EFFECT OF WHEAT AND CORN ON THE PROLIFERATION OF *CLOSTRIDIUM PERFRINGENS* TYPE A AND THE PREVALENCE AND IMPORTANCE OF *CLOSTRIDIUM PERFRINGENS* IN BROILER CHICKENS IN SASKATCHEWAN

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GENERAL ABSTRACT

This work was carried out in two phases. The first was to examine the effect that the cereals, wheat and corn and their major components have on the *in vitro* proliferation and alpha toxin production by *Clostridium perfringens* type A (CP). The second phase was to determine the prevalence of CP in broiler chickens in Saskatchewan and finally, what factors may be involved for the increased susceptibility of broilers on farms to CP, and what consequences to the production cycle affected flocks may have when infected with CP. In the first experiment, CP was grown *in vitro* in thioglycollate media (TG) alone, TG plus pancreatin and pepsin (TGE) or TG plus *in vitro* digested corn (C) or wheat (W) supernatant in a 2:1 ratio. Colony forming units (CFU) were counted and alpha toxin activity (U) was measured from each group using a commercially available kit. There was significantly more proliferation when CPA was grown in W compared to C, TG, or TGE. Alpha toxin production was significantly higher in C compared to W or TGE but no significant differences were found in U/CFU between TG and TGE, C or W. To further isolate the specific component in the corn and wheat that made the difference in the proliferation and alpha toxin production, a second experiment was conducted to examine this *in vitro* phenomena in either the digested or non-digested protein, fat or carbohydrate fractions of each. *In vitro* proliferation of CP was reduced when this bacterium was grown in digested corn gluten meal (CGM) compared to non-digested CGM, as well as digested or non-digested wheat gluten (WG) and the control media, thioglycollate (TG). Digestion of wheat and corn oil and starch reduced proliferation significantly, when compared to the non-digested components of these cereals. Alpha toxin production was increased when proliferation was inhibited in all cases. A dipeptide that has been identified in CGM hydrolysate, Alanine-Glutamine, was shown to reduce CPA proliferation when mixed with TG at 0.5%. The second phase of this study involved

examining the prevalence of CP in broiler chickens over the span of a full year. There were two sampling periods involving up to 41 barns per period. Prevalences of CP and anti-alpha toxin antibodies were evaluated in the birds at hatch and at slaughter. CP prevalence in the flock was also determined at mid-production and in starter feed samples. CP was present in 178/1440 (12.4%) of birds (57/738 (7.7%) for winter/spring and 121/702 (17.2%) summer/fall) between 18 – 28 days of age and occurred in 534/3000 (17.8%) of birds (208/1520 (13.7%) for winter/spring and 326/1480 (22%) for summer/fall) at slaughter. CP was not isolated from chicks at hatch. CP could be isolated from 76% of feed samples. Although not all birds had titres, 175/769 (22.8%) of hatched chicks and 303/1392 (21.8%) of broilers tested at slaughter peaked at an anti-alpha toxin antibody titre of 1:64. Finally, an evaluation of the influence of management practices on the occurrence of CP in broiler chicken production in the province of Saskatchewan was conducted over the same full year. Using a multilevel mixed model to account for unmeasured factors associated with the breeder flock, the broiler producer and the barn where the flock was raised, factors associated with the isolation of CP included anti-alpha toxin antibody (AATA) titres at hatch and at slaughter. Barn factors that influenced the frequency of CP isolation included relative humidity, CO_2, floor type and barn type. Feed form, feed supplier, contamination of starter feed and in feed antimicrobials also affected CP isolation. In addition there was a difference in CP isolation when bird strain was compared. There was a positive correlation between isolating CP from feces during mid-cycle production and isolating CP at slaughter. The effects of CP infection included an increase in lesion score with decreasing isolation of CP. There were no significant effects of CP infection on whole carcass or liver condemnations, total flock mortality or birds arriving dead at the plant, nor was there an effect on total weight gain or feed conversion. It is concluded that corn gluten can have an effect on the

proliferation and alpha toxin production of CP and that this component could explain the reduced incidence of necrotic enteritis (NE) in broiler chickens in the field. Further examining the effect of various corn and wheat varieties used in the production of poultry feed is warranted. CP was found to be present in broiler chickens in Saskatchewan, even with the incorporation of antimicrobials and/or anticoccidial medications in the feed. As well, there are a number of factors that can affect the isolation of CP from broiler chickens either at the barn level (mid production) or at slaughter, and these factors should be examined more closely in an attempt to complement other means to reduce the incidence of CP in broiler chickens and ultimately, reduce the incidence of NE.

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I dedicate this thesis to my late father, who always said he knew that I could do it.

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1. INTRODUCTION

Clostridium perfringens (CP) is believed to be ubiquitous in the environment, and along with *E. coli*, considered to be one of the first colonizers of the chick gut (Smith, 1965). Since 1961, CP has been designated as the bacterium responsible for causing necrotic enteritis (NE) (Parish, 1961a; Parish, 1961b; Parish 1961c). NE is considered to be an economically significant disease in poultry (Van der Sluis, 2000) with manifestations of acute mortality (Nairn & Bamford, 1967; Long, 1973), poor performance (Stutz & Lawton, 1984; Kaldhusdal & Hofshagen, 1992; Lovland & Kaldhusdal, 2001) and increased condemnations at slaughter (Lovland & Kaldhusdal, 1999; Lovland and Kaldhusdal, 2001). Although less well defined, it may be that subclinical NE can be overall more costly to the poultry industry than that of clinical NE. The exact mechanism of how CP infection develops into NE in the chicken remains unclear. However, NE has been shown to frequently occur in broilers experiencing mild to severe coccidiosis (Baba et al., 1997; Wages & Opengart, 2003).

Constituents of the broiler diet have been implicated as a reason for the apparent higher incidence of NE in chickens. Inclusion of high levels of animal protein in broiler diets has been shown to increase the recovery of *Clostridium perfringens* type A (CPA) from infected broilers suggesting that high levels of animal source protein may predispose to NE (Drew et al., 2004). As well, NE has been associated with diets consisting of high levels of wheat- or barley-based diets compared to a corn-based diet (Branton et al., 1987; Riddell & Kong, 1991; Kaldhusdal & Hofshagen, 1992; Takeda et al., 1995; Kaldhusdal & Skjerve, 1996; Branton et al., 1997). Previous work has shown that CPA has greater proliferation in wheat- and barley-based broiler grower supernatant compared to a corn-based supernatant (Annett et al., 2002). This study suggests that greater proliferation of this bacterium in wheat- or barley-based diet supernatant

may at least in part, explain the higher incidence of NE in broiler chickens fed a diet with a high proportion of wheat or barley. Alternatively, it may be that a high level of corn in broiler diets may reduce proliferation of CPA. The factors that influence the proliferation and alpha toxin production of CPA when grown in supernatants derived from corn or wheat remains elusive.

CPA produces a variety of toxins, and historically it has been suggested that the alpha toxin that this bacterium produces is responsible for the intestinal mucosal necrosis observed in affected chickens (Al-Sheikhly & Truscott, 1977; Fukata et al., 1988). Recently, however, the importance of alpha toxin and its association with development of NE has been questioned (Keyburn et al., 2006). Molecular work has revealed that there is another toxin, the netB toxin, produced by CPA that could be responsible for the development of NE in broiler chickens. Researchers have found though, that NE can be manifest in the absence of netB and in the presence of alpha toxin, leaving the latter as still a highly suspect cause of the disease (Songer and Cooper, 2010). However, the ability to reproduce the disease in the absence of a coccidial infection is still daunting to many workers in this field, and the ability of certain strains of CPA to produce lesions in infected chickens remains relatively unpredictable.

Currently, in Saskatchewan, CP is controlled in commercial poultry operations by the incorporation of antimicrobials and anticoccidial medications in the feed. With increased consumer awareness towards the possibility of transfer of antimicrobial resistance from animal to human pathogens, there is enhanced motivation for poultry producers to reduce or eliminate the utilization of antimicrobial growth promoters (AGPs) in poultry feed. In North America there is movement towards the withdrawal of prophylactic antimicrobials from poultry feed. As a result, it is anticipated that the occurrence of both clinical and subclinical NE will increase, as it did in the European Union, following the ban of many AGPs in poultry feed (Grave et al., 2004).

The prevalence of CP in broiler chickens with current management practices, some of which may demonstrate the reduction of CP infection and/or the occurrence of NE such as co-infection with coccidiosis (Al-Sheikhly & Al-Saieg, 1980; Shane et al., 1985), type of diet (Annett et al., 2002; Kaldhusdal & Skjerve, 1996; Truscott & Al-Sheikhly, 1977; Branton et al., 1987; Riddell & Kong, 1991; Kaldhusdal & Hofshagen, 1992; Takeda et al., 1995; Branton et al., 1997), seasonal effects (Kaldhusdal & Skjerve, 1996; Long, 1973), feed form (Branton, et al., 1987) and prevention through vaccination (Heier et al., 2001; Lovland et al., 2004) should be examined. With this information at our disposal, we would be able to better determine if management changes would affect the prevalence of CP and ultimately, the effect that these practices have on the incidence of either clinical or subclinical NE in broiler chickens raised in Western Canada once AGPs are removed from broiler diets. The following work was conducted in anticipation that the laboratory work that was performed – looking at how diet might affect CP proliferation and/or toxin production - could be extrapolated to, and be implemented in the field. Alternatively, the epidemiology portion of this study was performed as a method to determine if there are associations between certain measured factors that could be examined more closely in the laboratory setting.

2. LITERATURE REVIEW

2.1. *Clostridium perfringens*

Clostridium perfringens (CP) is a gram positive, spore-forming, anaerobic, large rod bacteria, which is present in the environment worldwide (Willis, 1969). This organism is divided into toxin types based on the major toxin that they produce. All CP bacteria produce alpha toxin. In CP type A (CPA) alpha toxin is the only toxin produced, type B produces beta and epsilon toxins; type C produces beta toxin; type D produces epsilon toxin and type E produces iota toxin (Rood & Cole, 1991). All CP can cause disease in farm animals, however, only CPA and CP type C have been reported to cause disease in poultry (Wages and Opengart, 2003). Crespo et al. (2007) found that all isolates, that were retrieved from necrotic enteritis- or ulcerative enteritis-suffering birds, and subsequently examined using multiplex PCR were CPA, regardless of avian species or disease history of the host. Similar findings were reported by Das et al. (2008), who examined two to three week old broilers that had died from NE. All isolates from this case were reported to be CPA. Interestingly, when Justin et al. (2002) examined the structural and kinetic properties of alpha toxin derived from an avian isolate, they found that the toxin had different substrate specificity and membrane binding properties compared to alpha toxins derived from other animal species.

