 2009). In subclinical cases mortality is rarely noted though a depression in animal production is seen, and, upon necropsy low level intestinal lesions may be noted (Helmboldt and Bryant 1971; Williams 2005). In some cases, a pale

coloured liver may be noted due to hepatitis caused by the same bacterium (Løvland and Kaldhusdal 1999). Subclinical effects are considered to have a greater economic impact as it is not easily diagnosed and may remained untreated, allowing the small associated effects to build up (McDevitt et al 2006).

3.4 Epidemiology

As a ubiquitous bacterium, *C. perfringens* is impossible to control completely but understanding the predisposing factors which can cause pathogenesis can help with designing plans to reduce outbreaks (Chalmers et al 2007). Several of these factors have been indicated including damage to the intestinal epithelium and different feed types which affect *C. perfringens* growth in the intestine while wet litter conditions may increase growth in the environment, increasing the potential for disease occurrence (Williams, 2005). There has been much debate as to the biggest factor in production of necrotic enteritis; studies remain inconclusive as results vary when different factors are tested (Van Immerseel et al 2009).

3.4.1 Intestinal Damage. Intestinal damage associated with rough feed and disease is considered a major precursor to necrotic enteritis pathogenesis by causing proteins to be released which *C. perfringens* may then use as growth substrate, potentially allowing the bacterial populations to levels significant to induce toxin production (Shane et al 1985; Collier et al 2008).

Coccidiosis is an intercurrent disease factor, that is, subclinical coccidiosis and necrotic enteritis outbreaks often occur at the same time (Shane et al 1985). Coccidiosis in avian

species is caused by different *Eimeria* species in the small intestine which may affect diverse portions of the intestine causing lesions on the intestinal wall (Williams 2005). Recent research does not point to lesion sites as directly causing *C. perfringens* proliferation but rather the mucus produced by the intestine as a method of protection, which could also be the case for other forms of intestinal damage (Collier et al 2008). Though coccidial vaccines have been attributed to decrease necrotic enteritis through virtue of coccidiosis control, it has also been shown that overdoses of coccidial vaccines may increase necrotic enteritis lesion scores in experimental settings; it is possible therefore that coccidial vaccines could encourage necrotic enteritis onset especially in animals already under intestinal stress (Gholamiandehkordi et al 2007).

Ingestion of rough feed or litter may also cause intestinal damage, though it has a lesser ability to act as a predisposing factor of necrotic enteritis is less obvious than coccidiosis does (Williams 2005). Should the chickens ingest rough particles, such as straw, scratches along the intestinal mucosa may occur which would create lesions similar to coccidiosis, which allow the bacteria to feed and grow (Branton et al 1997).

3.4.2 Nutritional Factors. Different feed types have shown to be associated with varying levels of disease (Branton 1997; Drew et al 2004). Most importantly, protein type and concentration seem to have a major effect on the occurrence of necrotic enteritis in a flock (Drew et al 2004; Olkowski et al 2006). Early work on necrotic enteritis has been associated with high concentrations of protein in feed and was originally described as a disease effecting cockerels on high protein feed (Long 1972). Originally there was no evidence as to one particular type of protein causing *C. perfringens* to proliferate. More

recently, some research has suggested that some amino acids may increase bacterial numbers whereas, others may actually reduce bacterial growth rate, an example is methionine which seems to have an antibacterial effect when fed at high levels (Dahiya et al 2007). Pathogenesis may be a result of protein source used in the bird's feed (Drew et al 2004). Animal proteins, such as fish and blood meal, are recognized as having a strong potential to induce necrotic enteritis and should be avoided to reduce *C. perfringens* production (Drew et al 2004).

Different cereals may affect *C. perfringens* growth, based on *in vitro* studies, *C. perfringens* may grow better in the intestine of birds fed barley and wheat because digestion of these cereals increases viscosity, therefore slowing transit time through the intestine allowing C. perfringens to proliferate (Annett et al 2002; Branton et al 1997).

3.5 Prevention and Treatment

Prevention is a more effective method of controlling clinical necrotic enteritis than a cure is as the animals tend to die within hours of becoming ill and there is often no time to administer medicines (Williams 2005).

3.5.1 Antibiotics. Sub-therapeutic doses of antibiotics have been used as a growth promoter that has the secondary effect of depressing necrotic enteritis in flocks (Long 1973). Antibiotic growth promoters (AGP) such as zinc bacitracin given at low levels are able to suppress *Clostridium perfringens* controlling necrotic enteritis lesions when given daily, birds which have already been infected may receive therapeutic doses of

antibiotics, such as lincomycin, to treat the birds and to stop further development of necrotic enteritis lesions (Wicker et al 1977; Hamdy et al 1983; Lanckriet et al 2010). When Norway first banned growth promoting antibiotics there was a sharp increase in the amount of necrotic enteritis outbreaks seen in the country, however disease incidence decreased when anticoccidial ionophores were approved for use in avian agriculture (Grave et al 2004).Ionophores are a type of antibiotic that interact with metal ions and act as a carrier to transport these ions across cell membranes (Butaye et al 2003). Traditionally, the main target of ionophores are intestinal protozoan parasites (coccidias) but has been shown to inhibit C. perfringens, as well (Butaye et al 2003; Lanckriet et al 2010). Countries which have banned human antibiotics for growth promotion are still able to use ionophores and are somewhat dependant on this product as a secondary method of control (Inborr 2001; Grave et al 2004).

3.5.2 Vaccination. To prevent the use of anticcocidial antibiotics and prevent coccidiosis outbreaks the use of coccidial vaccinations has become common place in organic poultry production systems; the vaccination prevent secondarily against necrotic enteritis by eliminating the potential for coccidiosis as a precursor (Williams 2005). During an infection from the coccidian group Eimeria, several cytokines such as IFN-γ and IL-6 are produced as well as a major boost in T-cell production largely consisting of cytotoxic populations that eclipse the role played by B-cells, thus it is important that vaccines activate the cell mediated immune system. (Lillehoj and Lillehoj 2000; Hong 2006). Once an animal is immune, Eimeria is still able to enter the intestine but will not progress further causing disease; low level maternal antibodies are produced though there is not a strong associated defense based on this (Dalloul and Lillehoj 2005).

3.5.3 Prebiotics and Probiotics. Probiotics are composed of live fed microbials including cultures such as bifidobacterium and lactobacillus, which transport to the large intestine and are able to benefit the host (Gibson and Roberfroid 1995; Netherwood et al 1999). To be an effective probiotic it must be able to withstand digestion, colonize the epithelial tissue and have positive effects on the host animal including promotion of health and production (Bezkorovainy 2001). Probiotics work multiple ways, the most common theory is competitive exclusion (CE) where the bacteria introduced by the probiotic takes up attachment sites on the mucosal surface and compete with pathogenic bacteria for receptors, decreasing direct contact of pathogenic bacteria with the cells (Patterson and Burkholder 2003; Verstegen et al 2005). Some probiotic cultures are also producers of volatile fatty acids and decrease the pH of the intestinal lumen making it difficult for pathogenic bacteria to survive (Nava et al 2005). The immune system may also be enhanced via probiotics through boosting heterophil and macrophage phagocytosis and numbers in the cecum and upregulation of interleukin(IL)-2 and interferon(IFN)-γ production (Farnell et al 2006; Chichlowski et al 2007; Dalloul et al 2005 b).
Prebiotics are feed ingredients resistant to digestion which help to induce beneficial effects on the host system without directly changing microbial status (Gibson and Roberfroid 1995). Mannan oligosaccharide and lignin prebiotics are able to promote positive commensal bacteria such as lactobacilli and bifidobacteria while decreasing invasion by pathogens such as E. coli through secondary effects similar to primary effects caused by probiotics (Baurhoo et al 2007). Prebiotics may cause change by promoting the immune system such as in the case of manna oligosaccharides (Nollet et al 2007). They may also cause changes to microbial populations through microbe agglutination by

adsorption of the microbes by MOS blocking their ability to bind to the intestinal surface and promote lactic acid producing bacteria, creating an environment unfavorable to pathogens due to decreased lumen pH (Gibson and Wang 1994; Spring et al 2000). Both pre and probiotics have been shown to increase the number of goblet cells found on the villus of the intestine which allows for greater mucus production during infection (Smirnov et al 2005; Solis de los Santos et al 2007).

4. Mannan oligosaccharides

Mannan oligosaccharides (MOS) are part of the feed additive group known as prebiotics; feed supplements that are able to promote health and/or increase production by stimulating beneficial bacteria in the gut (Gibson and Roberfroid 1995). MOS may also cause changes to pathogenic microbial populations and can cause agglutination of microbes which not only decreases the pathogenic effect but also allows for immune recognition and removal from the intestine through peristalsis and boosting phagocytic activity (Spring et al 2000; Cox et al 2006). The use of MOS in broiler production has been found to increase weight gain potentially due to thinner intestinal epithelium allowing for increased nutrient absorption (Ferket et al 2002).

Studies looking at the effect of MOS on bacterial populations of the gut have varying results. Some researchers have found no effect on anaerobic bacteria, especially in the cecum (Spring et al 2000; Yang et al 2007). Studies looking specifically at *C. perfringens* found decreased levels in the gut as well as decreased mortality associated with necrotic

enteritis (Hofacre et al 2003; Rehman et al 2009). Challenge based studies conducted with Salmonella found decreased colony levels in birds receiving 4000 ppm MOS in their diet (Spring et al 2000). Villi height was found to be greater in all areas of small intestine for both broiler chickens and turkeys fed MOS at concentrations varying from 0.05-0.4% of daily diet, though the exact area of effect differed between studies (Baurhoo et al 2007; Iji et al 2001; Fritts and Waldroup 2003). The duodenum was found to have significantly greater results for villi height, villi surface area and goblet cell concentration at earlier sampling periods in turkeys though the effect evened out by day 21, possibly meaning a decreased time to full gut maturity in birds fed MOS (Solis de los Santos et al 2007). MOS appears to reduce jejuna crypt depth in some cases, something which could affect gut maturity as the crypt is the site of goblet cell genesis and controls cellular turn over (Yang et al 2008). Other researchers found an increase in crypt depth when feeding MOS, potentially meaning greater gut maturity (Solis de los Santos et al 2007)

MANUSCRIPT 1 -

TOXINOTYPING OF NECROTIC ENTERITIS-PRODUCING AND COMMENSAL ISOLATES OF CLOSTRIDIUM PERFRINGENS FROM CHICKENS FED ORGANIC DIETS

1. Introduction

Environmental and dietary effects are contributing factors to *Clostridium perfringens* growth (Kocher 2003; Williams 2005). In chickens, the presence of intestinal damage produced by Coccidia and using high amount of wheat in the diet, are considered predisposing factors that increase *C. perfringens* proliferation and the incidence of Necrotic Enteritis (NE), through the production of extra-cellular toxins that degrade host tissue (Al-Sheikhly, & Al-Saieg, 1980; Annett et al 2002). The addition of antimicrobial growth promoters (AGP) in low doses to chicken feed has been shown to increase weight gain and feed efficiency (Castanon 2007). However, the use of AGP is thought to contribute to the selection of antibiotic-resistant bacteria and antibiotic residues that could contaminate the environment (Courvalin 1994; Teuber 1999; Witte 2001). As a consequence, the European Union and other countries have prohibited the use of antibiotics as AGP in complete feeds (Grave et al 2004). Nevertheless, the absence of AGP in the diets of chickens has significantly increased the incidence of NE (a reemerging disease), impaired feed efficiency and doubled the use of therapeutic antibiotics causing important economic loses to farmers (Casewell. et al 2003; Van Immerseel et al 2004; Grave et al 2006). In addition, there is no information available

regarding the strain characterization and their toxinotype in these new outbreaks of NE in organic production systems in order to better understand the potential mechanisms associated with the disease.