The toxins produced by CP are enzymatic products of genes. Whereas CPA has the plc gene for alpha toxin only, other types have genes for their respective toxins as well (Canard & Cole, 1989). Polymerase Chain Reaction can therefore, be used to rapidly identify CP type.

The CP alpha toxin gene is chromosomal, whereas other major toxin genes (β, ε, ι) are located on plasmids (Rood, 1998). It is believed that the alpha toxin gene can be induced by an

unknown environmental stimulus (Lyristis et al., 1994; Rood & Lyristis, 1995), and this stimulus could help to explain alpha toxin production and subsequent disease caused by CPA.

The alpha toxin produced by CPA is thought to be the primary pathogenic mechanism by which these bacteria cause disease (Al-Sheikhly & Truscott, 1977), and is described as the most potent toxin of CPA (Songer, 1997). Alpha toxin is a lecithinase (Phospholipase C), which splits lecithin (phosphotidylcholine) at the number three position producing phosphorylcholine and diglyceride. Since cell membranes are composed of lipid bilayers, numerous phosphotidylcholine molecules can be affected by the presence of the alpha toxin. The effect of enzyme activity is reduced integrity of the lipid bilayer membrane leading to altered ionic concentrations, ballooning degeneration and eventually necrosis. Current knowledge suggests that alpha toxin may only initiate the lipid bilayer splitting, and that this mechanism somehow stimulates the cell's own phospholipase C to cause more damage, leading to an autocatalytic self-destructing mechanism (Sakurai et al., 1993).

Other important, although minor toxins produced by CPA include theta toxin (perfringolysin) and kappa toxin (collagenase), both of which may play roles in the pathogenesis of disease caused by this bacterium. The kappa toxin may play a more important role in the development of necrotic enteritis (NE) than originally thought. When Al-Sheikhly & Truscott (1977a & 1977b) infused CPA supernatant into the intestine of broiler chickens, they assumed that the resulting necrosis was due to alpha toxin only. However, they did not evaluate the supernatant for the presence of either theta or kappa toxin. Other work (Fukata, et al., 1988) performed on germ-free chickens evaluated the effect of alpha toxin on incidence of mortality. The comparison was made using anti-alpha-toxin, and they found that there was higher, although not significant, mortality in 2 day old chicks, orally gavaged with CP compared to similar treated

birds that received anti-alpha-toxin serum. These authors only recorded mortality, and did not perform post-mortem examinations to confirm that death was due to treatment. As well, the presence of theta and kappa toxins was not determined, and their role in the pathogenesis of the disease remains unknown.

Subsequently, the importance of alpha toxin and its association with development of NE has been questioned (Keyburn et al., 2006). Using 11 groups of birds, of which two were *plc* mutants, these researchers reported a lack of correlation between alpha-toxin production and severity of disease. This is not surprising, considering the difficulty in reproducing NE without corresponding predisposing factors, such as co-infection with *Eimeria spp.* These conclusions were supported by the lack of clear differences in lesion scores in birds infected with *plc* mutants (lacking alpha toxin production ability) and wild type (*plc* producing ability). Although these data are suggestive that alpha toxin may not be an important factor in NE, the ability to produce NE lesions in infected birds is unpredictable.

In more recent work, 70% of CP isolates, from chickens with NE were reported to carry the netB gene (Keyburn et al., 2010). This gene was also present in CP isolated from healthy chickens. In this study, the netB gene was also demonstrated to produce the NetB toxin, which has now become highly correlated with the presence of NE in broiler chickens (Keyburn et al., 2006). Of interest is that netB has not been reported to be present in CP isolated from other avian species, although NE can also be manifest in other species such as wild and domestic geese (personal observation), Japanese quail and turkeys (Opengart, 2008).

When a mutant strain (cpa mutant) of CP was constructed so that it could be differentiated from wild-type strains, Coursodon et al (2010) found that they could detect alpha toxin from experimental host birds, as well from non-infected trial birds. These authors found a

direct relationship between NE lesion severity and the amount of alpha toxin detected, suggesting that alpha toxin does play an important role in the pathogenesis of NE. They also suggested that although broilers were inoculated with a CP mutant, it may be the resident gut CP that is producing increased levels of alpha toxin when tested.

De Cesare et al (2009) showed that a vast variation of ribotypes of CP can be isolated from either the same or different birds within or among varying flocks. This information may help to explain the differences in disease occurrence and severity when CP is isolated from either sick or apparently healthy birds in the field. Differing ribotype could very well explain the variation in other toxin production (e.g. Theta toxin) as well.

The importance of theta toxin activity has been demonstrated in a gas gangrene model, where mice were infected with either lethal or sub-lethal doses of CPA (O'Brien & Melville, 2004). In this study, the researchers found that only alpha toxin was necessary for the onset of myonecrosis when lethal doses of bacterium were administered to the host. However, if sub-lethal doses of the bacterium were given, then both theta toxin and alpha toxin were necessary to maintain CPA survival in the tissue. So, it may be possible that if certain ribotypes of CPA are lacking theta toxin activity (or perhaps another toxin of influence), then the ability of CPA to cause disease in the host may be altered. The influence of other intestinal microbes on the proliferation of CPA and their effect on activity or degradation of alpha toxin should thus be explored. It has been proposed that the alpha toxin may have differing configuration at the carboxy-chain thereby issuing variable toxicity in the enzyme (Titball et al., 2000). Others have suggested that the toxin activity of some strains with identical alpha toxin sequences may be due to differences in strain growth rate or expression rather than to differences in specific activity (Sheedy et al., 2004).

Lanckriet et al. (2010b) compared the protection potential of the supernatants of eight chicken strains of CPA, differing in level of alpha toxin production and the presence of the netB gene. They found that the supernatant from only one strain provided protection against NE when broiler chickens were challenged with virulent CPA. These workers concluded that NE is not determined only by the presence of alpha toxin or by the presence of the netB gene, and that the one protective supernatant in this trial was likely a result of the isolate producing some unknown combination of immunogenic compounds.

Kulkarni et al. (2007) immunized broiler chickens with various CPA immunogenic secreted proteins and found that when these same birds were inoculated with a mild or severe challenge of CPA, certain proteins offered superior protection against NE compared to others. More specifically, alpha toxin, hypothetical protein and pyruvate: ferredoxin oxidoreductase significantly protected the chickens against a heavy challenge, whereas glyceraldehyde-3-phosphate dehydrogenase and fructose 1, 6-biphosphate aldolase significantly protected the birds only against mild challenge. The fact that there are numerous secreted proteins that offered protection against challenge, suggests that alpha toxin may not be the only important protein involved in the protection and pathogenesis of NE.

Of all positively identified CPA, it is estimated that between 5% (Chakrabarti et al., 2003) to 7.3% (Bueschel et al., 1998) also have the presence of the enterotoxin gene (ent) which produces a toxin that is released upon bacterial sporulation (Rood, 1998). This toxin is responsible for approximately 75% of all human enterotoxemic food poisonings. The importance of this toxin in poultry production is its association with contamination of meat during processing and subsequent ingestion and illness in people (Rood, 1998). Craven et al. (2001) found that the proportion of CPA-positive carcasses from contaminated flocks ranged

from 8% to 68% (mean 30%), suggesting that enterotoxemia in humans could potentially be initiated from poultry products.

Reproducibility of NE has been an ongoing challenge for scientists wanting to investigate causes and prevention of this devastating disease. Recently, work focusing on toxins, other than alpha toxin, have been examined extensively. Ohtani et al. (2009) examined the possibility of CP cell-cell signaling to initiate and propagate toxin production. These workers found that the agrBDCp gene is involved in the positive regulation of alpha-, kappa-, and theta-toxin genes through quorum sensing that involves a two-component VirR/VirS system. In essence, these workers suggest that there are two or more genes involved in the pathogenesis of disease caused by CP, and that toxin production by a knock-out of one gene could be rectified by the products of a gene-positive strain in the same environment. This multiple gene system could potentially explain the various results observed by workers trying reproduce the disease in poultry, in that some CP isolates may not have a complete genetic make-up necessary to produce the toxins involved in clinical disease.

Other measures of virulence have recently been examined as well. When Martin & Smyth (2010) looked at the ability of disease and non-disease producing strains of CPA from chickens to adhere to extracellular matrix molecules (ECMMs), they found that the severe disease producing strains were capable of binding to collagen type III, IV and V, fibrinogen, laminin and vitronectin at higher levels than less severe disease producing strains, suggesting that the ability to adhere to ECMMs might work synergistically with other virulence factors (eg: NetB) with respect to the induction of NE. Cooper et al., (2010) examined the virulence of CPA that had been isolated from various avian and non-avian sources. They found that isolates originating from non-avian sources (human, bovine, porcine) and non-pathogenic avian-origin

CPA failed to produce disease in broiler chickens challenged with the isolates. This is in contrast to the isolate from a field case of NE, which was able to produce disease in the experimental hosts. These authors concluded that NE strains likely have poultry-associated virulence attributes, such as poultry colonization factors and NetB toxin, which allows for the development of disease.

2.2. Disease in Poultry Caused by *Clostridium perfringens* Type A

Currently, there are four recognized diseases associated with CPA in poultry and a fifth form of disease in broiler chickens caused by CPA has recently been described (Dinev, 2010). The first is a severe, often fatal condition called NE. Since 1961, CPA has been designated as the bacterium responsible for causing NE (Parish, 1961a; Parish, 1961b; Parish 1961c). This form of the disease has been reported to affect up to 37% of conventional flocks, causing increased mortality, reduced feed conversion and growth rate (Hofshagen & Kaldhusdal, 1992; Hofshagen & Stenwig, 1992, Hofacre et al., 2003; Wages & Opengart, 2003). It is believed that some mechanism, whether a direct stimulus to CPA, or an alteration in co-existing microflora, allows the proliferation of CPA, which subsequently produces toxins and causes necrosis in the jejunum and ileum of the host. On gross pathological examination, the gut is swollen, often gas-filled, with hemorrhage and fibrinous exudates covering the mucosa. In severe cases, the mucosa forms a diphtheric membrane which can be easily epilated from the underlying submucosa. On histopathological examination, a section of gut taken from an affected bird shows a moderate to severe, mixed, heterophilic and mononuclear cellular infiltrate, proteinaceous exudates admixed with necrotic tissue and numerous long, rod-shaped gram positive bacteria, in the gut lumen, lining the villi. Occasionally, there are also large vacuolated foci in the mucosa and submucosa, interpreted to be areas of gas produced by CPA. Lovland et al. (2003) found a correlation

between higher anti-alpha toxin antibody (AATA) levels in broilers from flocks that had suffered from clinical necrotic enteritis (CNE) or CP-associated cholangiohepatitis (CPH) compared to broiler from flocks with low levels of these lesions. Therefore, the ability to measure AATA could be a method to determine if the flock has suffered from this disease. McCourt et al. (2005) looked at 310 broilers over a five to six week grow-out period and found a high proportion of birds to have no CP alpha toxin antibody. The differences in measuring techniques and the cut-off point for titers is a possible explanation for the differences observed here. However, one cannot rule out the possibility that McCourt et al. (2005) collected serum from birds that had very little exposure to CPA.

Another form of this disease manifests as inflammation of the liver (Onderka et al., 1990; Hutchison & Riddell, 1990; Sasaki et al., 2000). Both Onderka et al. (1990) and Sasaki et al. (2000) were able to reproduce this condition in broiler chickens when the bile duct was ligated and when CP organisms were inoculated into the hepatoenteric bile duct. It is believed that there is an initial infection in the gut of the bird and that following enteric infection, CPA ascends the bile duct and infects the liver causing fibrosing cholangiohepaptitis (Onderka et al., 1990). This condition is believed to occur with higher frequency in flocks previously diagnosed with clinical NE. As a result, CPA contributes to economic losses, both to the producer and to the processor, by increasing the number of livers or whole carcasses condemned because of cholangiohepatitis (Lovland & Kaldhusdal, 1999; Lovland and Kaldhusdal, 2001).

Subclinical NE has been suggested as an insidious, but important manifestation of CPA infection (Stutz & Lawton, 1984; Kaldhusdal & Hofshagen, 1992; Lovland & Kaldhusdal, 2001). Numerous reports have cited that CP infection can affect production parameters such as condemnation (whole bird and liver), total mortality, weight gain and feed conversion (Elwinger

et al., 1992; Hofshagen & Kaldhusdal, 1992; Kaldhusdal et al., 2001; Hofacre et al., 2003). Stutz & Lawton (1984) found that the number of CP organisms in the gut elicited a negative effect on weight gain, when chicks were fed either a diet containing various carbohydrates (66.1% glucose, sucrose or fructose) or a practical diet (Chick Chow, Ralston Purina). A potential criticism of this paper is that the authors examined CP only, and did not look for other microbial causes of growth depression. Kaldhusdal & Hofshagen (1992) found a correlation between increased numbers of CPA in the gut of broilers and increased feed conversion ratios. These authors also associated the increased number of CPA organisms with the reduction in intestinal streptococci and lactobacilli, and believe that the increase in these latter bacteria could impede the development of subclinical NE. Subsequent to this, Kaldhusdal et al. (1999) reported that there was an association between the number of CPA and mild intestinal lesions, without disease-specific mortality in the challenged birds. When Lovland & Kaldhusdal (2001) examined 100 broiler flocks with high levels of CPA-associated hepatitis and compared them to 100 broiler flocks with low levels of CPA-associated hepatitis, they found that production parameters such as 35 day live weight gain and feed efficiency were significantly lower, and mortality was significantly higher in the flocks with high levels of CPA-associated hepatitis.

In an attempt to further characterize subclinical NE, Olkowski et al. (2006) infected 14 – 21 day old broiler chickens with a strain of CP that was isolated from a field case of NE and evaluated intestinal CP numbers as well as gross and histopathological lesions resulting from the challenge. These experimental birds were fed a high protein diet containing 40% meat meal or 40% fish meal and control birds were fed a 23% wheat-soybean diet. None of the birds in the trial exhibited gross lesions typical of field cases of NE. However, these workers describe histopathological changes that could account for the reduced performance in flocks diagnosed

with subclinical NE. Of interest, is that there were also lesions described in un-infected control birds. The presence of lesions in birds that were not infected further confounds the understanding of the disease caused by CP. The possibility of these trial birds being CPA positive carriers prior to the study was not reported. In this particular paper, the level of fishmeal fed to the birds could have accounted for some of the lesions described, as Tišljar et al. (2002) describes erosions attributed to this feed ingredient. There was no statistical analysis done by Olkowski et al. (2006) to offer a significant comparison of histopathological lesions between infected and non-infected control birds.

A fourth form of disease in poultry recognized to be caused by CPA is gangrenous dermatitis. In this condition, CPA causes extensive edema and emphysema beneath the skin (Wages & Opengart, 2003). In some cases, the subcutaneous tissue is similarly affected, and often the adjacent musculature is hemorrhagic and necrotic (Wages & Opengart, 2003). In this form of disease, the bacterium infects previously injured tissue which has subsequently become anoxic providing conditions suitable for anaerobic growth. The CPA proliferates in anoxic tissue, produces and releases alpha toxin, which subsequently causes tissue necrosis. The manifestation of this condition is thought to be similar to CPA-associated gangrene in humans (Stevens et al., 1997).

Very recently, a new form of disease produced in poultry by CPA was described (Dinev, 2010). The author reports an outbreak of necrotic gastritis associated with CPA in broiler chickens. Clinical signs are described as a sudden increase in mortality with typical macroscopic lesions observed only in the ventriculus. CPA was isolated from all gastric samples investigated. However, the role of CPA in the development of this condition in broiler chickens remains unclear, and the presence of CPA in the specimens could be secondary to a primary massive

gastric insult. Further investigation into the pathogenesis of CP-associated primary ventriculitis is warranted.

2.3 Occurrence of *Clostridium perfringens* Type A in Poultry

It is believed that CPA is ubiquitous to the environment, occurring in feces, soil, dust and litter (Wages & Opengart, 2003). In a study in Brazil, CPA was found to be present in 42% of ration samples and 30% of water samples, thereby offering a mode of contamination for poultry (Schocken-Iturrino et al., 2009). It is also thought that CP is a common inhabitant of the chicken intestinal tract (Johansson & Sarles, 1948; Shapiro & Sarles, 1949), being one of the first colonizers of the chick intestine, along with *E. coli* (Smith, 1965). Timms (1968) examined three groups of healthy chickens aged 18 days, 7 weeks and 5 months and found a mean level of less than log10 1.7 (range 0.0 – 2.8) colony forming units (CFUs) in the small intestine of all age groups. CP had the lowest recovery compared to all other bacteria (*E. coli*, *Streptococci*, *Lactobacilli* and *Bacteroides*) isolated in this study. The number of birds that tested positive for CP was not provided. Shane et al. (1984) found that although 75% of chickens over a five week period had "perfringens-like" organisms in their intestines, only 5% of the total were positively identified as *Clostridium perfringens*. These authors did not type the *Clostridium perfringens* that were isolated. Lovland et al., (2004) showed that there was an increase in cecal CP with increasing age. However, the peak of CP infection may occur at the ages of three to four weeks – a time often associated with a feed change in the flock, and changes in feed sources have been attributed to increased incidence of NE (McDevitt et al., 2006). This period also corresponds to the *Eimeria spp* oocyst peak period. Hence, if samples are collected at that time, an increased potential for isolating CP during that same period could occur.

When Kalender and Ertas (2005) examined 45 day old broiler chickens from eight flocks, they were able to detect CPA in 5% of the intestinal samples collected at an abattoir in Turkey. However, other workers (Tschirdewahn et al., 1991; Miwa et al., 1997 and Craven et al., 2001) have reported CPA prevalence from 75 to 95%. Further to this, Craven et al. (2001) found that processed poultry meat was also contaminated with CPA. In addition to the reported contamination rate of 8% to 16%, in processed carcasses, Craven et al. (2003) demonstrated that ribotypes of CPA found on the contaminated carcasses were, in some cases, the same ribotype as those found on the broiler breeder farm. These authors concluded that at least some CPA contamination found on processed broiler carcasses originated from the breeder operations and were likely transmitted through the hatchery and grow-out operations.

A study was conducted to examine the prevalence of netB gene in CPA isolates from Canadian retail grocery chicken samples (Nowell et al., 2010). In addition to looking for the netB gene, this group also tested the samples for cpb2, cpe and tpeL genes. They were able to isolate CPA from 42/64 fresh samples and 16/24 frozen samples (total of 183 isolates). Of these, they found that 20% of the chicken samples had the netB gene, tpeL was identified in three isolates, 93 isolates had the cpb2 gene, and cpe was not identified in any of the isolates. This initial survey of the grocery poultry was done because the effect of NetB toxin on human health has not yet been determined, and its occurrence in food destined for human consumption could have strong implications for control and treatment of NE if it is determined that this toxin can cause disease in people.

It has been shown that there are numerous subtypes of CP (Engstrom et al., 2003). The reports of different prevalence of CP from various sources could be a result of the age at which the host was tested. Pedersen et al. (2003) infected chicks with various doses of CP at 1, 9 and

22 days of age. They found that with increasing age at inoculation, there was an inverse relationship, in days, in which the inoculated isolate could be recovered from the host. They proposed that the test strains were being replaced by the naturally occurring strains of CP in all groups, with resistance to new strain colonization increasing with bird age.

When Barbara et al. (2008) examined the ability to re-isolate various PFGE- types of CPA from inoculated broiler chickens, they found that the only the NE-producing strain could be isolated during the challenge phase, whereas up to 7 other types could be identified at the post-challenge phase of the trial. They suggested that the NE-producing strains had some unidentifiable infective advantage over the non-NE-producing strains. In addition, they proposed that the NE-producing strains could be producing bacteriocins that could inhibit the non-NE strains. The inhibiting effect of bacteriocins was not identified to occur between the non-NE strains.

Johannson et al. (2010) examined the genetic diversity and prevalence of netB in CP isolated from a broiler flock affected by mild necrotic enteritis. From this single flock, they found 32 different pulse field gel electrophoresis (PFGE) patterns among 88 CPA isolates, indicating a high genetic diversity. In 3/7 (43%) of the birds with NE, more than one CPA genotype was detected. There was a different PFGE pattern of CPA isolated from birds compared to those isolated from litter. These authors hypothesized that the plasmid carrying the netB gene could be transferred between different CPA genotypes, and in doing so, could increase virulence with every *in vivo* transfer. They also suggested that mild NE differs from severe NE with regard to CPA genotype diversity.

Further work to support findings of high genetic variability of CPA was cited when the genetic analysis of CPA was carried out by Lepp et al. (2010). These workers concluded that NE

is caused by several novel virulence factors. When they examined the genomic sequences of 7 unrelated NE-producing CPA, they found that although the genes for alpha toxin are present on the chromosome, the genes for NetB were present on a plasmid. In addition, these workers could also identify 3 highly conserved NE-associated loci in the 7 NE-producing CPA isolates. As a result, they suggested that poultry NE is caused by several virulence factors in addition to the previously identified agents such as alpha toxin and NetB.

2.4 Predisposing Factors for Development of Necrotic Enteritis

2.4.1 Damage to intestinal tract

Necrotic enteritis is associated with damage to intestinal mucosa, which is presumed to provide a favourable environment for CPA proliferation. Damage to the intestine can occur when birds ingest high fibre litter or have a primary coccidial infection (Al-Sheikhly, & Al-Saieg, 1980; Shane et al., 1985; Wages and Opengart, 2003).