C. perfringens types A, B, C, D and E are currently recognized as the major pathogenic contributors and are associated with the production of one or more of six different major lethal toxins (Granum, 1990; Songer, 1996). Types A and C are the only types linked to the disease observed in avian species (Tschirdewahn., *et al* 1991; Cooper & Songer, 2009). The *C. perfringens* strains that infect avian species produce many extracellular toxins, however toxins α (cpa), β (cpb), β-2 (cpb2), enterotoxin (cpe) and netB are the most significant (Engström, *et al* 2003; Martin & Smyth, 2009). The phospholipase C enzyme *cpa,* was originally thought to be the main factor associated with pathogenesis of necrotic enteritis, however recent work has indicated that *cpa* toxin is not an essential virulence factor in NE in chickens (Keyburn *et al* 2006). Beta toxin is present in *C. perfringens* type C found in poultry (Smedly *et al*, 2004). Toxins β, β -2 and NetB are thought to be pore forming in nature causing increased permeability (Shatursky *et al* 2000; Keyburn *et al* 2008). Toxins *β-2* and NetB may be found in several bacterial isolates and have been indicated as possible factors of pathogenesis though incidence of these genes varies (Bueschel *et al* 2003; Keyburn *et al* 2006). Enterotoxin is linked to certain diarrheas, as the major factor in food borne contamination and causes cell death by changing membrane permeability (Cornillot *et al* 1995; McClane *et al* 2000; Brynestad & Granum, 2002). Studies of chickens from conventional systems found that Type A and C isolates are the most common in sick birds, many of which were also

found to have the *cpb2* gene, though toxin expression was detected in only half of the birds (Crespo *et al* 2007). Additionally, *C. perfringens* was also found in 10-15% of healthy birds (Shane, *et al* 1984; Gholamiandekhordi, *et al* 2006). However, the expression and production of C. *perfringens* toxins can be associated with the quorum sensing-related gene *luxS*, which can synthesize AI-2 protein. This protein facilitates pathogenic bacteria signal interactions during *C. perfringens* infections and induces toxin production including alpha toxin (Ohtani *et al* 2002; Vandeville *et al* 2005; De Keersmaecker *et al* 2006). To understand the pathogenesis of NE in organic systems, it is important to develop an *in vivo* passage model that allows consistent production of strain behavior, clinical signs and lesions of NE. To date, consistent reproduction of the disease by oral inoculation with *C. perfringens* has not been successfully achieved despite the ability of bacteriocins from NE-producing strains of C. *perfringens* that displace non-NE strains from the gut of chicks (Olkowski *et al* 2006; Barbara *et al* 2008). Nevertheless, new experiments have been carried out to reproduce the infection using outbreak strains focusing more on virulence factors such as NetB-producing strains (Cooper & Songer, 2009). We hypothesized that the *C. perfringens* colonies which cause necrotic enteritis infection in organic systems is the same as that found in conventional systems. The present study tested whether challenge with *C. perfringens* isolates from outbreaks on organic farms would reproduce the illness and examines the interaction between toxinotype and the pattern of illness observed.

2. Materials and Methods

2.1 Bird sample collection and culture of field isolates

Intestinal samples from 25 chickens of 25-32 days of age were collected from flocks experiencing necrotic enteritis outbreaks on two commercial farms using antibiotic free certified feeds provided by the Organic Producers Association of Manitoba, Winnipeg, Canada. Necropsy was conducted on 25 animals that showed signs of disease including depression, ruffled feathers, and low weight gain. Two centimetres of intestinal sections from duodenum (distal), jejunum (proximal to Meckel's diverticulum) and ileum (proximal to ileo-caecal junction) were collected for histology and fixed in 10% phosphate-buffered formalin for at least 24 h, after which they were transferred to 50% ethanol solution for 15 minutes followed by 70% ethanol until they were embedded in paraffin. Sections of 5 μm were cut and stained with haematoxylin and eosin. The samples were analysed by standard procedures with light microscope emphasizing gut architecture and tissue damage. Bacteriological samples from the duodenum, jejunum and ileum were cultured on triptose soya agar with 5% sheep blood agar (Becton Dickinson, Mississauga, Canada) for 24 hours in an anaerobic environment at 35°C. Primary isolates were identified by characterizing colony morphology, gram staining, and reverse CAMP (Hansen & Elliot, 1980). Isolates were preserved in preservation media (Brucella Broth, BBL, Mississauga, ON) and 50% buffered glycerol (1 M $MgSO_4$, 1 M Tris pH 8 and H_2O) and kept at -80^{0}C until use.

2.2 Toxinotyping by PCR

Nucleic acid extraction of the intestinal lumen content was performed using a RNeasy mini kit (Qiagen, Mississauga, Canada). DNA extraction procedures were carried out according to the manufacturer guidelines. DNA aliquots were diluted 10 times to minimize PCR inhibition (Vilei *et al* 2005). All primers were obtained from University of Calgary, Calgary, Alberta, Canada (Table 1). The genotyping for *C. perfringens* was based on a procedure described by Garmory et al (2000) and Keyburn et al (2008) with some modifications. Briefly, two multiplex, M1 (*cpb2, cpa, cpb*), M2 (*etx, cpIo, cpe*) and one single for *netB* PCR reactions were prepared using equal concentrations of primers. Each 25 µL PCR reaction contained 12.5 µL of PCR Master Mix [100µM Tris-HCl, pH8.3 (at 25ºC); 500 µM KCl; 15 µM $MgCl_2$; 0.01%(w/v) gelatine, and 5 U of Taq DNA polymerase , 4 µM dNTPs] (Promega, Madison, WI, USA), 0.30 µM of each *cpe*, *cpb*, *cpb2*, *etx*, *cpa* and *cpIo* oligos and 50µM for netB oligo primers. 2-5 µL of DNA template were added to each reaction. The PCRs were performed in a model 2720 Thermal Cycler (A&B Biosystem, Foster City, CA,USA) using the following thermocycling program: 95ºC for 1 min, 39 cycles consisting of 95ºC for 45 sec, 53ºC for 3 sec, and 72ºC for 1min, and one final extension step at 72ºC for 10 min. Electrophoresis was performed on a 1.5% agarose gel with ethidium bromide using standard procedures.

TABLE 2. PCR toxin primer sequences and products

Toxin/ Protein	Gene	Primer sequence (5'-3')	Fragment sizes (bp)	Gene bank access	Reference
Alpha	cpa	F: GCTAATGTTACTGCCGTTGA R: CCTCTGATACATCGTGTAAG	324	X17300 X17300	Garmory *et al*. (2000)
Beta	cpb	F: GCGAATATGCTGAATCATCTA R: GCAGGAACATTAGTATATCTTC	195	X83275 X83275	Garmory *et al*. (2000)
Beta2	cpb2	F: AGATTTTAAATATGATCCTAAC R: CCAATACCCTTCACCAAATACTC	567	L77965 L77965	Bueschel *et al*. (2003)
Enterotoxin	cpe	F: GGAGATGGTTGGATATTAGG R: GGACCAGCAGTTGTAGATA	233	X71844 X71844	Garmory *et al*. (2000)
NetB	netB	F: GCTGGTGCTGGAATAAATGC R: TCGCCATTGAGTAGTTTCCC	384	FJ189503 FJ189503	Keyburn *et al.* (2008)
Epsilon	etx	F: GCGGTGATATCCATCTATTC R: CCACTTACTTGTCCTACTAAC	655	X60694 X60694	Garmory *et al*. (2000)
Iota	cpIo	F: ACTACTCTCAGACAAGACAG R: CTTTCCTTCTATTACTATACG	446	X73562 X73562	Garmory *et al*. (2000)
AI-2	luxS	F: ACAAAGGTTAAGGCACCATATGT R: ACCTGTTTTGCATGACTCTTAGCT	385	NC003366 NC003366	Ohtani *et al.* (2002)

2.3 Capillary 16S ribosomal DNA gene sequencing comparison and phylogenetic tree

The region of 16S ribosomal DNA was amplified using standard PCR conditions. The following primers were used to run the PCR assay using the methodology described by Weisburg *et al* (1991): 27f 5'AGAGTTTGATCMTGGCTCAG3' and 1100 r 5'GGGTTGCGCTCGTTG3'. Forward primers were fluorescently labelled (WellRED D4dye, Sigma-Proligo, St. Louis, MO) to allow detection of the fragments by capillary electrophoresis. PCR reaction contains 12.5 ul of PCR Master Mix (Promega, Madison, WI, USA), Primers 5uM each and DNA template (0.2 ug) for a total volume of 20µL. Samples were run in a preheated thermal cycler for 35 cycles with: 30 s at 94^{o}C, 30 s at 54^{o}C, 30 s at 72^{o}C. 10 µL of DNA amplicons were analysed on a 1.5% agarose gel. PCR bands profiles were verified by 16S rDNA gene sequence determination and phenotypic identification. 16S rDNA sequencing of C*lostridium perfringens* was performed at the University of Calgary, University Core DNA Services using an Applied Biosystems 3730 and 3730xl DNA Analyzers. The isolates collected in this experiment were compared to the whole *C. perfringens* genome sequences found in the NCBI database.

Sequences alignment was performed using a BioEdit Sequence Alignment Editor (Hall, 1999) and tree visualization was generated by UPGMA algorithm using MEGA version 4 (Tamura *et al* 2007). The three isolate sources included necrotic enteritis affected birds from field outbreaks (wild type), healthy birds prior to NE virulence testing model inoculation of *C. perfringens* (natural flora) and chickens displaying mild or no signs of disease after inoculation of *C. perfringens* (post challenge).

2.4 *C. perfringens* isolation from NE virulence testing model

The ability of the *C. perfringens* field outbreak isolates to induce NE was evaluated by an NE virulence testing model using chickens fed organic feed. Two hundred and fifty Ross broilers were experimentally raised to mimic the conditions of the natural outbreak. The birds were floor raised in groups of 25 per pen and fed an antibiotic free certified organic starter diet containing 23% crude protein and 50% wheat. At day 14, birds were fed a certified organic grower diet containing 20% crude protein and 60% wheat. Both diets were purchased from the manufacturer who supplied feed during the field outbreak. On day one all the animals were vaccinated against coccidia, using an oral Coccidiosis vaccine (Immucox® Guelph, ON, Canada). The vaccine pucks were evenly split at 1 puck for every 100 birds. Two representative field outbreak isolates of *C. perfringens* were initially grown in 5mL of Schaedler's anaerobic broth (Oxoid® Missisauga, ON, Canada) at 37^0 C in anaerobic conditions for use in the challenge study. The amount of media was increased over the course of 8 days and cultures were split to allow for growth until sufficient amounts of bacteria were obtained (Olkowski et al 2006). Using an esophageal cannula birds were orally challenged on day 14 with 1 ml of *C. perfringens* culture at 3×10^{10} CFU/bird. Five birds from each pen were euthanatized at day 11 (pre challenge) to determine normal flora; the remaining birds were euthanatized at day 22 (post challenge) in accordance with the Animal Ethics Use Protocols of the University of Manitoba approved by the Animal Ethics Committee. Approximately 10 grams of ileal and cecal digesta were collected, mixed and the isolation of *C. perfringens* was carried out as described previously.

3. Results

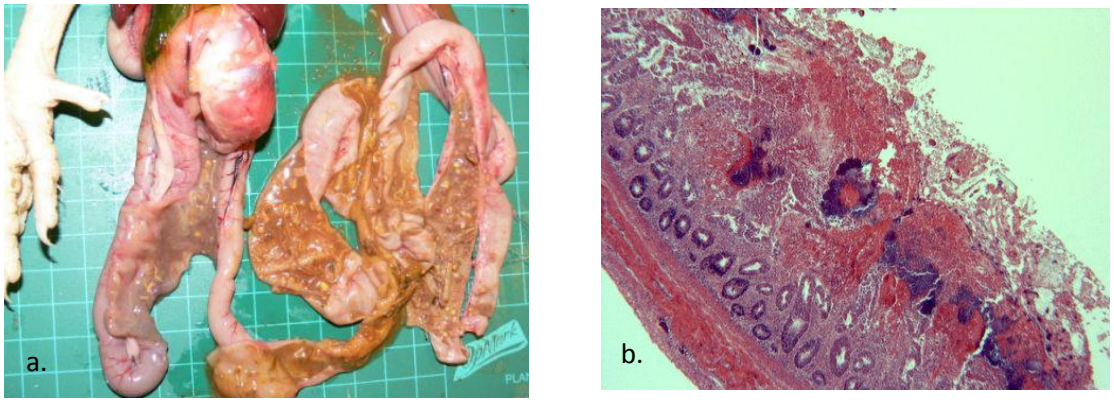
3.1 Post-mortem, histology and sample culture analysis

3.1.1. Outbreaks. To determine the characteristic lesions of natural cases of NE, pathology was performed on animals that, at time of necropsy, displayed depression, ruffled feathers, inappetence and dark diarrhea. Macroscopically, necropsy of the affected birds revealed dehydration, small intestine thickened and ballooned with gas, intestinal mucosa with dipheritic membrane, necrotic areas in the distal intestine with thickened intestinal walls associated with the Turkish towel effect typical of NE (Fig. 1a). Histopathology of the duodenal and jejunal sections showed necrosis of villous tips or entire villi, down to the crypts with many large bacilli in this necrotic and cellular debris (Fig. 1b). Also, there were various coccidial stages including oocysts throughout the cellular debris and enterocytes of the viable tissue. Parasitological and bacteriological findings indicated high levels of *Coccidia* and *C. perfringens* that correlated with the histological analysis which led to a final diagnosis of Coccidiosis and NE.