Intestinal coccidial infection, caused by *Eimeria spp*, in floor-raised broilers is common. There is a recognized correlation between NE infection in broilers and co-infection with coccidial organisms. Baba et al. (1997) orally inoculated 4 day old chicks with either 10^8 CFU of CPA, or 2 X 10^4 oocysts of *E. necatrix*, or a co-infection (10^8 CFU of CPA plus 2 X 10^4 oocysts of *E. necatrix*) with both microorganisms. They found that the NE mean lesion score was 0.4/12 in the CPA alone inoculated group compared to 5.8/12 in birds in the CPA + *E. necatrix* inoculated group, while the *E. necatrix*-infected group had an intermediate score of 2.6/12, leading them to conclude that when occurring together, coccidia and CPA have a synergistic effect on intestinal lesions.

Hermans and Morgan (2007) examined the risk factors associated with NE in broiler chickens in the United Kingdom. In addition to showing that there was an association between

season and wet litter, their work supported previous studies and showed that there was a strong association between coccidiosis in the flock and the occurrence of NE.

As such, coccidial organisms are often used in studies where workers wish to reproduce NE in broiler chickens, with the intent to develop a model that can be utilized to more clearly understand the disease. In an attempt to develop an optimal NE model, Wu et al. (2010) examined the effect of the incorporation of fishmeal into the diet and *Eimeria spp* infection in broiler chickens. They found that although the diet higher in fishmeal (250 or 500 g/kg) resulted in higher numbers of CP in the intestinal tract; mortality was significantly increased only when fishmeal diets were combined with *Eimeria spp* following CP inoculation. This suggests that there may be a synergistic effect between fishmeal and coccidial organisms, but the lack of fulminant NE without the initial damage to the intestinal tract caused by *Eimeria spp* suggests that intestinal damage to the mucosal lining of the gut is still one of the most important factors for the development of this disease in poultry when these birds are exposed to various strains of CP.

Pedersen et al. (2008) co-infected broiler chickens with coccidial vaccine (10 times the normal dose) and CP in an attempt to produce disease in the birds. There was no mortality as a result of NE in any of the birds. Subclinical NE was produced in the birds receiving both the vaccine and CP; no intestinal necrosis was observed in control birds or in those receiving only coccidial vaccine. In contrast to previous work, these authors found no significant difference in broiler body weight between the four treatment groups. In addition to enumerating CP retrieved from the intestinal samples, these workers also performed PFGE on the isolates. They found 24 different PFGE patterns in CP derived from chickens and 24 different patterns from feed samples that were also investigated during this study. This result alone, emphasizes the importance of

determining the pre-infection load of experimental birds, as well as assuring that exposure to different strains from the environment are minimized when performing infection studies. Finding this number of isolates, included with the challenge strain, further emphasizes that in addition to the strains of CP capable of infecting poultry there are a multitude of other factors that could influence each strain in its predisposition to form fulminant NE. In addition, this could explain why there are apparent differences in pathogenicity when isolates are reintroduced into naïve hosts during experimental infection studies. In such cases, there may be several strains infecting one NE-affected host, but when CP is isolated from that host, the isolate may very well be a non-pathogenic strain, instead of the preferred, disease-causing strain, and thereby unable to re-reproduce disease in laboratory settings. Further, one cannot rule out the possibility of mutational events that could alter the pathogenicity of various CP strains.

The creation of a NE model without the co-infection of *Eimeria spp* has been difficult to develop. Cooper & Songer (2010) report the development of such a model using various strains of CP to produce classical NE lesions in inoculated broiler chickens. They examined the effects of 11 strains of CP isolated from various sources. PCR was performed on these strains to establish the genotype (all were type A) and to determine if each had the cpb2 and/or netB genes, in addition to alpha toxin. Three strains were cpb2 and netB negative; three were cpb2 and netB positive; five stains were cpb2 positive and netB negative. They inoculated broiler chickens with each strain and compared gross duodenal and jejunal lesion scores post-infection. They concluded that virulence varies from strain-to-strain and that both netB positive and netB negative strains would produce disease in the host. When examining the strains, they reported that *in vivo* passage may increase virulence in the strain, whereas, *in vitro* passage may reduce

virulence. In addition, they reported that although some strains have the cpb2 gene, it did not extrapolate to the active production of beta-2 toxin.

2.4.2 Diet

Diets containing fishmeal can predispose poultry to NE (Truscott & Al-Sheikhly, 1977). These authors fed broiler chicks a 50% fishmeal diet for 14 days, then fasted the same chicks for 20 hours prior to feeding them CP-inoculated (10^8 CFU/gram) standard feed. There was a considerable increase in mortality in fishmeal-fed chicks (8/25) compared to standard-fed chicks (2/25) prior to CP challenge. It is interesting to note that Truscott & Al-Sheikhly (1977) were able to induce mortality due to NE in birds from all groups, and this could be due to the stress of fasting prior to infecting with CP. Fasting could lead to alterations in the gut microbial equilibrium or to intestinal mucosal deterioration, thereby reducing essential protective barriers, and again, providing a suitable environment for CPA growth. Gizzerozine present within fishmeal can lead to the formation of erosions in the alimentary tract of birds (Tišljar et al., 2002). When feed high in fishmeal is offered to birds following a prolonged fast, gizzerozine may act synergistically with feed-deprived mucosa to produce considerable primary lesions in the intestine, enhancing proliferation of CPA.

There are numerous reports to suggest that the incidence of CPA-associated NE is higher in broiler chickens fed wheat-, barley- or rye-based diets compared to a corn-based diet (Branton et al., 1987; Riddell & Kong, 1991; Kaldhusdal & Hofshagen, 1992; Takeda et al., 1995; Kaldhusdal & Skjerve, 1996; Branton et al., 1997). More specifically, when Branton et al. (1987) were examining the effect of milling method in wheat and corn, they found that mortality attributed to naturally-occurring CPA-associated NE was significantly higher in broilers fed a wheat-inclusion diet, hammer-milled (101/350) or rolled (76/420), compared to broilers fed a diet containing corn alone (12/420) or a corn/wheat mixture (12/350 and 44/350). Later, Branton

et al., (1997) examined the effect of adding complex carbohydrates (pine shavings, guar gum and pectin) to wheat-based broiler diets and comparing the incidence of NE in *E. acervulina* and CPA-infected broiler chickens to those chickens offered an unaltered corn-based diet or a wheat-based diet. It was determined that chickens consuming corn diets yielded the lowest lesion scores (0.533), and the highest lesion scores were found in chickens fed the wheat diets (1.833). When guar gum or pine shavings were added to wheat diets, they yielded intermediate results (1.270 and 1.133 respectively). Of interest in this paper is that when pectin was added to the wheat diet, the intestinal lesion score was not significantly different (0.793) than lesions examined in birds fed the corn-based diet. Riddell & Kong (1991) evaluated numerous rations in an attempt to explain the increase in CPA-associated NE observed in broilers fed a wheat-based diet. These authors compared mortality and weight gain in CPA-inoculated broiler chickens placed on diets based on either corn, corn + glucose, corn + guar gum, wheat, wheat + pentosanase, wheat + pectin, rye, barley, or oat groats. They concluded that mortality due to NE was higher among chickens fed rations based on wheat, rye, barley and oat groats than among chickens fed corn-based rations. However, when pectin was added to a wheat diet, mortality was identical to that recorded in birds fed the corn ration (0/30), but there was a significant reduction in weight gain in the wheat/pectin fed birds (1301 +/- 33 g) compared to the corn-fed birds (1739 +/- 34 g). This study did not have any non-infected controls to compare with infected test birds. Kaldhusdal & Hofshagen (1992) found that when broiler chickens were naturally infected with CPA there was an observed increased occurrence of NE in birds on a diet containing a large amount of barley compared to diets containing corn.

In a large scale retrospective study, Kaldhusdal & Skerjve (1996) examined the occurrence of NE and its association with broilers fed wheat, barley or corn-based diets in

Norway over a 20-year-period from 1969 to 1989. They found that there was a higher incidence of NE diagnosed in broilers the years when barley or wheat constituted the majority of broiler feed, than when corn was a major constituent of broiler feed.

In an experimental infection with CPA, Takeda et al. (1995) found that when leghorn chickens were fed 50% rye in their diet, there was a significant increase in the number of CPA organisms that could be retrieved from their ceca four, six and eight days after feeding began, compared to CPA numbers retrieved from chickens fed a corn-based diet. Of interest in this paper, is that the leghorn chickens did not develop signs or lesions consistent with NE, and it may be worthwhile to compare layer with meat strains to determine if there is an inherent susceptibility to NE.

Currently, the mechanism that increases the incidence of NE in broilers maintained on wheat- or barley-based diets is not known. However, part of the answer may be due to increased viscosity, associated with components such as beta glucans in barley and xylans in wheat. These components slow digesta transit time which may lower oxygen tension in the small intestine and potentially allow CPA to proliferate locally, produce toxins and induce disease. Xylanases and β-glucanases are commonly added to wheat and barley diets respectively, to reduce intestinal viscosity, and ultimately improve chick performance. As a result of normalizing the viscosity of digesta, enzymes may therefore prevent excess proliferation of anaerobic bacteria in the intestine. However, when Hock et al. (1997) examined the effect of the inclusion of enzyme, antibiotic or enzyme plus antibiotic in wheat-based diets, they found that there was no reduction in clostridial CFU in either the ileal or cecal contents when compared to contents examined from broilers fed an enzyme-free control diet.

Drew et al. (2004) correlated dietary protein source with the proliferation of CP in broiler chickens. These authors found that feeding a fishmeal protein resulted in an increase in the recovery of CP from both ileum and cecum in 28 day old CP-infected broiler chickens compared to broilers fed a diet with soy protein. The diets used in this study were all formulated with corn. In addition, they found significant differences in CP CFU between protein concentrations (230 g/kg vs 400 g/kg), within the same protein source. However, diets with the lowest protein also had the highest corn content, and one could conclude, therefore, that corn actually reduced the proliferation of CP, as opposed to high protein increasing CP. It would be interesting to see if these results could be replicated using a wheat-inclusion diet as opposed to a corn- inclusion diet.