3.1.2. NE virulence testing model. Clinical signs were mild in all groups and were characterized by depression, ruffled feathers, and inappetence. Necropsy revealed distended intestine, edema and enteritis areas in the middle and distal small intestine; there was no evidence of macroscopic changes associated with thickness or intestinal

tissue damage. Histopathological analysis showed extensive oedema, lymphocytic infiltration, mild villi necrosis and various coccidia stages in the enterocytes in all intestinal sections.

FIGURE 1. Macro and microscopic lesions of natural cases of Necrotic Enteritis



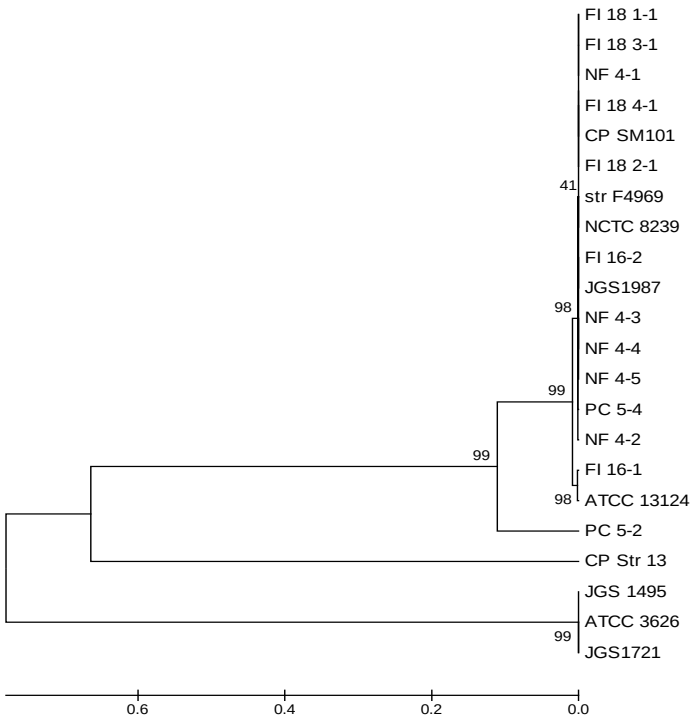
a. Small intestine thickened and ballooned with gas, intestinal mucosa with dipheritic membrane, necrotic areas in the distal intestine with thickened intestinal walls associated with the Turkish towel effect typical of Necrotic Enteritis. b. Intestinal section showing villi necrosis, with many large bacilli lining the necrotic area and cellular debris.

3.2 Genotype, toxinotype and phylogenetic tree

3.2.1 Phylogenetic analysis of cultures. The majority of the 13 isolate strains showed a high level (97-100%) of similarity with two of the three whole genome sequences of *C. perfringens* found in the gene bank (GenBank/DDBJ/EMBL) strains SM101, American Type Culture Collection ATCC 13124; Accession no. NC 008262 and NC 008216 respectively (Fig 2). In order to obtain a highly reliable results in the phylogenetic analysis the sequences from whole genome project were also added in the analysis, including strain ATCC 3626, JGS1495, JGS1721, JGS1987, National Collection of Type Cultures (NCTC) 8239 and Strain F4969 (incomplete sequence data).

Whole genome sequence SM101 was highly related to all natural flora isolates, five of six field isolates and one of two post challenge isolates. As well, unfinished whole genome sequences of strain F4969, NCTC 8239 and JGS1987 were highly related. The data were supported by bootstrap analysis showing values higher than 60 (Figure 2). Field Isolate 16-1was highly related to ATCC 13124 (bootstrap value of 98). It was the only isolate found within this group. Post challenge isolate 5-2 stood alone as it was not as highly related to any of the other trial isolates though its lineage relates to Strain 13.

FIGURE 2. Phylogenetic Analysis of *Clostridium perfringens* Isolates of 16S rDNA sequences.



Level of similarity relation between *C. perfringens* isolates from outbreaks, normal flora and post-challenge and three sequences of *C. perfringens* deposited in the gene bank are shown. FI=Field Isolate NF=Normal flora PC=Post Challenge, all others refer to strains from the NCBI database.

3.2.2 *C. perfringens* toxinotyping All field isolates were positive for *cpa*, *cpb2*, *netB* and *luxS* (Table 2). Normal flora isolates (collected before inoculation in the NE virulence testing model) were all positive for *cpa* and showed varying degrees of positive reaction for *cpb2*, *cpIo* and *netB*. Over half of the normal flora isolates were *C. perfringens* type A; three isolates found to contain *cpIo* belonged to *C. perfringens* type E. Isolates collected after the NE virulence testing model were all positive for *cpa*, though only some showed positive results for *cpb2* and *netB.* The *luxS* gene was positive to all the strains regardless of their source.

TABLE 3. Summary of the Clostridium perfringens PCR Toxinotyping profile according different sources and correlation with clinical status.

Wild type from organic farm outbreaks of Necrotic Enteritis									
Isolates ID	cpa	cpb	cpb2	cpe	netB	etx	cpIo	luxS	Illness
FI 16-1	+	-	+	-	+	-	-	+	+
FI 16-2	+	-	+	-	+	-	-	+	+
FI 18 1-1	+	-	+	-	+	-	-	+	+
FI 18 2-1	+	-	+	-	+	-	-	+	+
FI 18 3-1	+	-	+	-	+	-	-	+	+
FI 18 4-1	+	-	+	-	+	-	-	+	+
Normal flora isolates									
NF D2	+	-	-	-	+/-	-	-	+	-
NF 4-1	+	-	+	-	+/-	-	+/-	+	-
NF 4-3	+	-	+	-	+	-	+/-	+	-
NF 4-4	+	-	+	-	-	-	+/-	+	-
NF 4-5	+	-	+/-	-	+/-	-	-	+	-
Post-challenge/passage isolates									
PC 5-2	+	-	+	-	+/-	-	-	+	+
PC 5-4	+	-	+/-	-	+/-	-	-	+	-

Toxin type: Alpha (cpa), beta (cpb), beta 2 (cpb2), enterotoxin (cpe), NetB, Epsilon (etx), Iota (cpIo), AI-2 (luxS).

+/- Weak positive results. FI=Field Isolate NF=Normal flora PC=Post Challenge

4. Discussion

In the present study, the genotype of NE-producing strains of C. *perfringens* from an organic production system was determined. Also, the capacity of these strains to induce and reproduce NE was examined using a virulence test model.

C. perfringens SM101, Strain F4969 and NCTC 8239 are all Type A bacteria known to carry the cpe gene. Type E JGS1987 is also related to this large group. Isolates NF4-3 and 4-4 are Type E based on toxinotype and were found to have a similar lineage as JGS1987 though Type A isolate FI16-2 was found to be more directly related. SM101 was the only completed whole genome in this group, a Type A originating from a human food poisoning isolate (Myers et al 2006). SM101 did not consistently cause disease under experimental conditions, which could explain its relation to all five natural flora isolates which did not have disease causing effects (Myers et al 2006). Five of six field isolates were also found in this group, all of which are Type A like SM101. None of the experimental isolates in this group were positive for cpe which may be found in SM101 strains associated with food poisoning (Myers et al 2006).

Field isolate 16-1 belongs to whole genome sequence ATCC 13124, taken from human gas gangrene and having great disease causing potential (Myers et al 2006). Both described are type A *C. perfringens* and neither were shown to carry cpe genes. Despite similarities in genotype, FI16-1 was divergent from related field isolates found in the SM101 groups. It seems that this infection was produced by a bacterial population unique in comparison to others collected.

Isolates NF 4-4 and PC 5-4 were taken from the same pen and were highly related. PC 5-2 does not have a corresponding NF isolates as no *C. perfringens* could be cultured. It could be that challenge boosted *C. perfringens* levels to where they were detectable, oddly, the isolate was not highly related to that which was used to inoculate the birds but appears to be an original strain most highly related to Strain 13, a soil borne bacterium with disease causing potential. This strain was most likely already present at the time of challenge or was introduced soon after (Shimizu et al 2002). JGS 1496, ATCC 3626 and JGS 1721 strains are all whole genome sequences currently in the works and are the divergent group, a logical outcome as they are *C. perfringens* types not found in chickens or within the isolates studied in this research.

C. perfringens Type A commonly occurs in North America and is one of two types associated with NE (Songer, 2006). Alpha toxin was found in all bacterial cultures from the field isolates, normal flora and post challenge isolates. In this study, alpha toxin was recurrently found to be the only major toxin pointing to *C. perfringens* Type A as the main type noted, apart from few exceptions that are described later in this manuscript.

An interesting finding was that *cpIo* was detected at low levels in normal flora isolates. To our knowledge, this is the first report indicating the presence of *C. perfringens* Type E in poultry. The finding of Type E in the presumed normal flora could explain the lack of disease found in these three groups pre challenge. It should also be noted that the room used for experimentation was previously used to house bovine species which could carry Type E *C. perfringens* (Itodo et al 1986). Toxin gene *cpIo* was not found in the field or post challenge isolates.

Toxin genes for *cpb*, *cpe*, and *etx* were absent in all field strains,normal flora and post challenge isolates. Toxins *cpb* or *etx* are associated with *C. perfringens* types other than type A and would have changed the toxinotype noted. Toxin gene *cpe* is known to cause human food poisoning and may be found in type A and C *C. perfringens* but is not commonly seen in poultry (Songer, 1996; Tschirdewahn *et al* 1991; Gholamiandekhordi *et al*, 2006).

It has been suggested that potentially virulent toxins cpb2 and netB could contribute to disease effects in field isolates (Keyburn *et al* 2008; Bueschel *et al* 2003). In the present work, these genes were also found in varying degrees in normal flora and post inoculation isolates. However, no obvious NE was noted in the birds used for normal flora samples despite the presence of at least one of these toxin genes in all of the isolates. Only one of two birds in the post inoculation group showed signs of enteritis, one of them having *cpb2* and low levels of *netB* expression. The evidence of the level of virulence of these toxin genes remains inconclusive. Our results agree with those of previous studies, which suggested that *cpb2* and *netB* did not necessarily have a direct correlation with the incidence of NE (Gholamiandekhordi *et al* 2006; Martin & Smyth 2009). Positive PCR results for both *netB* and cpb2 toxin genes in the field isolates could play a role in pathogenesis and may have enhanced necrosis in the intestine. However, some studies have shown that β -2 toxin may not be significantly associated with NE (Keyburn *et al* 2008; Gholamiandekhordi *et al* 2006; Bueschel *et al* 2003). It should be noted that the occurrence of a gene does not necessarily determine phenotype of toxin production which may be a stronger determinant of virulence (Abildgaard et al 2010).

Positive findings for luxS were expected based on the level of conservation of this gene between bacterial types (Brassler, 1999). As a quorum sense related gene, *luxS* is reliant on predisposing factors to increase colonization by causing a shift in the gut ecology and bacterial numbers before allowing toxin production. In the case of α toxin it was found that *luxS* had only a slight stimulatory effect and was not completely dependent on *luxS* for toxin production (Ohtani *et al* 2002). However in our results using NE-producing strains there was no difference between luxS findings in normal flora versus NE-producing strains.