Further to this, Dahiya et al. (2007) examined the influence of glycine on the growth of CPA in broiler chickens. These authors concluded that glycine was an important determinant of CPA growth in the intestinal tract of broiler chickens. However, they attributed lesion scores in the intestines of infected broilers to CPA, but did not have uninfected, glycine-treated controls. This comparison would have permitted exclusion of the substantial amino-acid imbalance as a cause for lesion appearance. In a subsequent study, Papp et al. (2009) looked at the response of broiler chickens to different experimental diets when challenged with CP. They found that in CP-challenged birds, 4.77% glycine in the diet would produce significantly more severe intestinal lesions scores and a greater whole blood chemiluminescence (WBCL) response compared to broiler chickens fed diets with 0.76%, 2.1% or 3.43% glycine. The authors suggested that the reason for the elevated WBCL was the increased glycine in the diet increasing CP growth, which resulted in both an increase in lesion score in the intestine caused directly by increased CP and the stimulation of a protective response (increased production of white blood

cells) by the host in relation to the increased pathogen load secondary to increased glycine in the diet. Quantification of CP was not performed prior to experimental infection, nor at the end point of the study, therefore, it may not be possible to assume that the results observed in this work were a result of increased CP numbers in the intestinal tract of the broiler. As well, the results provided were reflective of challenged birds only, and no results were reported for this experiment with respect to non-challenged birds. Providing the results for the non-challenged birds would help to rule out the possibility that the high glycine in the diet was the cause for the increase in lesion score and WBCL.

Wilkie et al. (2005) examined the effect of various protein sources and amino acid profiles on intestinal levels of CP in broiler chickens. They found that a positive correlation between glycine content in the diets and ileal and cecal CP numbers in CP-challenged broilers. Their results also show that when CP was grown in medium supplemented with various amino acids, methionine, leucine and alanine, that growth was significantly reduced compared to the control media. These authors also reported that birds that were fed fish meal, meat/bone meal, feather meal and potato protein concentrate had significantly higher intestinal CP counts than the birds fed corn gluten meal, soy or pea protein concentrate or the control diet. In addition, there was a significantly higher *Lactobacillus* count in the birds fed corn gluten meal when compared to those on the meat/bone meal diet. This observation could also be interpreted as *Lactobacillus spp* inhibiting the growth of CP.

Palliyeguru et al. (2010) examined the effects of dietary protein concentrates on the incidence of subclinical, spontaneously-occurring NE and growth performance in broiler chickens. They found that birds fed potato-protein diets had lower weight gains and feed intakes compared to soy-protein and fish-protein diets. They also reported a higher incidence of necrotic

lesions in the proximal small intestine compared to soy-based diet fed chickens. They attributed part of the explanation for the difference observed in the potato vs. other diets on the lower lipid content and the higher trypsin inhibitor activity in the potato-protein diet. Because trypsin cleaves alpha toxin and beta-2 toxin, the reduced presence of this enzyme in the gut could explain the increased presence of lesions caused by these toxins. These authors found no significant differences in CPA cfu/g from mucosal scrapings taken from birds in all treatment groups. This finding correlates well with McReynolds et al. (2004) who reported that they could find no relationship between the incidence of NE and the total CPA population of the jejunum. Palliyeguru et al. (2010) also suggested that the higher level of aromatic amino acids (e.g. tyrosine) in the potato diet could have contributed to the initial lesions in the intestine, leading to more significant NE-like lesions at post-mortem inspection. It has been reported that the bacterial degradation of aromatic amino acids produces phenolic and aromatic compounds that are toxic to the intestinal epithelium (Smith & Macfarlane, 1997).

Potato proteins have been cited to be antimicrobial in nature. The ethanol-water extract of this tuber demonstrated *in vitro* inhibitory growth to CP and *Escherichia coli* (E. coli) (Lim et al., 2004). Providing juice obtained from potato to human volunteers resulted in higher growth of Bifidobacterium and *Lactobacillus* while there was a corresponding decrease in growth of CP and *E. coli* (Lee, 2005). Ohh et al. (2009) fed broiler chickens three different levels of potato protein (PP) and compared coliform and aerobic bacteria to that of birds fed diets that contained either avilamycin or a basal diet without antimicrobials. They found that PP at 0.75% inclusion in the diet was as effective as avilamycin for reducing bacterial load. In addition, these workers found that weight gain, feed intake and feed conversion ratio were also linearly improved with increased PP inclusion in the diet. These workers attributed the beneficial results observed in

this study to the antimicrobial activity of proteinase inhibitors found in the water-soluble protein fraction of the potato tuber.

Gutierrez del Alamo et al. (2009) examined the effect of wheat starch digestion rates on broiler performance. They found that starch digestion rates varied among cultivars and this subsequently affected broiler performance. In addition, these workers found that wheat crude protein content negatively correlated with starch digestion rates. They measured *Clostridium perfringens* and *Lactobacillus spp* in cecal chyme and compared the numbers between chickens that had been provided various wheat varieties in their experimental diets. No significant differences were observed in bacterial counts measured and wheat cultivars. However, had the researchers examined counts in the small intestine, where disease is typically manifest, they may have observed differences in the counts, as there was substantial differences in proximal jejunum protein digestion between wheat cultivars.

Amerah et al., (2008) examined the influence of particle size and grain type on broiler performance. They found that birds fed corn-based diet had larger gizzards, deeper crypts and longer gut length than those fed wheat based diets. These differences in physical structure of the digestive tract could also account for some of the differences observed in broiler trials when CPA have been inoculated into hosts and fed corn-or wheat-based diets. A well-developed gizzard can increase gut motility (Ferket, 2000) and cholecystokinin release that will increase pancreatic enzyme secretion. The increase in pepsin activity could improve protein digestion, thereby affecting protein availability and/or form for CPA, resulting in differences in proliferation of this bacterium.

Engberg et al. (2004) examined the influence that feeding whole wheat to broilers would have on the numbers of CPA. They found that there was a tendency to have reduced numbers of

ileal and cecal populations of CPA in these whole wheat fed-birds compared to those fed pelleted diet. When Bjerrum et al., (2005) looked at the influence that feeding whole wheat would have on certain bacterial counts in the intestine, they found that CPA was significantly reduced when supplemental wheat was fed whole compared to a pelleted form. These authors hypothesized that the difference in CPA counts with various feed forms might be due to different rates of digestion when comparing starch to protein in whole versus pelleted form, which thus altered the rate at which CPA could be acquiring the nutrients in the lower gut.

In vitro work performed by Annett et al. (2002) showed that when CPA was grown in corn-based supernatant, there was a significant reduction in the number CFU compared to when this organism was grown in wheat- or barley-based diet supernatant, suggesting that there is a component in corn that inhibits the proliferation of CPA compared to the other grains examined. Part of this could perhaps be explained by different make-up of various components within each cereal (protein, starch and lipid), and the possible inhibition of one of these components to the proliferation of CPA.

Corn gluten meal (CGM) has been recently approved as an environmentally friendly herbicide for lawns (Pest Management Regulatory Agency, Canada, 2003). The herbicidal mechanism is believed to be due to the inhibition of new root growth (Christians, 1993). In addition, to affecting new root growth, when Mediterranean fruit fly larva were provided diets consisting of various protein sources, they pupated when fed, among other proteins, wheat gluten but failed to pupate when fed corn hydrolysate, (Chan et al., 1989). CGM hydrolysate has been analyzed by high pressure liquid chromatography (Liu & Christians, 1994). These authors could identify certain dipeptides (gln-gln, ala-asn, ala-gln, gly-ala, and ala-ala) present in the hydrolysate, and suggest that these dipeptides are the effective agents that provide CGM with its

root-inhibiting activity. Alanine-glutamine, one of the aforementioned dipeptides, has been used as parenteral therapy to human patients. Fuentes et al. (2004) report that incorporation of ala-gln into total parenteral nutrition (TPN) of patients suffering from secondary peritonitis, significantly reduced infectious morbidity, compared to patients that did not have the dipeptide added to their TPN. Glutamine is has been credited with being a source of energy for enterocytes, and could explain the reduced morbidity in patients supplemented with the ala-gln dipeptide. It is possible, however, that the presence of these dipeptides found in CGM hydrolysate, could also inhibit bacterial growth.

It has been shown that different strains of CPA can produce varying amounts of alpha toxin (Rood, 1998; Shimizu et al., 2002), and this could be one explanation for the variable incidence of NE when CPA is isolated from infected broilers. Alternatively, there could be some factor within the digesta of the host, directly related to diet, that affects CPA proliferation and subsequent alpha toxin production.

Bacteroides fragilis have been shown to require a novel N-Aceyl Mannosamine Epimerase to utilize sialic acid (Brigham et al., 2009). *Clostridium spp* have demonstrated their ability to utilize sialic acids (Walters et al., 1999), and variation in components from one cereal to another, and/or CPA strain may influence the ability of this bacterium to utilize sialic acid, and thereby proliferate.

McReynolds et al. (2007) fed broiler chickens diets containing 0, 2.5 and 4.5% lactose, and then administered CPA via oral gavage for three consecutive days at 17 days of age. These workers demonstrated that although no significant difference could be detected in the bacterial populations, there was a significant decrease in lesion score and in mortality in birds fed 2.5% lactose compared to those birds fed the 4.5% lactose or the lactose-free diet. The reason for this

effect was not determined, but it has been hypothesized that the increase in carbohydrates in the diet could inhibit adherence of the bacteria to epithelial cells and/or could reduce the pH of the intestine following fermentation of this sugar.

Essential oils (EOs) have been shown to inactivate bacteria (Briozzo et al., 1988; Paster et al., 1990; Dorman & Deans, 2000) *in vitro*. Mitsch et al., (2004) examined how two different blends of essential oils (thymol, eugenol, curcumin and piperin vs thymol, carvacrol, eugenol, curcumin, and piperin) might affect the proliferation of CPA in broiler chickens. They found that both oil combinations significantly reduced CPA populations in the gut when tested at 14, 21 and 30 days. Contrary to this, Abildgaard et al. (2010) found that CPA levels in the host ileum and cecum were not significantly reduced in CPA-infected birds when they were fed a blend of essential oils including thymol, eugenol, curcumin,and piperin (CRINA® Poultry) at dietary concentrations of 0, 100 or 200 mg/kg of feed. As well, they could find no improvement in production parameters (growth rate and feed conversion ratio) in the EO-supplemented broilers compared to the control birds. One of the many proposed mechanism for the effect of EOs on bacteria is that the hydrophobicity of the EOs enables them to partition in the lipid of the cell membrane, rendering them permeable (Burt, 2004).

Fatty acids have also been reported to have antimicrobial compounds (Cañas-Rodriguez & Smith, 1966). Medium chain fatty acids (MCFA) have been described to have various effects against CP (Skřivanová et al., 2005). These workers aimed to determine the susceptibility of CP to C2-C18 fatty acids *in vitro*. They found that lauric acid (C12) had the highest activity towards CP. In more recent work, Timbermont et al. (2010) looked at the control of CPA-induced NE in broilers with butyric acid, fatty acids and essential oils. They found that the *in vitro* minimal inhibitory concentration of lauric acid on CPA was 0.063 mg/ml. This effect was mirrored *in*

vivo, when NE lesion scores were significantly reduced in birds given MCFA in combination with butyric acid or with butyric acid and essential oils (thymol, cinnamaldehyde, eucalyptus). They reported that butyric acid alone did not affect CP proliferation, but attributed a beneficial effect to the host by way of this compound's anti-inflammatory effects (Place et al., 2005), reinforcement of the colonic barrier through increased mucin and host antimicrobial peptide production (Barcelo et al., 2000; Schauber et al., 2003), decreased intestinal epithelial permeability (Mariadason et al., 1997; Peng et al., 2007) and enhanced nutritional source for epithelial cells (Kien et al., 2007). Therefore, the level of butyric acid and lauric acid in a diet could perhaps influence the susceptibility of a flock to developing either subclinical or clinical NE.

The possibility that volatile fatty acids (VFAs) may impact the presence of CP in broiler ceca cannot be ruled out. Van der Wielen et al. (2000) found that there was a natural decrease in the numbers of Enterobacteriaceae in the ceca of broiler chickens with increasing age. They attributed this reduction to an increase in VFAs as the bird aged. The potential for VFAs to have a negative effect on the ability to isolate CP from the ceca should not be overlooked.

Di-cation minerals such as zinc, calcium (Moreau et al., 1988) and magnesium (Ananthanarayan & Paniker, 1981) are important minerals for the activity of CPA alpha toxin. Therefore, it would seem likely that availability of these ions from various diets may influence the manifestation of disease in host animals or in relation to the development of a repeatable model for NE. The inclusion or concentration of these minerals, not only in the diet, but also in the water may then, influence the development of NE, particularly in rural communities where water quality (e.g. total dissolve solids) may be questionable.

2.5 Prevention of Necrotic Enteritis

2.5.1 Antimicrobial growth promotants

Currently, NE is being controlled by the incorporation of antibacterial growth promotants, such as virginiamycin (George, et al., 1982) and bacitracin (Prescott et al., 1978), as well as anticoccidials (to prevent predisposing coccidial infection) such as salinomycin (Engberg, et al., 2000) and monensin (Elwinger et al., 1998) into broiler diets, through the growing period. When bacitracin (20 ppm), salinomycin (60 ppm) or both were incorporated into a standard broiler diet there was a significant reduction in the number of CPA per gram of digesta collected from the ceca and rectum, compared to broilers fed the same diet without the antimicrobials (Engberg, et al., 2000). These authors also examined the effect of the antimicrobials on *Lactobacillus salivarius*, and found that only when bacitracin and salinomycin were combined, was there a reduction in this bacterium. The significance of this remains unclear. However, Fukata et al., (1991) consider *Lactobacillus acidophilus* (LA) to be a beneficial bacterium to the intestinal tract of the host. They found that there was a reduction of CPA recovered from germ-free chicks when these birds were co-infected with LA and CPA, compared to mono-infection with CPA. Whether *L. salivarius* is equally as effective at reducing proliferation of CPA as LA has not been examined.

The possibility of bacteria acquiring resistance to antimicrobials is of concern, especially if resistance develops towards antimicrobials used to treat humans. With the suspension of the use of antimicrobial growth promotants from poultry feed in Europe, there has been a reported increase in the occurrence of NE in broiler chickens (Kaldhusdal & Lovland, 2000). As a result, there has been a corresponding increase in the use of antimicrobials to treat NE following a break. Lanckriet et al., (2010) examined the effect of anticoccidials and antimicrobials to treat NE in broiler chickens. Although amoxicillin, tylosin and lincomycin were found to be effective

in the treatment of NE, they also reported that there was a tendency for resistance to develop to lincomycin. This is of concern because this antibiotic is one that is used in human medicine. They also reported that while salinomycin, lasalocid and narasin (70 ppm) were effective in treating NE, maduramicin and narasin (50 ppm)/Nicarbazin (50 ppm) were not.

2.5.2 Competitive exclusion products

The use of competitive exclusion products (Aviguard®, Broilact®, Mucosal Starter Culture™) have also been examined as a method to prevent CPA proliferation and the development of NE (Fukata et al., 1991; Hofacre et al., 1998; Craven et al., 1999; Kaldhusdal, et al., 2001). When Aviguard® (an unidentified competitive exclusion product) was provided to coccidial-infected, 20 day-old chicks challenged with approximately 5 X 107 CFU of CPA, there was a significant reduction in intestinal gross lesion scores and mortality, due to NE, in the Aviguard®-treated birds (Hofacre et al., 1998). Craven et al. (1999) could show a significant reduction in CPA infection only when Mucosal Starter Culture™ (MSC) was given at hatch, to CPA-challenged day-old chicks that were stressed with a diet containing 50% rye. However, Kaldhusdal et al. (2001) could not find significant improvements in performance or reduction in mortality or NE in broilers treated with Broilact® compared to untreated control chickens. La Ragione et al. (2004) demonstrated that a single oral dose of *Lactobacillus johnsonii* F19785 given to 20 day old specific pathogen free White Leghorn chickens reduced colonization and persistence of these birds that were subsequently challenged with CPA 24 hours later.

Bacillus licheniformis was tested for its ability to prevent NE in CPA-challenged broilers (Knap et al., 2010). They found that CPA-challenged birds had decreased growth rate and feed efficiency and that birds provided with the *B. licheniformis* spores performed significantly better. They also found that the *B. licheniformis* treatment was similar in performance and lesion score to birds treated with Virginiamycin (15g/ton). Possible explanations for the reduction of disease

in birds given *B. licheniformis* is that the vegetative cells of this genus are known to be enzyme producing, such as proteases, which could hydrolyze alpha toxin produced by CPA. Alternatively, Bacillus-produced bacterocin has been demonstrated to have activity against *Clostridium spp*., (Teo & Tan, 2005) thereby reducing proliferation of CPA. Interestingly, both Bacillus and Clostridium organisms possess the same luxS gene, and Kaper & Sperandio (2005) reported that the luxS-mediated system enhances CPA to express alpha toxin. *B. licheniformis* may then influence the response of CPA in a way so that the expression of toxin is not activated (Knap et al., 2010).

2.5.3 Vaccination

Vaccination against *Clostridium perfringens* has been investigated for several years due to the alpha toxin effects on humans and its development into gas gangrene. As alpha toxin appears to be one of the major factors in the development of NE in poultry, any work on its effect elsewhere should be acknowledged. Stevens et al. (2004) investigated the effect of immunizing mice with the C-domain of alpha toxin and its prevention of gas gangrene in these animals. They concluded that immunization with this compound would significantly improve survival of infected hosts compared to sham-inoculated mice, and that vaccination with this antigen could be a viable strategy for the prevention of fulminant gangrene. The possibility of using this method to help in the prevention of NE in poultry should not be overlooked.

Prevention of various diseases has been successful with the use of vaccination products. To be utilized by the poultry industry, vaccines should be easy to transport, store and administer to a large number of individuals. In addition, the vaccine should provide rapid protective immunity and be economical. In recent years, development of vaccines for the prevention of NE has also been researched.

The utilization of recombinant vaccinia virus that expressed the C-domain of CPA alpha toxin was shown by Bennett et al. (1999) to offer protection to mice against a lethal dose of α-toxin. This vaccine also induced higher anti-alpha toxin antibodies to be produced in the vaccinates as well.

In ovo vaccination and vaccination at hatch are common practice in attempts to prevent disease in poultry. However, this method needs to be examined further since Mast and Goddeeris (1999) studied the immunoglobulin production in chickens vaccinated at various ages. They found that when chicks were immunized with bovine serum albumin (BSA) *in ovo* or at one day of age, antibodies (IgM and IgG) were not detectable at 10 days post-immunization. However, when chicks were immunized at 12 days of age, IgM and IgG responses were high and had a normal kinetic pattern. They concluded that vaccination at one day of age did not activate the B-cell response, and therefore the late embryonic and neonatal chickens do not have a completely developed immune function. This conclusion needs to be taken into consideration when vaccines to be utilized at the hatchery are being developed.

Thompson et al. (2006) explored the possibility of orally immunizing broiler chickens against NE. In this study, they found that some alpha-toxin negative mutant, but not avirulent strains, would offer protection against challenge with CP alpha-toxin positive strains. The authors concluded that there are likely immunogens other than alpha-toxin that are important in the development of protective immunity against NE. Further, Kulkarni et al., 2006 reported that immunity tends to develop if the host has been exposed to a virulent strain of CP but no immunity develops when the strain is avirulent.

When Kulkarni et al., (2010) vaccinated broiler chickens with attenuated *Salmonella* Typhimurium (ST) carrying constructs that expressed either CPA alpha toxoid (AT) or

Hypothetical protein (HP), they found that these orally- immunized birds produced serum IgY and mucosal IgA and IgY responses against both CPA and *Salmonella* antigens. AT birds that were subsequently provided a moderate CPA challenge were significantly protected against disease, whereas, those birds vaccinated with ST carrying HP were significantly protected against both moderate and severe CPA challenge. In addition, they also showed that there was an increase in weight gain in the AT-vaccinated birds when given either a moderate or a severe CPA challenge. This work further supports the possibility that alpha toxin is responsible for NE, but also supports the possibility that other proteins produced by CPA (eg: HP) could be responsible for, and/or contribute to the development of NE.

The effect of hen-egg antibodies (HEA) on CP colonization of the gastrointestinal tract of broiler chickens was examined by Wilkie et al. (2006). The premise behind this work was to determine if egg antibodies derived from hyperimmunized hens using a CP bacterin, would reduce colonization in CP-challenged birds. They found that CP colonization was not reduced, and that the administration of HEA might actually exacerbate NE. The authors speculated that the reason for the increase in NE lesions in the gut with addition of HEA could possibly be due to agglutination of the bacterial cells at specific sites along the intestine. Further work needs to be done in this area to confirm that the administration of HEA is not beneficial in the reduction of NE in commercial broilers.

2.5.4 Other

There are numerous studies revering the effectiveness of plant extracts on bacteria. This area of research has become of more interest of late because of the increased reports of antimicrobial resistance towards known antimicrobials. Shah et al. (2004) examined the antimicrobial effect of the ethanolic extract from the Australian Medicine Plant (Eremophila duttonii). They found that the extract had *in vitro* antimicrobial activity towards *Clostridium perfringens, C. sporognes* and

Listeria monocytogenes. In addition, these workers stated that this compound did not have cytotoxic effects. Other work that has demonstrated the effectiveness of various plant extracts on CP include dried Muscadine Pomace (McDougald et al., 2008) and lupulone, derived from *Humulus lupulus* (Siragusa et al., 2008). Further work is needed in this area to explore natural compounds that will have antimicrobial effectiveness against common pathogens, either in preventing disease and in treatments of disease, especially since there are fewer and fewer antimicrobials permitted for use in animal production.