In the field cases, classic signs of NE typically seen upon necropsy included the well characterized Turkish towel effect and focal necrosis in the middle and distal small intestine (Broussard *et al* 1986; Kaldhusdal & Hofshagen, 1992). The isolation of both *Coccidia* and *Clostridium perfringens* confirmed a simultaneous infection which implies that coccidiosis is a common precursor to NE, suggesting that similar origins are seen in organic and conventional systems (Al-Sheikhly & Al-Saieg, 1980).

Experimental reproduction of acute necrotic enteritis has proven difficult in many cases (Thompson et al 2006; Olkowski et al 2006, Gholamiandehkordi et al 2007). It has been shown that *in vitro* passage can reduce virulence of a strain due to genetic modification (Chalmers et al 2007). Predisposing factors associated with necrotic enteritis have been used in challenges to increase the potential for disease replication with varying results (Olkowski et al 2006; Chalmers et al 2007; Gholamiandehkordi et al 2007). For example, mucosal damage, caused by the coccidia vaccine, could affect intestinal microbiotic features triggering endogenous virulence factors which facilitate necrotic enteritis (Gholamiandehkordi et al 2007).The outcome of the NE virulence testing model in this

experiment had similar outcomes to that seen by Thompson et al. (2006) where the inoculation isolate was taken recently from an outbreak and went through minimal *in vitro* passage yet did not accomplish an acute case of necrotic enteritis. This study differed from others where necrotic enteritis was achieved in that the birds were given liquid inoculations rather than cultures mixed with feed (Chalmers et al 2007; Cooper & Songer, 2010).

The presence of large numbers of bacteria in the necrotic debris of the mucosa, which is a characteristic of NE, was similar to what has been shown before (Olkowski et al 2006). Therefore, our model should be considered successful.

In conclusion, the *C. perfringens* strains isolated from organic farm outbreaks, normal flora and birds challenged with NE-producing strains were found to be similar to those found in conventional systems based on toxinotype characterization and phylogeny analysis. Based on these findings it seems that the effect of a NE outbreak is equally as challenging in organic as in conventional systems with an added risk due to the absence of prophylactic measures. Therefore, more research into non-antibiotic prophylactic strategies may be beneficial to reduce the potential for NE outbreaks. In addition, further analysis is needed to establish a much better correlation between gene expression of toxin and the pathogenic capacity of *C. perfringens* strains from organic systems.

MANUSCRIPT 2 -

THE EFFECT OF PREBIOTIC SUPPLEMENTED ORGANIC FEED ON GUT HEALTH AND IMMUNITY IN BROILERS CHALLENGED WITH NECROTIC ENTERITIS PRODUCING ISOLATES OF *CLOSTRIDIUM PERFRINGENS*

1. INTRODUCTION

Necrotic enteritis is an emerging disease caused by *Clostridium perfringens* infection of the intestine (Van Immerseel 2004). It has been associated with concurrent disease as well as feed type (Williams 2005). In order to fight off diseases such as necrotic enteritis, birds must have a strong immune system partially hinging on a healthy gut environment. Necrotic enteritis is often associated with coccidian infection as a precursor (Shane et al 1985). The lesions caused by coccidiosis serve as a source of nutrients to *Clostridum perfringens,* allowing for bacterial growth to a level where toxins may be produced via quorum sensing, a form of bacterial communication dependant on high population concentrations (Johansson and Sarles 1948; Helmbolt and Bryant 1971).

Gut health is important part of complete health, homeostasis and production in an avian system and can be described in a variety of ways including microbial population, gut morphology and immune system ability (Choct 2009). *Clostridium perfringens* is ubiquitous yet potentially pathogenic, though it is able to cause necrotic enteritis, it may also be part of the normal flora in the gut without causing disease (Abildgaard et al

2009). A variety of factors have been described which affect C. perfringens growth and pathogenecity including high protein feeds and intestinal damage (Shane et al 1985; Drew et al 2004).

Gut morphology is important for both digestive and immune reasons; villi increase surface area important for digestion and absorption while removing bacteria in the mucus by moving it out of the intestine (Stokes et al 2001). Villus architecture integrity affects nutrient absorption as well as movement of the lumen contents including potentially harmful bacteria reducing potential for attachment and infection (Stokes et al 2001). Longer villi and crypt depth speak to gut maturity whereas; simultaneous short villi and deep crypts have been associated with toxins in the intestine (Nabuurs et al 1993; Yang et al 2007). Deep crypts may lead to more secretory cells and fewer absorbtive cells as well as rapid tissue turnover which may be positive for the immune system but is negative for digestion and production in high levels (Choct 2009). The protective mucus layer is produced by goblet cells which originate in the intestinal crypt and aids in lubrication and transportation as well as protection, more goblet cells does not account for more mucus produced at all times but creates the opportunity for increased production in times of stress (Gaskins 2001).

Cecal tonsils are areas of highly organized lymphoid tissue found at the transition from the ceca to the rectum, made up of T-cell and B-cells capsules separated by connective tissue (Casteleyn et al 2010). Cecal tonsils mature quickly, with germinal centers having formed in reaction to immune stimulation within 2 weeks post hatch (Jeurissen et al 1989). Germinal centers are important to gut homeostasis and immunity as they are areas

of major pathogenic recognition and immune reaction (Berek et al 1991). Germinal centers are areas of dense immune cell populations formed after antigen stimulation in peripheral lymphatic tissue, largely consisting of B-cells though T-cells and follicular dendritic cells (FDC) are also important parts of the cellular matrix (Berek et al 1991; Butch et al 1993). FDC's are surface cells that pull antigens in for further immune recognition and effecting B-cell populations (Tews et al 1997). As the germinal centre matures the population of B-cells found changes; an increased number of cells are associated with immune stimulation, specifically antigen stimulated B-cells (McLennen 1994). Unlike in mammals, chickens do not have well defined dark and light zones though follicular dendritic cells are found at the center and rapidly dividing B-cells tend to be sitting at the periphery in the early stages of maturation (Hainke et al 2010).

Toll Like Receptors are a part of the innate immune system known as pathogen recognition receptors (PRR) which recognize generalized molecular patterns presented by pathogens called pathogen-associated molecular patterns (PAMPs) causing a chain reaction which stimulates the immune system to react (Creagh and O'Neill 2006). Each TLR recognizes certain PAMPs known as its 'ligand'; some recognize just one, while others can recognize multiple sometimes in conjunction with other TLRs (Cormican et al 2009; Akira et al 2001). TLR2 and TLR4 are able to recognize portions of the bacterial cell of gram positive and gram negative bacteria, respectively (Fukui et al 2001; Keestra et al 2008). Duplicate genes have been recognised in chickens in TLR2 which have slightly varied ligands associated with gram positive bacteria such as *C. perfringens* (Cormican et al 2009; Higuchi et al 2008). Chicken TLR2 receptors work in conjunction with duplicate TLR1 receptors to recognize and respond to triacylated and diacylated

lipopeptides (Keestra et al 2007; Higuchi et al 2008; de Zoete et al 2010). In chickens, TLR4 is best known for its ability to recognize gram negative cell wall related lipopolysacchirides (LPS) (Keestra & von Putten 2008).

Mannan oligosaccharides are prebiotics that boost gut health by increasing beneficial bacterial populations as well as cause agglutination of some pathogenic bacteria (Chee et al 2010; Spring et al 2000). Much interest is being given to prebiotics as prophylactic immune stimulators since antibiotic bans have become more common due to fears of antibiotic resistance.

The use of MOS in broiler production has been found to increase weight gain, decrease *C. perfringens* levels in the gut as well as decrease mortality associated with necrotic enteritis (Yang et al 2007; Hofacre et al 2003; Chee et al 2010). Though the actual effect of MOS on *C. perfringens* is unknown, it has been theorized that MOS may decrease *C. perfringens* levels in a similar manner of control as with *Salmonella* species, via bacterial agglutination or blocking the binding site stopping the bacterium from colonizing the gut (Oyofo et al 1989; Hofacre et al 2003).

MOS has been shown to effect gut morphology in a variety of ways, the results being most obvious in the jejunum (Iji et al 2001). Villi height was found to be greater in several investigations though the exact area of the intestine affected differed between studies (Baurhoo et al 2007; Iji et al 2001; Fritts & Waldroup 2003). Decreased time to full gut maturity has been described in the duodenum of birds fed MOS (de los Santos et al 2007). MOS appears to reduce crypt depth in some cases, other researchers found an

increase in crypt depth when feeding MOS, potentially meaning greater gut maturity (Solis de los Santos et al 2007; Yang 2008).

We hypothesize that the inclusion of MOS into the diets of broilers challenged with *C. perfringens* will improve gut health by effecting gut morphology, immunity related to TLR 2 and 4, spleen and bursa activity, as well as intestinal colonization by *C. perfringens.*

2. Methods and Materials

2.1 Animal Trial

One hundred and fifty Ross broiler chickens were experimentally raised to mimic the conditions of the natural outbreak. The birds were floor raised in groups of 15 per pen and fed an antibiotic free starter diet described in Tables 4 and 5. MOS was added after base diet production in a small bowel mixer working from lowest to highest concentrations at levels of 2, 4, 6 and 8 g/kg except in one diet which acted as the control and remained untouched. On day one all the animals were vaccinated against coccidia, using an oral Coccidiosis vaccine (Immucox® Guelph, ON, Canada). The vaccine pucks were evenly split at 1 puck for every 100 birds.

TABLE 4. Base diet ingredients

Ingredient	% in Diet
Soybean Meal	32
Wheat	56.41
Soy Oil	6
Biophos	3.2
Broiler mineral	.5
Broiler Vitamin	1
DL-Meth	0.09
Limestone	0.8

TABLE 5. Base diet nutrients

Nutrient	Amount
Energy (kcal/kg)	3138.8
Protein (%)	23.01
Calcium (%)	1.053
Phosphorus (%)	.801
Met (%)	.499
Met(cys) (%)	.938
Lys (%)	1.171

2.2 Microbiology Two representative field outbreak isolates of *C. perfringens* were initially grown in 5mL of Schaedler's anaerobic broth (Oxoid® Missisauga, ON, Canada) at 37^0 C in anaerobic conditions for use in the challenge study as described (Brady et al 2010). The amount of media was increased over the course of 8 days to allow for growth until sufficient amounts of bacteria were obtained (Olkowski et al 2006). Using an esophageal cannula birds were orally challenged on day 14 with 1 ml of *C. perfringens* culture at 3×10^{10} CFU/bird except for one group which was did not receive challenge or MOS in their diet and is referred to as 'control' from here on. Five birds from each pen were euthanatized at day 11 (pre challenge) to determine normal flora; the remaining birds were euthanatized at day 22 (post challenge) in accordance with the Animal Ethics Use Protocols of the University of Manitoba approved by the Animal Ethics Committee. Approximately 10 grams of ileal and cecal digesta were collected and mixed. The

isolation and 16S phylogenetic analysis of *C. perfringens* was carried out as described previously (Brady et al 2010).

2.3 Histological Analysis

Immediately after collection sections of the duodenum, jejunum, ileum and cecal tonsils were preserved in 10% neutral buffered formalin and left for 48 hours before switching to 50% ethanol for 15 minutes followed by 70% ethanol until processing. The sections were stained with Alcian Blue Periodic acid-Schiff (AB-PAS) pH 2.5. The samples were studied at a 16x zoom using a Zeiss microscope and ImageJ version 1.42q programming to calibrate and measure histological slides. Five samples of different animals from each treatment were analyzed for villus height, width and area, crypt depth and goblet cell number. Approximately five measurements were taken and averaged from each sample to determine a representative sample measurement used in statistical analysis using mixed procedure.

2.4 Relative Organ Weight.

Broilers were weighed immediately after euthanasia by cervical dislocation. Weights of the bursa and spleen were taken at the time collection and measured to the nearest milligram; these weights were expressed as a percentage of body weight at time of collection for statistical analysis using mixed procedure (Qureshi et al 1997).