Lysozyme, a natural antimicrobial protein has long been noted as having antibacterial properties, and is an important part of the innate immune system in animals. It exerts its bacteriocidal activity on gram positive bacteria by hydrolyzing the cell wall peptidoglycan. Lui et al. (2010) examined the effect of exogenous lysozyme on CP colonization in broiler chickens. These workers fed lysozyme to broilers that had been infected with CPA, and compared production parameters as well as disease characteristics with those birds that were not treated. They found that providing exogenous lysozyme to the diets of CPA-infected broilers significantly reduced the numbers of CPA in the ileum, NE lesion scores, inhibited the overgrowth of *E. coli* and *Lactobacillus* in the ileum and improved feed conversion ratio. Supporting work by Zhang et al. (2010) demonstrated that the incorporation of a lysozyme-based antimicrobial blend into diets fed to broilers challenged with CPA reduced the negative effects of the disease, and the ability of this compound to control disease was not significantly different than that observed in birds provided with a commonly used antibiotic growth promotant (bacitracin methylene disalicilate at 55 g/MT) to control NE. Since lysozyme is commercially extracted from hen eggs, the possible utilization of this compound to control NE may be of value in the future.

Miller et al., (2010), demonstrated the improved health and production parameters in birds orally vaccinated with lytic bacteriophages. The authors isolated these bacteriophages from the environment and demonstrated their ability to destroy a genetically diverse population of CPA. They went on to test the effectiveness of the phages in CPA-challenged broiler chickens, and reported a reduced mortality of 92% compared to untreated chickens. Subsequent to this, the authors demonstrated that this phage treatment was effective when administered through drinking water or through feed, bestowing its virtues to be potentially applied in the field.

2.6 Importance of CPA and Its Effect on Poultry in the Future

The current issue worldwide is the gradual elimination of antimicrobial and anticoccidial growth promotants from animal feed because of the fear that bacteria could develop resistance to antimicrobials used to treat human infections. At present, NE is considered one of the most threatening emerging diseases in the broiler industry worldwide and as such will continue to be considered an economically significant disease in poultry (Van der Sluis, 2000) because of the development of acute mortality (Nairn & Bamford, 1967; Long, 1973), poor performance (Stutz & Lawton, 1984; Kaldhusdal & Hofshagen, 1992; Lovland & Kaldhusdal, 2001) and increased condemnations at slaughter (Lovland & Kaldhusdal, 1999; Lovland and Kaldhusdal, 2001). An economic analysis examining the effect of subclinical NE (SNE) on broiler chickens raised in Canada was conducted by Skinner et al. (2010). Although this study was examining SNE in Canada, these authors expressed their costs in US dollars from the perspective of poultry producers. The assumptions made in this case were that the average flock size was approximately 20,000 birds, with final weights ranging from 4.63 to 7.94 lb; the incidence of SNE was estimated to occur at 20%. They found that in Canada, the cost of subclinical NE was

estimated to be $878.19 to $1480.52 US per flock. When most producers raise up to six flocks per year, the cost of this disease can be staggering. In a recent study conducted in Jordan, Gharaibeh et al., (2010) found that a great proportion of the 67 CPA isolates that they tested were considered resistant to lincomycin, erythromycin and tilmicosin, but these same isolates still showed susceptibility to penicillins, phenicols, quinolones and tetracyclines. Since NE in Western Canada is currently being controlled with antimicrobials and anticoccidials, it is of utmost importance to find alternative methods to control this disease in poultry once these antimicrobials are no longer available. It has been suggested that the occurrence of both clinical and subclinical NE will increase, as it did in the European Union, if there is a similar ban of AGPs in poultry feed (Grave et al., 2004) in North America. In Western Canada, broilers are fed primarily wheat or barley-based diets. As discussed previously, NE occurs with higher frequency in broilers fed a wheat or barley-based diet, compared to a corn-based diet. Therefore, when antimicrobials are banned, NE could be potentially devastating to Western Canadian poultry producers. In addition, poultry producers in Saskatchewan have management practices that differ considerably compared to other poultry producing areas. For example, broiler chickens in this province are not typically vaccinated against Marek's disease at the hatchery. The practice of *in ovo* or day of hatch antibiotic injections has not, in the past, been carried out in this province. This can leave the producers with higher first week mortality compared to other regions. In addition, Saskatchewan and other Canadian prairie producers have to deal with extreme temperature fluctuations not experienced elsewhere. This fluctuation in temperature places more emphasis on the producer to maintain a steady environment in the barn with respect to temperature and humidity. As these parameters change, so can they then affect the potential for the flock to become stressed (chilling, over heating, excess moisture in the litter etc). If for

example, there is a sudden increase in temperature in the winter (eg: during a Chinook where temperatures can change from – 25^{o}C to + 10^{o}C in a matter of hours) and the heating and ventilation system needs to be changed rapidly to accommodate this. A study on the prevalence of CPA and its consequences to the producer or the processing plant has not been previously examined in the Province of Saskatchewan. Because of the unique production characteristics employed by Saskatchewan broiler producers, an evaluation of the influence CPA is having on production now, could potentially give this group a pro-active viewpoint of how to reduce the impact of NE if prophylactic antimicrobials are banned in Canada.

Because of the prevalence of wheat-based broiler chicken diets in Western Canada, along with the looming expectation of the withdrawal of in-feed prophylactic antimicrobials, the examination of why NE is more prevalent in broiler chickens fed a wheat-based diet compared to a corn-based diet is explored in the subsequent work. The goal is to examine, *in vitro*, the effects of corn and wheat on the proliferation and toxin production of CPA. In addition, the major components of cereal will be examined to narrow, further, the compound that either positively or negatively affects the proliferation of CPA. Although the feed components appear to be a primary element for the development of NE in broiler chickens, this disease does in fact appear to be multifactorial. In light of the complexity of this condition, an examination into several management factors surrounding the production of broiler chickens in Saskatchewan were examined. These data will assist with the determination of the prevalence of CP in the province, as well as make correlations toward the presence of this bacterium in the bird at the farm and at slaughter, and also determine the effect that this bacterium has on flock production parameters.

3. EFFECTS OF WHEAT AND CORN ON *IN VITRO* PROLIFERATION OF *CLOSTRIDIUM PERFRINGENS* TYPE A AND ITS ALPHA TOXIN PRODUCTION

3.1 Abstract

Clostridium perfringens type A (CPA) produces alpha toxin, one toxin that is believed responsible for mucosal necrosis in birds affected by necrotic enteritis (NE). NE is more prevalent in broilers fed a wheat-based diet compared to a corn-based diet. CPA was grown *in vitro* in thioglycollate media (TG) alone, TG plus pancreatin and pepsin (TGE) or TG plus *in vitro* digested corn (C) or wheat (W) supernatant in a 2:1 ratio. Colony forming units (CFU) were counted and alpha toxin activity (U) was measured from each group using a commercially available kit. There was significantly more proliferation when CPA was grown in W compared to C, TG, or TGE. Alpha toxin production was significantly higher in C compared to W or TGE but no significant difference in U/CFU between TG and TGE, C or W. These data suggest that CFU alone may not be sufficient to predict potential NE in broilers infected with CPA.

3.2 Introduction

Necrotic enteritis (NE), a common disease of poultry worldwide, is caused by *Clostridium perfringens* type A (CPA) when it is present in high numbers in the small intestine (Wages & Opengart, 2003). The reason for excessive proliferation of this bacterium in the intestine of some birds remains unclear. Factors associated with increased incidences of NE include damage to the intestinal mucosa due to co-infection with *Eimeria spp*., the ingestion of fishmeal (Truscott & Al-Sheikhly, 1977; Tišljar et al., 2002) or high fibre material, such as litter (Wages & Opengart, 2003). Diet has also been implicated with a higher incidence of NE in chickens. Inclusion of high levels of animal protein in broiler diets has been shown to increase the recovery of CPA from infected broilers suggesting that high levels of animal source protein may predispose to NE

(Drew et al., 2004). As well, NE has been associated with diets consisting of high levels of wheat- or barley-based diets compared to a corn-based diet (Branton et al., 1987; Riddell & Kong, 1991; Kaldhusdal & Hofshagen, 1992; Takeda et al., 1995; Kaldhusdal & Skjerve, 1996; Branton et al., 1997). Previous work has shown that CPA has greater proliferation in wheat- and barley-based broiler grower supernatant compared to a corn-based supernatant (Annett et al., 2002). This study suggests that greater proliferation of this bacterium in wheat- or barley-based diet supernatant may at least in part, explain the higher incidence of NE in broiler chickens fed a diet with a high proportion of wheat or barley. Alternatively, it may be that a high level of corn in broiler diets may reduce proliferation of CPA.

CPA produces an alpha toxin. This alpha toxin is a phospholipase C (plc), which is believed responsible for the intestinal mucosal necrosis observed in affected chickens (Al-Sheikhly & Truscott, 1977; Fukata et al., 1988). However, the importance of alpha toxin and its association with development of NE has been questioned (Keyburn et al., 2006). Using an undisclosed number of specimens, these researchers reported a lack of correlation between alpha-toxin production and severity of disease. This is not surprising, considering the difficulty in reproducing NE without corresponding predisposing factors, such as co-infection with *Eimeria spp*. This research was supported by comparing the lesion scores in birds infected with plc mutants (lacking alpha toxin production ability) and wild type (plc producing ability). Although these data are suggestive that alpha toxin may not be an important factor in NE, the ability to produce NE lesions in infected birds is unpredictable. As diet appears to influence the occurrence of NE, it was of interest to explore the production of alpha toxin by CPA when this bacterium was grown in digested wheat or corn supernatant. This study was performed to

determine if there were differences in proliferation of CPA when grown in supernatants from digested wheat and corn and to evaluate the alpha toxin production from each.

3.3 Materials and Methods

3.3.1 Cereals

Whole corn and wheat used in broiler diets at the University of Saskatchewan were ground in a Retsch ZM100 grinder (F.Kurt Retsch Gimbh & Co. KG. Haan, Germany) using a 1 mm screen.

3.3.2 *In vitro* digestion of the cereals

In vitro digestion of the cereals was performed according to previously published methods (Annett et al. 2002) with minor modifications. All incubations were carried out at 40^{o}C in a Forma Scientific Orbital Shaker (model 4520, Forma Scientific Inc. Morietta OH). To mimic crop digestion as a moderately acidic environment, 20 g of each cereal and 40 ml of 0.03 M HCl were mixed in a 175 ml conical centrifuge container and pH was measured to assure that it was in the range of 5.0 and 5.2, and then this mixture was incubated for 30 min at 200 rpm. Following this, 10 ml of 1.5 M HCl was added and pH measured (range 1.5 to 1.9); then 60,000 U pepsin (Sigma, St. Louis MO) was added and the mixture incubated for 45 min at 200 rpm. Next, 14 ml of 1.0 M NaHCO3 (final sample pH range of 6.49 to 6.51) and 59.1 mg of 8 X pancreatin (Sigma, St. Louis MO) were added and the mixture was incubated for 2 h at 120 rpm to mimic intestinal digestion. Solids were removed by centrifugation at 22 100 x g for 12 min (Beckman J2-MC, Beckman Instruments Inc., Palo Alto California 94304) and then the supernatant was filtered through gauze. The pH of the supernatant was measured and equalized to approximately 6.5 with 1.0 M NaOH followed by freezing at -20^{o}C until used.