2.5 Cloning for qRT-PCR

Primers used for cloning were chosen based off of existing qRT-PCR primers as described by Kogut et al (2005; personal communication). The following primers were used for the TLR2 clone forward primer 5' AAGCAGGATAAACAGCCTGCA and reverse primer 5' GAACCCGTGACCTTTCACCT using the following thermocycling program: 95ºC for 7 minutes, 35 cycles consisting of 94ºC for 45 seconds, 52ºC for 45 seconds, and 72ºC for 1 minute, and one final extension step at 72ºC for 7 minutes which produced an amplicon 1030 base pairs in length. The primers used for the TLR4 clone were forward primer 5' CCACCGCGCTGACTCTTG and reverse primer 5' GGACCTGAACCTGGAGTCAAA using the following thermocycling program: 95ºC for 5 minutes, 40 cycles consisting of 94ºC for 30 seconds, 57ºC for 30 seconds, and 72ºC for 30 seconds, and one final extension step at 72ºC for 7 minutes which created an amplicon 180 base pairs in size. A PCR was run using the primers separately with DNA extracted from chicken intestinal sections collected during a past experiment. The PCR product was run on a 1% agarose gel, cleans bands which matched the expected amplicon size were extracted using a sterile scalpel and purified using the Promega Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). This product was ligated and transformed using pGEM-T easy vector system kit (Promega, Madison, WI, USA) following recommended protocol. Color screening using X-Gal-IPTG plates was used to indicate colonies where proper ligation and transformation took place. The colonies found positive after screening underwent DNA extraction and were tested by running a

PCR of the clone using the qRT-PCR primers, ensuring a band as expected was produced on a 0.9% agarose gel.

2.6 qRT-PCR

Sections from the duodenum, jejunum and ileum were immediately placed into liquid nitrogen then placed in a -80 C freezer for long term storage. RNA extraction was conducted using Tri Reagent (Ambion, Mississauga, ON.) following company protocol.

For a relative quantitative measurement, a standard curve of each target gene was acquired using clones of individual target genes followed by a log10 series dilution. Each experiment was performed in duplicate with the same plasmid dilution used as a calibrator. Applied Biosystems' High Capacity cDNA Reverse Transciption Kit (Applied Biosystems, Carlsbad, CA, USA) was used to create cDNA. A Step One Real Time PCR machine was used for qRT-PCR using SybrGreen PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) use based on company procedure. All primers chosen for qRT-PCR were described by Kogut et al (2005; personal communication). TLR2 primers used for qRT-PCR were 5' AGGCACTTGAGATGGAGCAC and 5' GGACAATACCCGGTCCAAAT using the thermocycling program: 95ºC for 10 min, 40 cycles consisting of 95ºC for 10 seconds, 59ºC for 20 seconds, and 72ºC for 15 seconds creating a product 294 base pairs in length. TLR4 primers used for qRT-PCR were forward 5'GTCCCTGCTGGCAGGAT and reverse 5'TGTCCTGTGCATCTGAAAGCT using the thermocycling program: 95ºC for 10 min, 45 cycles consisting of 95ºC for 10 seconds, 58ºC for 30 seconds, and 72ºC for 15 seconds produced a product 74 base pairs

in length. Gene activity was defined based on relative expression corrected for efficiency based on the house keeping gene beta-actin.

3. Results

No variation in feed efficiency, weight gain or feed intake was noted between treatment levels (Table 6).

Microbiology Bacterial cultures taken from this experiment were compared to NCBI isolates using 16S sequencing techniques (Figure 4). The isolates were largely separate from the main group, with the exception of two post challenge (PC) isolates from the current experiment. Though highly related overall, most isolates were found to be separate from the known genomes. This is interesting as a similar result was only associated with one isolate in the last manuscript. It is not clear whether this may be related to the use of a new feed source or PCR sample quality.

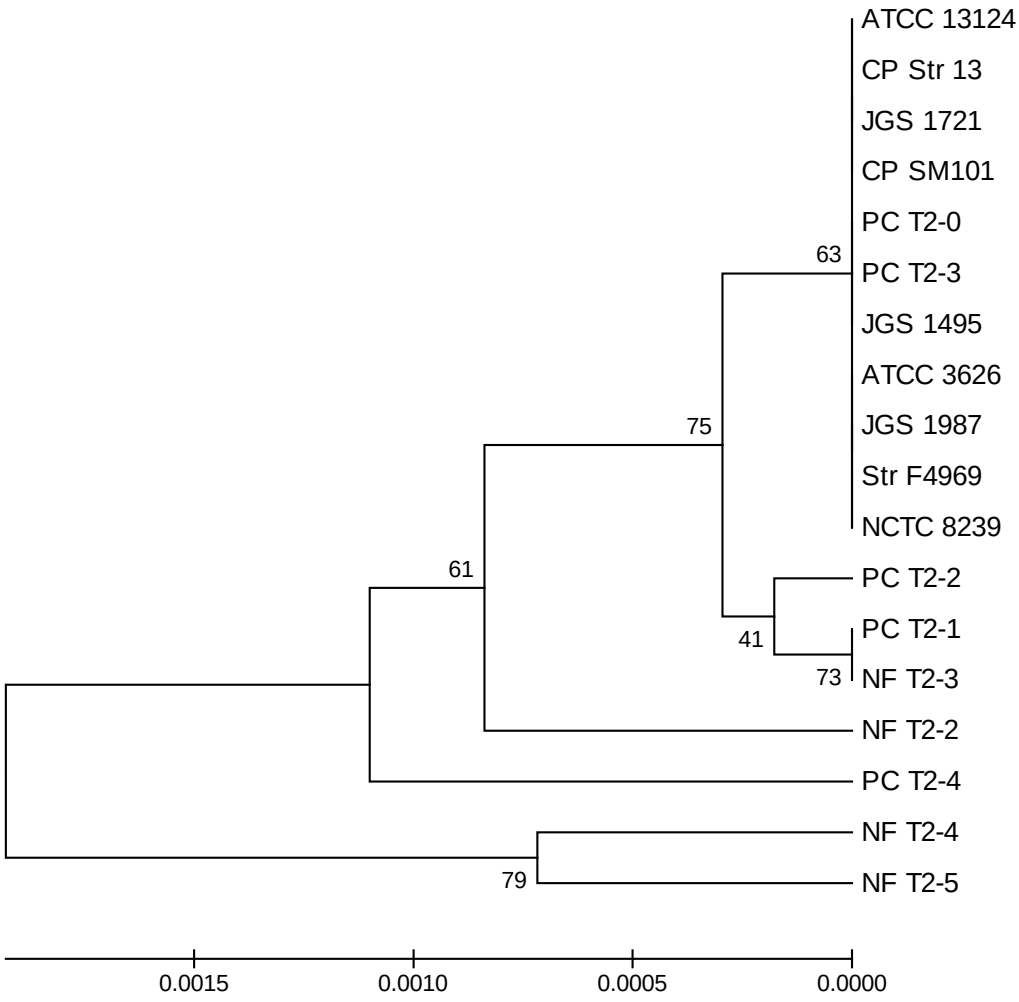
Toxinotype of the isolates collected from the experiment showed that most were found to be positive for cpa but not cpb, cpIo or etx meaning they were Type A *C. perfringens* with the exception of one belonging to the pre inoculation group fed 4g/kg MOS which was also positive for etx and was therefore type D. None of the NF isolates were cpb2 positive, though all but 8g/kg MOS post-challenge were, also, 6g/kg MOS level group is netB negative despite all other isolates being positive for this toxin gene. LuxS, a quorum sensing gene highly conserved in many bacterial species, was positive in all isolates.

Table 6. Effect of MOS on Feed Efficiency, Weight Gain and Feed Intake in Broilers Challenged with *C. perfringens*

	Least Square Means						P-Values		
Variable	Control	0g/kg	2g/kg	4g/kg	6g/kg	8g/kg	Trt	Day	Trt*Day
Feed Efficiency	1.65	1.11	1.35	1.37	1.25	1.30	0.95	0.86	0.60
Weight Gain	105.86	125.86	104.14	104.17	115.08	98.53	0.15	<0.0001	0.74
Feed Intake	169.02	123.24	143.59	145.22	135.52	128.34	0.11	<0.0001	0.83

p-values below p=0.05 were found to be significant

FIGURE 3. Phylogenetic Analysis of *Clostridium perfringens* 16S rDNA Sequencing Data in Broilers Before and After *C. perfringens* Challenge



NF – natural flora, PC – post challenge; 0, 1, 2, 3, 4, 5: control, 0, 2, 4, 6, 8 g/kg MOS, respectively. All other isolates were whole genome sequences taken from the NCBI database for comparison. Bootstrapping values determine the efficiency of the UPGMA algorithm used for clustering analysis.

TABLE 7. Summary of the *Clostridum perfringens* PCR Toxintopye Profile Before and After *C. perfringens* Challenge

Isolate	cpa	cpb	cpb2	cpe	cpIo	etx	netB	luxS	Type
				Challenge					
Field	+	-	+	-	-	-	+	+	A
				Normal flora isolates					
0g/kg	+	-	-	-	-	-	+	+	A
4g/kg	+	-	-	-	-	+	+	+	D
6g/kg	+	-	-	-	-	-	-	+	A
8g/kg	+	-	-	-	-	-	+	+	A
				Post-challenge isolates					
0g/kg	+	-	+	-	-	-	+	+	A
2g/kg	+	-	+	-	-	-	+	+	A
4g/kg	+	-	+	-	-	-	+	+	A
6g/kg	+	-	+	-	-	-	+	+	A
8g/kg	+	-	-	-	-	-	+	+	A
Control	+	-	+	-	-	-	+	+	A

Note: *C. perfringens* could not be isolated from the treatment group 2g/kg pre challenge
Toxin type: Alpha (cpa), beta (cpb), beta 2 (cpb2), enterotoxin (cpe), NetB, Epsilon (etx), Iota (cpIo), AI-2 (luxS).
+/- Weak positive results. Field Isolate describes the *C. perfringens* used for challenge.

All treatment groups showed signs of enteritis post-challenge (PC) on day 22; the 4g/kg MOS treatment group saw somewhat increased signs of disease as hemorrhagic enteritis was noted. The signs noted included enteritis, intestinal hepatic inflammation and distension of the proventriculus.

Histology. Histological measurements of villi height (Figure 4), varied in the area of the ileum where the height at 8g/kg MOS was significantly lower at Day 22. There was also a tendency toward greater villi heights on Day 22 at 2 g/kg as shown in Figure 4. Significant interaction effect was seen in villi width where both MOS level and day effect

played a part in changing this measurement of gut architecture (Figure 5). Goblet cell per unit area was significantly different in the area of the duodenum where much lower levels were noted on day 11 at 0 and 8 g/kg (Figure 7). Crypt depth remained unaffected within this experiment (Figure 6). Differences between day 11 and day 22 were significant in all measurements of gut morphology. All numerical data is shown in tables 8 and 9.

Spleen to bursa ratios were unaffected by MOS levels (Figure 8, Table 10).

Lymphocyte numbers in the germinal centres pre inoculation were not found to be significantly different between treatments (Figure 9). Post inoculation treatment MOS level of 6g/kg was found to have significantly lower lymphocyte numbers per square millimetre.

The results of qRT-PCR for a significant difference in the ileum post inoculation where the treatment with 4g/kg MOS inclusion had higher TLR2 gene expression levels. Pre inoculation ileum effects and both pre and post inoculation cecal tonsil levels were under affected. No significant difference was seen between pre and post inoculation TLR2 levels in either the ileum or cecal tonsil. Results of the qRT-PCR testing TLR4 expression showed variation in the preinnoculation ileum values between treatments 2g/kg and 4g/kg MOS. Though no significance was noted in the post inoculation expression of TLR4, 2g/kg MOS tended to have a greater expression level (Figures 10, 11, 12 and 13).

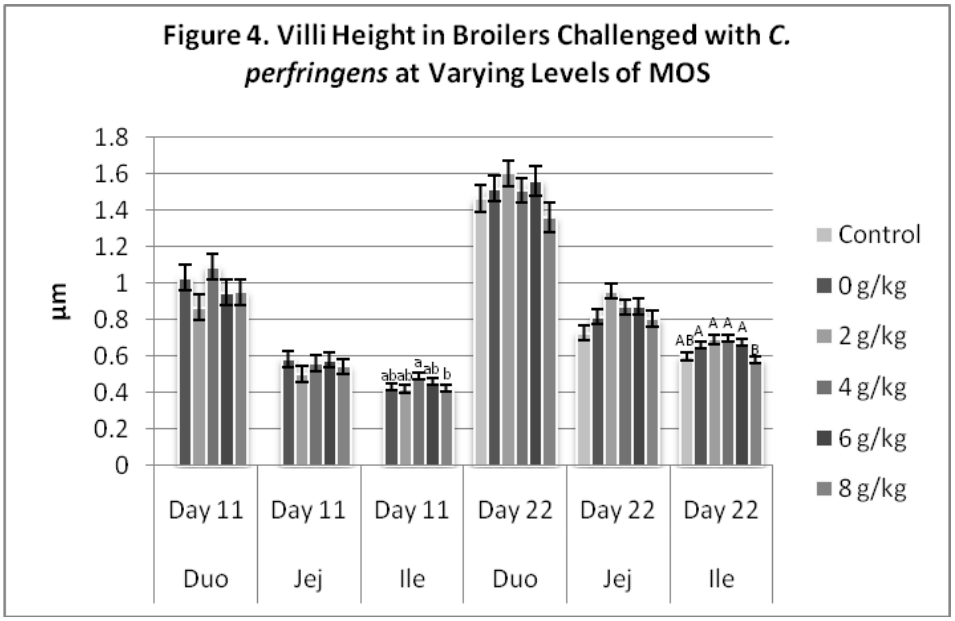


Figure 4. Villi height in broilers given varying levels of MOS on Day 11, pre-challenge with *C. perfringens* and Day 22, post-challenge. Duo= duodenum, Jej= jejunum, Ile= ileum. Control on Day 22 may be compared to 0g/kg on Day 11.
Bars with different subscripts differ (p=<0.05)

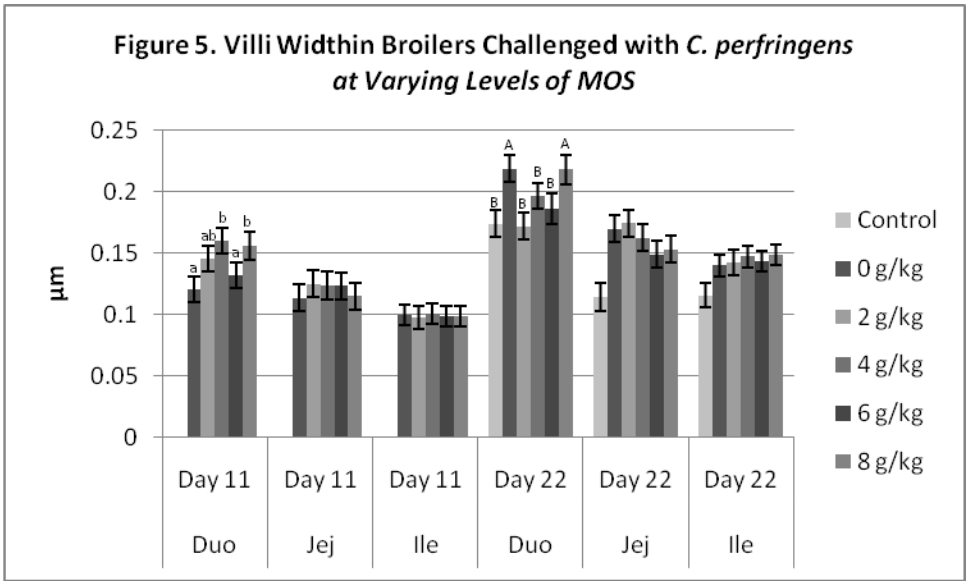


Figure 5. Villi width in broilers given varying levels of MOS on Day 11, pre-challenge with *C. perfringens* and Day 22, post-challenge. Duo= duodenum, Jej= jejunum, Ile= ileum. Control on Day 22 may be compared to 0g/kg on Day 11.
Bars with different subscripts differ (p=<0.05)

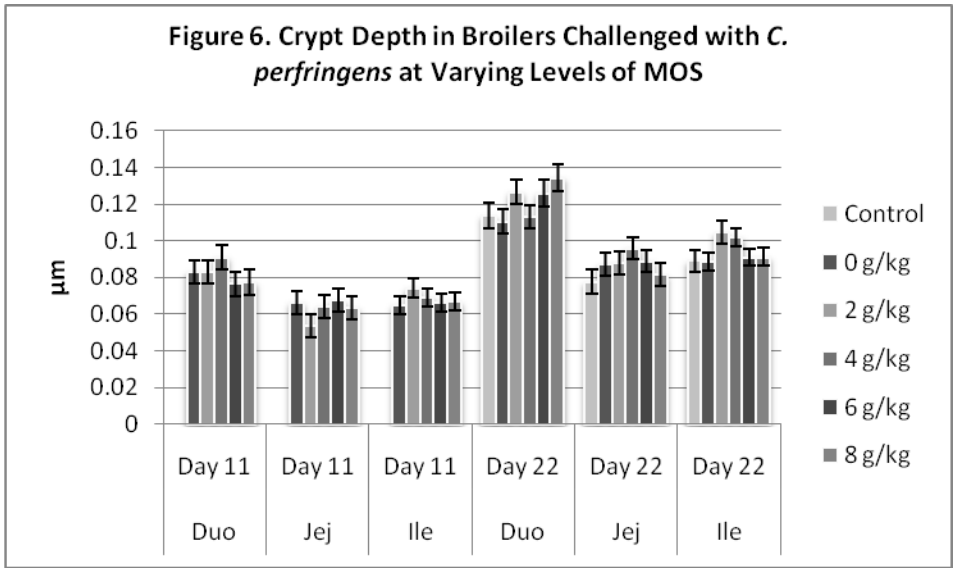


Figure 6. Crypt Depth in broilers given varying levels of MOS on Day 11, pre-challenge with *C. perfringens* and Day 22, post-challenge. Duo= duodenum, Jej= jejunum, Ile= ileum. Control on Day 22 may be compared to 0g/kg on Day 11.
Bars with different subscripts differ (p=<0.05)

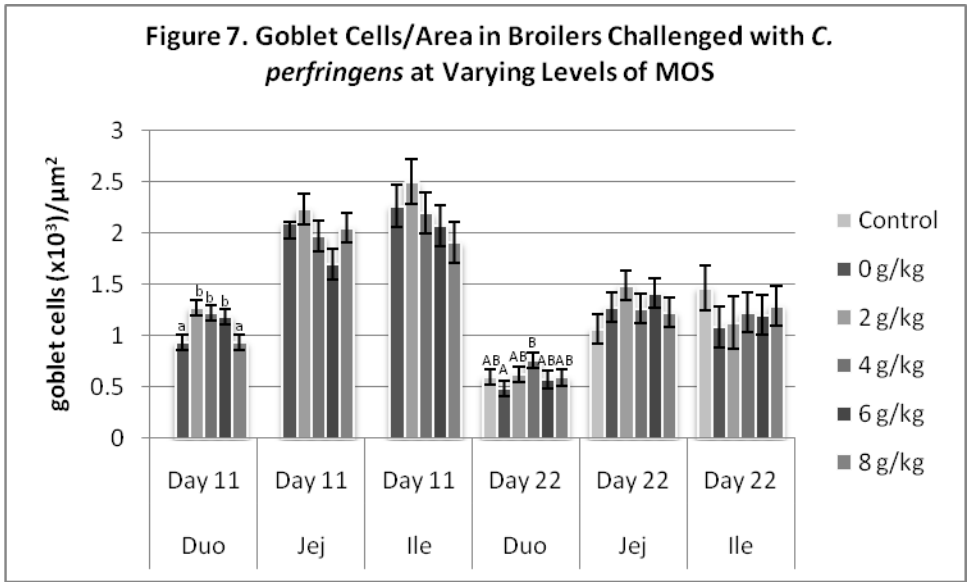


Figure 7. Goblet Cells/Area in broilers given varying levels of MOS on Day 11, pre-challenge with *C. perfringens* and Day 22, post-challenge. Duo= duodenum, Jej= jejunum, Ile= ileum. Control on Day 22 may be compared to 0g/kg on Day 11.
Bars with different subscripts differ (p=<0.05)

Table 8. Effect of MOS and sampling location on villous height, width, crypt depth and goblet cell/area in broilers challenged with *C. perfringens*

	Treatment																	
Variable	Control			0g/kg			2g/kg			4g/kg			6g/kg			8g/kg		
	D	J	I	D	J	I	D	J	I	D	J	I	D	J	I	D	J	I
VH, mm	1.465	0.073	0.599	1.275	0.710	0.545	1.238	0.732	0.559	1.294	0.717	0.592	1.258	0.722	0.569	1.163	0.684	0.500
VW, mm	0.174	0.114	0.116	0.170	0.142	0.117	0.158	0.147	0.121	0.173	0.142	0.120	0.157	0.138	0.122	0.188	0.133	0.124
CD, mm	0.114	0.078	0.094	0.095	0.078	0.078	0.105	0.069	0.089	0.103	0.080	0.086	0.097	0.078	0.077	0.108	0.072	0.079
GC/VA	0.598	1.066	1.464	0.766	1.621	1.782	0.964	1.670	2.054	0.991	1.586	1.770	0.881	1.512	1.803	0.787	1.665	1.601

Table 9. P-values for villous height, width, crypt depth and goblet cell/area in broilers challenged with *C. perfringens* at varying levels of MOS

	Day						P value								
Variable	11			22			Treatment			Day			Trt*Day		
	D	J	I	D	J	I	D	J	I	D	J	I	D	J	I
VH, mm	0.979	0.557	0.430	1.504	0.846	0.651	0.544	0.088	**0.001**	**<.0001**	**<.0001**	**<.0001**	0.127	0.234	0.195
VW, mm	0.144	0.153	0.099	0.220	0.161	0.138	**0.042**	0.013	0.264	**<.0001**	**<.0001**	**<.0001**	**0.001**	0.774	0.957
CD, mm	0.083	0.063	0.069	0.119	0.086	0.089	0.394	0.352	0.106	**<.0001**	**<.0001**	**<.0001**	0.102	0.646	0.612
GC/VA	1.1237	2.0165	2.2142	0.6258	1.1818	1.402	**0.016**	0.768	0.464	**<.0001**	**<.0001**	**<.0001**	0.0978	0.3762	0.7514

Table 10. Effect of the interaction of treatment and day on Spleen:Bursa as a percent of body weight in broilers challenged with C. perfringens at varying levels of MOS

Variable	Treatment						Day		P values			
	Control	0%	0.02%	0.04%	0.06%	0.08%	11	22	SEM	Trt	Day	Trt*Day
S:B	3.3812	3.0992	3.3171	3.1099	3.3943	3.38	2.99	3.39	0.00192	0.5981	0.103	0.9724

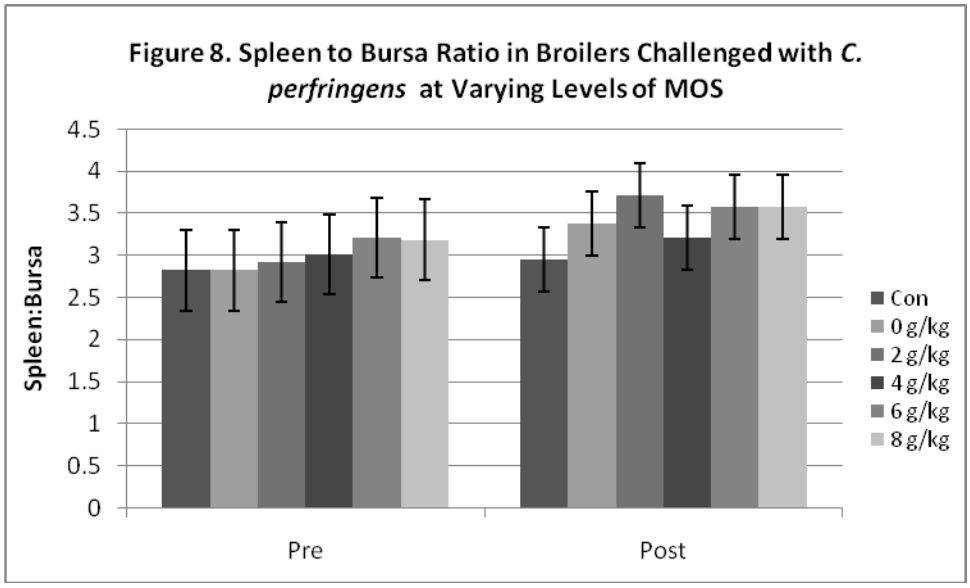


Figure 7. Spleen: Bursa in broilers given varying levels of MOS on Day 11, pre-challenge with *C. perfringens* and Day 22, post-challenge. Duo= duodenum, Jej= jejunum, Ile= ileum. Control on Day 22 may be compared to 0g/kg on Day 11.
Bars with different subscripts differ (p=<0.05)

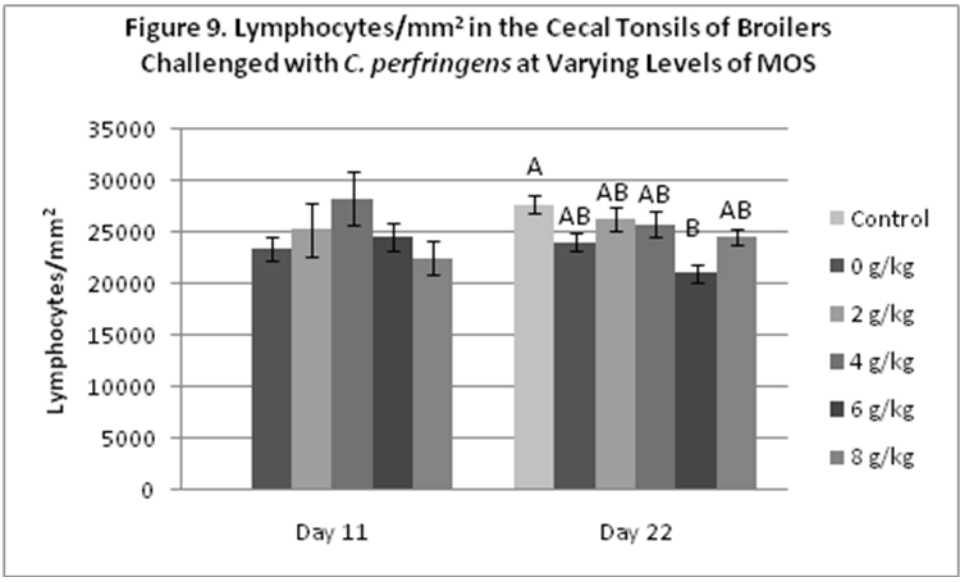


Figure 9. Lymphocytes/mm^2 in broilers given varying levels of MOS on Day 11, pre-challenge with *C. perfringens* and Day 22, post-challenge. Duo= duodenum, Jej= jejunum, Ile= ileum. Control on Day 22 may be compared to 0g/kg on Day 11.
Bars with different subscripts differ (p=<0.05)

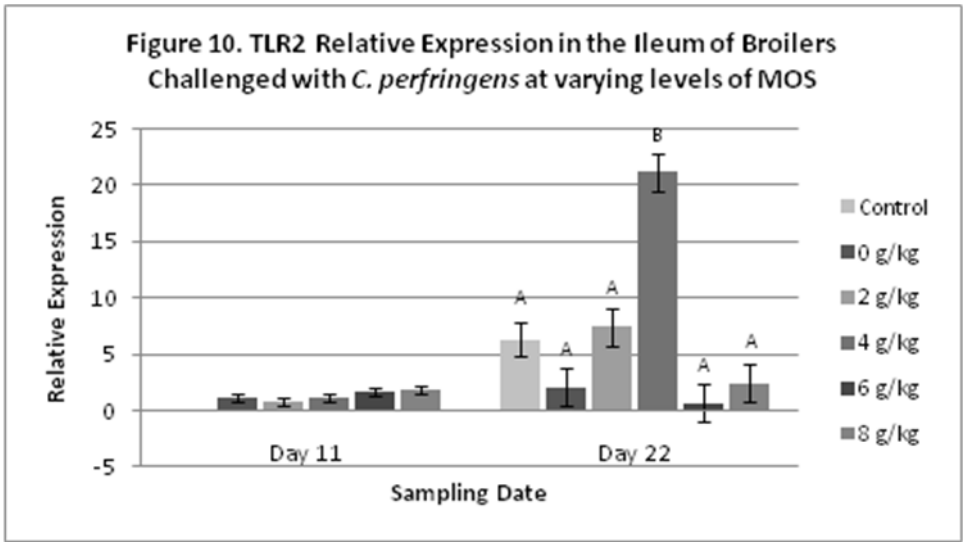


Figure 10.TLR2 Relative Expression in the ileum of broilers given varying levels of MOS on Day 11, pre-challenge with *C. perfringens* and Day 22, post-challenge. Control on Day 22 may be compared to 0g/kg on Day 11.
Bars with different subscripts differ (p=<0.05)

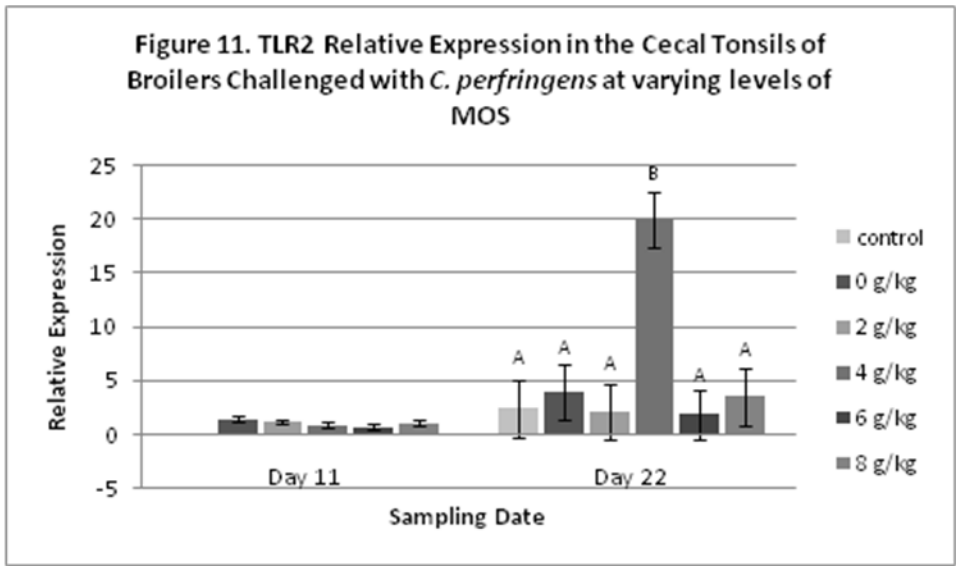


Figure 11.TLR2 Relative Expression in the cecal tonsils of broilers given varying levels of MOS on Day 11, pre-challenge with *C. perfringens* and Day 22, post-challenge. Control on Day 22 may be compared to 0g/kg on Day 11.
Bars with different subscripts differ (p=<0.05)

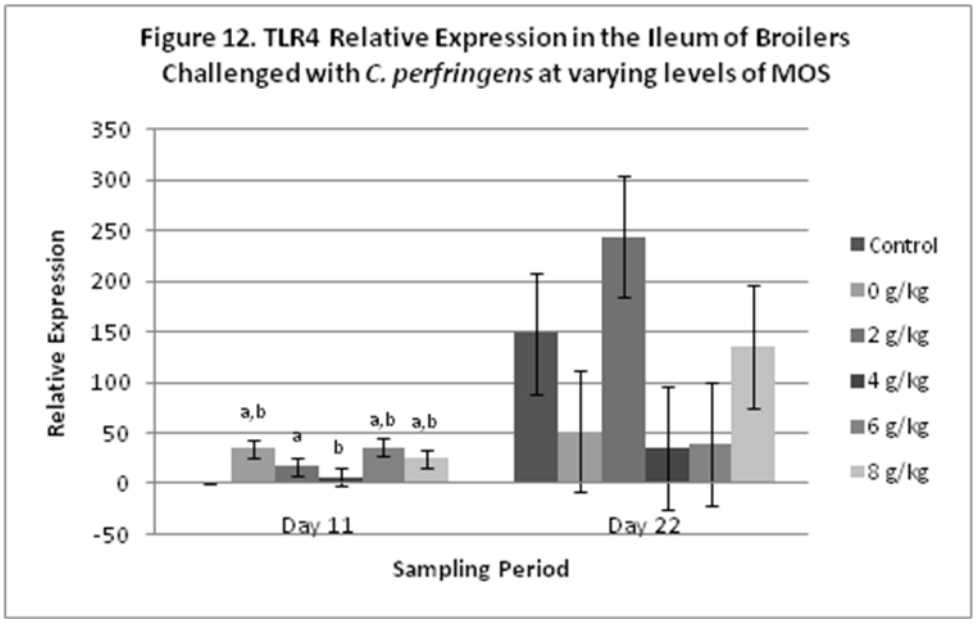


Figure 12.TLR4 Relative Expression in the ileum of broilers given varying levels of MOS on Day 11, pre-challenge with *C. perfringens* and Day 22, post-challenge. Control on Day 22 may be compared to 0g/kg on Day 11.
Bars with different subscripts differ (p=<0.05)

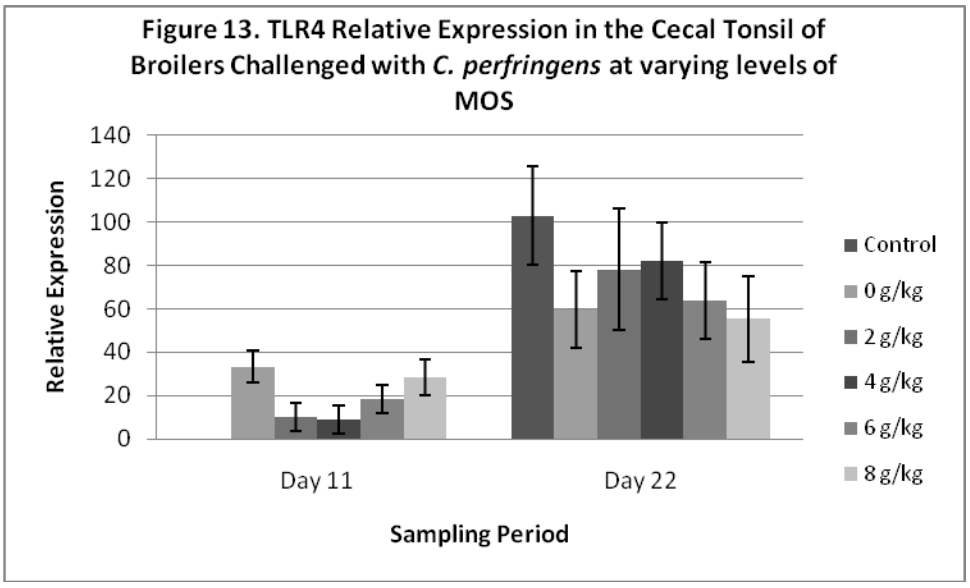


Figure 13.TLR4 Relative Expression in the cecal tonsils of broilers given varying levels of MOS on Day 11, pre-challenge with *C. perfringens* and Day 22, post-challenge. Control on Day 22 may be compared to 0g/kg on Day 11.
Bars with different subscripts differ (p=<0.05)

4. Discussion

No significant difference in feed efficiency was noted between treatments, this same lack of effect was also noted by Hofacre et al (2003). On the other hand, many researchers have found that MOS does tend to increase body weight and improve feed efficiency, this effect is often most obvious in the first three weeks (Parks et al 2001; Yang et al 2007; Yang et al 2008). It seems that the effect of MOS is highly dependent on other inputs such as management and feed.

Most isolates were found to be Type A, the most common form found in poultry, though Type C is also found (Tschirdewahn et al 1991). Type D was found in 4g/kg MOS treatment group pre inoculation, this is more commonly associated with ruminants (Itodo et al 1986). It is more likely that this type was already present in the experiment room which was previously used for cattle allowing for colonization of the birds through contact with the floor. The post inoculation 8g/kg MOS was cpb2 negative, this is interesting as it is both different from the natural flora (NF) findings as well as the feild isolate (FI) used for inoculation. The natural flora isolate treatment of 6g/kg MOS was the only isolate found to be netB negative, this changed after challenge, probably due to a different population colonizing the gut after the influx of new *C. perfringens* isolations. This is interesting as pathogenic forms tend to displace non-pathogenic forms and netB has been shown to contribute to pathogenecity (Barbara et al 2008; Keyburn et al 2008). Low levels of enteritis were noted in all treatment groups post challenge. The decreased

pathogenic effects of *C. perfringens* despite being from a known disease causing source is consistent with Manuscript 1 as well as with other authors who also noted this difference (Olkowski et al 2006).

An increase in length and width may attribute to increased nutrient uptake and digestion (Yang et al 2007). Villi height variation in the ileum is a similar result to that seen in turkeys given MOS (Solis de los Santos et al 2007). Though, it is the opposite effect noted by other researchers where villi height was notably increased in the duodenum and/or jejunum but not ileum (Savage et al 1997; Baurhoo et al 2007; Iji et al 2001). Villi height also showed a tendency to be greatest on day 22 at 2g/kg MOS, the recommended standard (Rosen 2007; Yang et al 2007). This tendency was more obvious post challenge, possibly due to the increased potential to produce an effect during times of stress. Villi width was influenced by the interaction effect of day and MOS level. Information on MOS effect on villi width is limited but one researcher did note significance at 0.11% MOS, thus a treatment effect may have been found had we gone above 8g/kg (Savage et al 1997).

Goblet cell per unit area describes the level of potential mucin production which may act to defend the intestinal surface from pathogens. Differences noted in the goblet cell per unit area were seen at day 11 in the duodenum at the lowest and highest levels of MOS additive. Reverse peristalsis in the duodenum allows this section of intestine to maximize its use of nutrient and additives such as MOS which may explain why the effect is only seen in the duodenum (Petersen et al 1999).

Crypt Depth changes can signal different things, an increased crypt depth could mean a greater gut maturity and potential for gut health by mucin production but could also be negative in that it may mean rapid tissue turn over, fewer absorptive cells and has been associated with toxin levels (Yang et al 2007; Choct 2009). In this trial there were no significant differences in crypt depth between MOS groups though the crypt depth did increase as expected over time due to gut maturity.

The only variation in lymphocyte numbers was a dip at 6g/kg post infection, generally, lymphocyte proliferation would be expected in the hindgut post challenge rather than a decrease. The decrease in lymphocyte numbers at 6g/kg post-infection may demonstrate control of C. perfringens through agglutination or the blocking of bacterial binding sites which thereby decreased germinal center activation and lymphocyte proliferation expected during bacterial challenge. Lymphocyte stimulation associated with MOS in weaned piglets has been noted, physiological variation between mammalian and avian species may account for the lack of systemic effects found in this trial (Notchi et al 2009).

Differences in spleen and bursa weights may indicate an effect on B and T lymphocyte populations as these are primary immune organs from which these cells originate (Qureshi et al 1997). No effect on spleen to bursa ratio was noted between MOS groups meaning that B and T lymphocyte production was not markedly increased. This demonstrates that MOS does not cause systemic effects on the adaptive immune system which would have caused lymphocyte proliferation in the primary and secondary immune organs.

TLR2 was chosen for evaluation based on its ability to detect ligands from gram positive bacteria such as *C. perfringens* (Fukui et al 2001). TLR2 tended to have the most variation post inoculation, there was very little difference in TLR2 production in pre inoculation results. It is interesting to note that there was very little change to TLR2 levels between sampling times as one could expect changes during immune maturation. Though many TLRs are up regulated during CP infection, TLR2 types 1 and 2 are more strongly so than others not directly in defence of gram positive bacteria such as TLR4 (Lu et al 2009). Post infection, TLR2 tended to be greatest in the treatment receiving 4g/kg MOS. This effect was seen in the ileum for TLR4 at 2 g/kg, industry standard. In the ileum pre-inoculation, TLR4 expression at 2g/kg and 4g/kg MOS were significantly different from each other, possibly because gram negative bacteria were more efficiently controlled by MOS at 4g/kg in the ileum pre-inoculation. 4g/kg TLR4 levels remained low post inoculation, whereas the levels for 2g/kg spiked post inoculation, though this may mean 2g/kg MOS was less able to suppress gram negatives it could also be due to a pen effect causing variation in the resident microflora. It was surprising to see that TLR4 had higher relative expression levels than TLR2 as *C. perfringens* is gram positive and therefore contains a TLR2 ligand. It has been theorized that *C. perfringens* does not have to attach to the epithelium to cause damage; this means necrotic enteritis could occur without stimulation of tissue based TLR2 and may account for the low levels seen in this experiment (Olkowski et al 2008). It is expected that Gram negative bacteria in the intestine may be causing TLR4 upregulation. As this effect was noted pre and post infection, this does not necessarily point to full blown infection caused by gram negative bacteria as the intestine is in a constant state of inflammation and may only be the effect

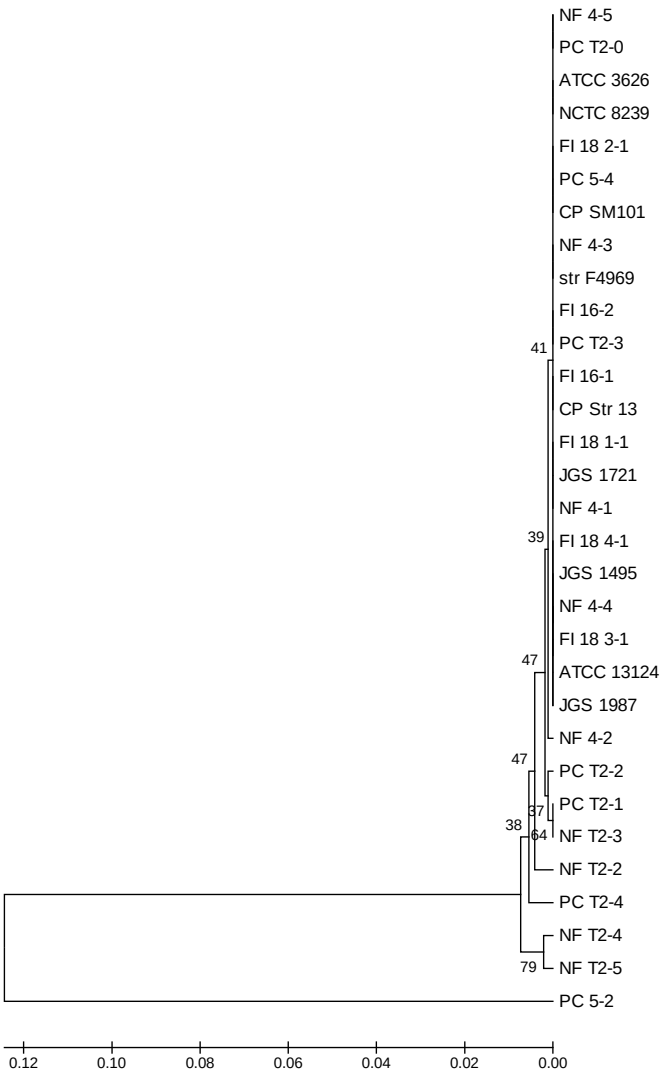
of higher levels. It also seems that the effects by MOS on the intestinal immune system are localized effecting only the lumen and perhaps tissue in direct contact.

GENERAL DISCUSSION and CONCLUSIONS

To understand a complete picture of the bacterial relationships between the two studies, a phylogenetic tree containing both sets of results was compiled (Figure 15). The largest group arranged by 16S sequencing contained all NCBI genome isolates as well as most of the isolates from the past experiment and two post challenge isolates from the current experiment. Of the isolates contained in this group from the current experiment only one was from the treatment group of 6g/kg MOS and the other a control which received neither MOS nor the bacterial challenge and can therefore be considered a model of the natural flora for that group of broilers. Natural flora and post challenge isolates from treatment 4g/kg MOS of the past experiment were not highly related to the main group. This post challenge isolate was described as separate from the main group in the last paper which coincides with the current information. The natural flora isolate had not been shown as separate previously but it is possible that the greater number of isolates for comparison allowed for more specific results, excluding this isolate out of the main group. In the other isolates, some pairs were formed but were an unusual mix of PC and NF isolates where none of these pairs were from the same treatment. It is likely that a mix of *C. perfringens* isolates were originally available in the gut, but were largely displaced when competitive pathogenic forms of *C. perfringens* were introduced. It should be noted

that only two of the PC isolates took on strong genetic resemblance to the field isolate used for inoculation, possibly meaning they were the only two treatment groups were sufficiently colonized or that these isolates already lived in the gut but were then increased in levels post inoculation.

FIGURE 14. 16S Phylogenetic Analysis of *C. perfringens* isolates from both Manuscript 1 and 2.



NF: Natural Flora, FI: Field Isolate, PC: Post Challenge; T2: Manuscript 2 isolates

Positive isolation of Types D and E *C. perfringens* were related to past use of room by other species. In a commercial setting this would be extremely unlike but speaks to the importance of farm history as a factor in pathogenesis. As a spore forming bacteria, *C. perfringens* can be very difficult to remove from the environment, increasing chances of repeat exposure (Craven et al 2010). All post-challenge isolates were Type A *C. perfringens* and netB positive. NetB has been associated with pathogenic forms of *C. perfringens* which have been shown displace non-pathogenic strains; this might explain the increase in isolates containing this gene (Keyburn et al 2006; Barbara et al 2008). In this case, netB was found in both the natural flora and post challenge isolates, as no signs of disease were noted in the natural flora isolates, this research does not support findings that netB is sufficient to induce necrotic enteritis (Keyburn et al 2006).

The differences between pre challenge and post challenge analysis of C. perfringens isolates via both toxinotyping and phylogenetic analysis shows that a change in the microbial population did occur, thus this methodology was sufficient to direct gut microbial populations.

This research does not support the hypothesis that MOS has a direct effect on *C. perfringens*; based on our findings, it is more likely this effect may again be dependent on outside factors (Yang et al 2007; Hofacre et al 2003). Assessment based on *C. perfringens* enumeration may have helped to define the results of MOS on the diet of challenged broilers. As noted in Manuscript 1, necrotic enteritis is difficult to reproduce experimentally which may have portrayed the results inaccurately.

The effect of MOS on various areas of the gut varied between measurements and areas of the intestine. What caused a reaction in one area did not necessarily cause the same effect in the rest of the intestine. This inconsistency may be associated with infection or with the MOS supplementation; future studies may be able to determine which exact input was responsible for this.

Though MOS was associated with the upregulation of TLR2 at 4g/kg it was insufficient to boost toll-like receptor 4, lymphocyte stimulation in the cecal tonsils or drive change in the spleen and bursa. Based on this and the lack of consistent change in gut morphology and *C. perfringens* isolates between treatments shows that MOS was insufficient to drive major changes in gut health and immunity. It appears that any effect found in this case could be more directly related to luminal effects and potentially changes in the adjacent tissue but no systemic effects could be noted. As *C. perfringens* does not need to attach to the intestine to cause damage it is possible that the increases in TLR2 are from macrophages returning to the tissue from the lumen for antigen presentation (Olkowski et al 2008). Being able to separate the source of TLR2 would help to define which parts of the immune system play the biggest role in changes caused by MOS.

Due to the great variation in MOS outcomes noted between this and other studies, it seems that MOS supplementation is highly dependent on other effects. It has been found that the effectiveness of MOS hinges on effects such as major diet ingredients, based on the variation between studies this seems to very likely be the case (Yang et al 2008).

A recurring issue found in literature review as well as unpublished trials taken on by this group is that it is extremely difficult to reliably reproduce necrotic enteritis under experimental conditions. The lack of reproducibility makes it difficult to properly assess outcomes in other sources of NE work and compare it to our own. As well, to understand the pathogenesis of NE in organic systems it is important to develop an *in vivo* model that allows reproducible results associated with the production of both strain behavior and clinical signs and lesions of NE.

It would be worthwhile to study numeric changes in *C. perfringens* populations which receive MOS before and after challenge and compare to unchallenged control groups in order to better determine the effect of MOS on necrotic enteritis control. As well, a more in depth look at 4 g/kg MOS, which lead to significant effects in this work, and 2 g/kg , which lead production improvements found by other researchers, would be warranted to determine if these effects are highly concentration dependant. Further studies looking at MOS as a part of the feeding regime from a more holistic point of view could help to maximize the positive effects it produces as they appear to be greatly dependant on outside factors.

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