3.3.3 Bacterial proliferation

Clostridium perfringens was isolated from a broiler with clinical necrotic enteritis. Confirmation that this bacterium was type A was carried out by multiplex polymerase chain reaction as

described in Annett et al. (2002). The culture was kept frozen in 800 µl aliquots at -80^{o}C following one *in vitro* passage. For proliferation, 100 µl of a thawed sample was inoculated into 100 ml of sterile thioglycollate (TG) and incubated for 13 h at 37^{o}C under mild agitation (90 rpm). From this, 100 µl was placed into six replicates of either 6 ml TG, 6 ml TG plus 0.5 mg pancreatin and 408 U pepsin (TGE), or eight replicates each of 2 ml thawed corn supernatant plus 4 ml TG (C) or 2 ml thawed wheat supernatant plus 4 ml TG (W). The inoculum was mixed, then incubated anaerobically at 40^{o}C for 4 h at 120 rpm. Following incubation, serial dilutions in sterile 0.85% NaCl were performed and 20 µl placed on blood agar plates containing neomycin (1 µg/ml), which were incubated anaerobically at 37°C overnight and colony forming units (CFU) counted. This experiment was performed in triplicate.

3.3.4 Alpha Toxin Analysis

Culture remaining from each proliferation replicate was centrifuged at 3,210 x g (Beckman CS-6, Beckman Instruments Inc., Palo Alto, California 94304) for 5 minutes to remove bacteria and the supernatant was frozen at -20^{o}C until used. For analysis, the supernatant of cultures that had CFU/ml closest to the median value for each trial was used. Four samples were selected from each trial where CPA had been grown in the C and W supernatant and at least one sample from each trial for CPA grown in TG or TGE. The supernatant was thawed and 1.8 ml placed in a 10K MicrosepTM Omega Centrifugation filter (Pall Life Sciences, Ann Arbor, MI, 48103-9019) and centrifuged at 7 250 x g (Beckman J2-MC) for 60 to 165 minutes. Alpha toxin was measured in the volume-equalized retentate using an Amplex red phosphotidylcholine-specific Phospholipase C assay kit (A-12218, Molecular Probes, Eugene OR) read on a Fluoroskan Ascent plate reader (Thermo LabSystems, Finland).

3.3.5 Protein analysis

One sample each of whole cereal and supernatants from digested corn and wheat were freeze dried and percent protein was determined using a LECO FP-528 Protein/Nitrogen analyzer (LECO Corporation, St. Joseph, MI, 49085-2396). The analysis was completed twice per sample and the mean value between the two recorded.

3.3.6 Mineral analysis

To determine if there were substantial differences in major mineral constituents between the corn and wheat varieties utilized in this work, minerals in whole cereals were analyzed using AOAC 935.13a/993.14 methods.

3.3.7 Statistics

The Kruskal-Wallis non-parametric analysis of variance and the Mann-Whitney rank-sum test for analysis between two samples were used to evaluate statistical significance of C. perfringens proliferation and alpha toxin production among the various types of media. Means were considered significantly different when $p < 0.05$.

3.4 Results

3.4.1 Protein and mineral analysis

Mineral constituents of the corn and wheat used in this study and the protein content of the supernatants derived from the corn and wheat digestion are shown in Table 3.1. The di-cations, Fe, Mn and Zn were all substantially higher in the wheat sample (23, 28 and 49 ppm) compared to the corn (<1, <1 and 10 ppm respectively). In addition, there was a higher percentage of Mg and S in the wheat sample (0.140 and 0.178) compared to the corn variety (0.080 and 0.067,

Table 3.1. Protein And Mineral Content Of Corn And Wheat* used for the assay

	Corn	Wheat
Protein (%)		
Whole Cereal	4.50	16.64
Supernatant	2.18	6.41
Mineral (ppm)		
Cu	<1	1
Fe	<1	23
Mn	<1	28
Zn	10	49
Mineral (%)		
Ca	<0.01	0.03
P	0.22	0.37
Mg	0.08	0.14
K	0.28	0.38
S	0.07	0.18
Na	<0.01	0.03

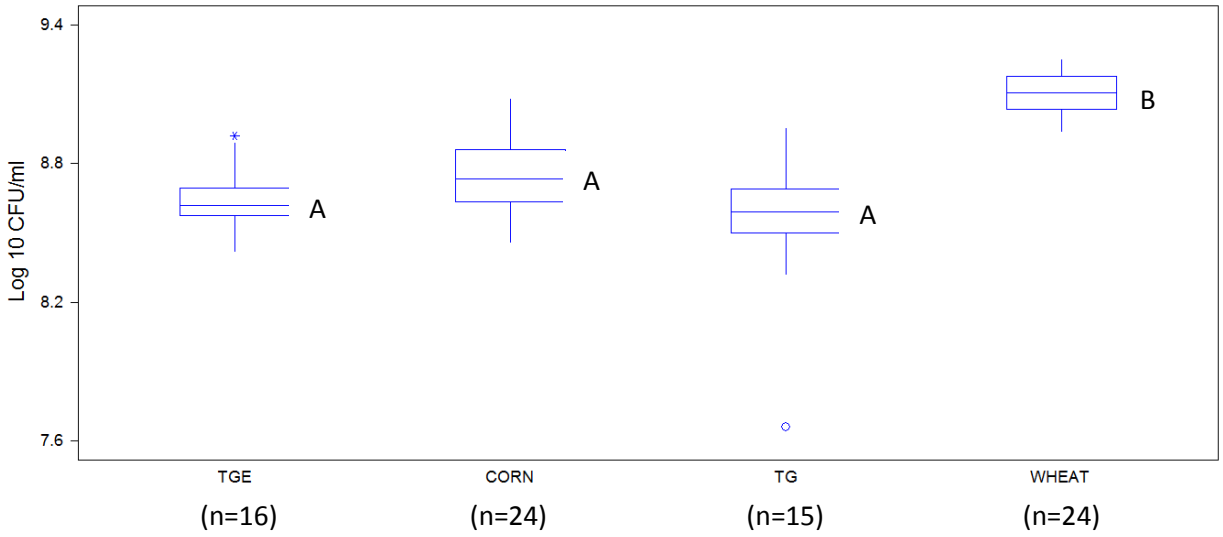
*As is basis.

respectively). The protein content in the whole cereal was notably higher in the wheat (16.6%) compared to the corn (4.5%).

3.4.2 Proliferation

Proliferation of CPA (CFU/ml) from three combined trials is shown in Figure 3.1 and Table 3.2. In all three individual trials (data not shown), there was significantly more proliferation when CPA was grown in wheat supernatant compared to the other three mediums. The difference in CFU/ml between CPA grown in corn supernatant compared to the controls (TG and TGE) approaches significance ($p = 0.054$ and $p = 0.080$ respectively), and this pattern was similar in all three individual trials.

Figure 3.1 Clostridium Perfringens Type A Proliferation In Unsupplemented Or Media Supplemented With Digested Wheat Or Corn Supernatant



TGE = TG plus pancreatin and pepsin

1. Area within the boxes represent the middle half of the data. Horizontal line within the box represent the median value. "*" indicates possible outlier.
2. Median values with different letters (A,B) indicate significant differences ($p<0.05$)

Table 3.2. *C.perfringens* Proliferation In Unsupplemented Media Or Media Supplemented With Digested Wheat Or Corn Supernatant

	Corn (n = 24)	Wheat (n = 24)	TGE (n = 16)	TG (n = 15)
Median (Log10 CFU/ ml)	8.74^{A}	9.10^{B}	8.62^{A}	8.59^{A}
First quartile (Log10 CFU/ ml)	8.61	9.02	8.56	8.48
Third quartile (Log10 CFU/ ml)	8.87	9.17	8.71	8.71

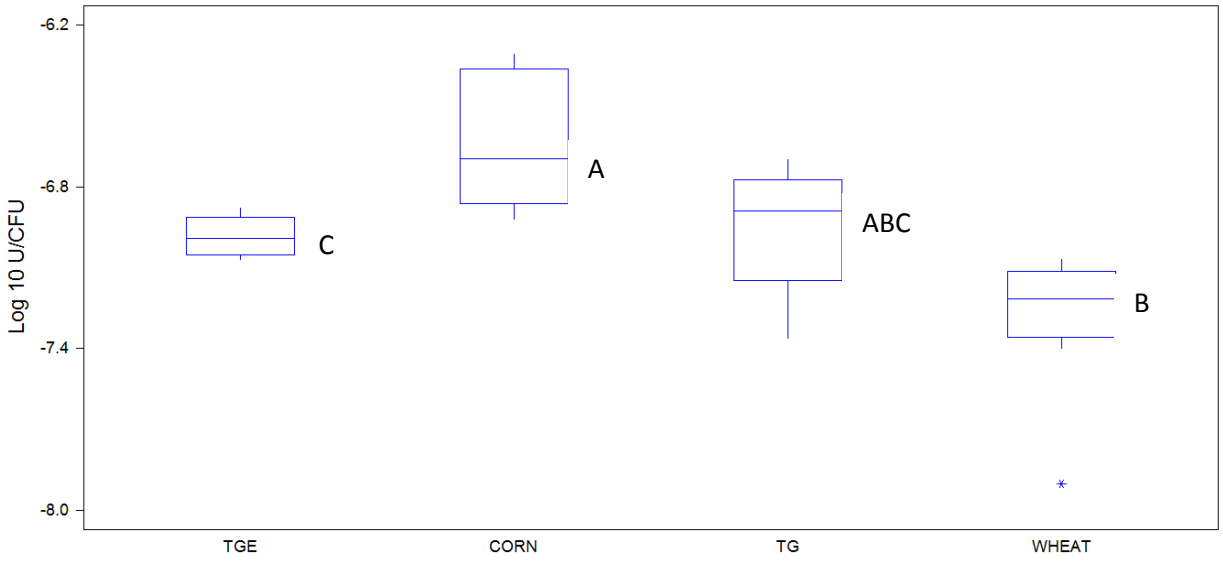
^{A,B} median values with different superscript letters are statistically different ($P<0.05$).
TGE = TG plus pancreatin and pepsin.

3.4.3 Alpha toxin production

Alpha toxin production (U/CFU) when CPA was grown in various media is shown in Figure 3.2 and Table 3.3. There are significant differences in alpha toxin production per CFU when CPA

was grown in corn compared to wheat or TGE, but no difference in U/CFU between TG and the other media.

Figure 3.2 Alpha Toxin Production Of *C. Perfringens* Type A When Grown In Unsupplemented Media Or Media Supplemented With Digested Wheat Or Corn Supernatant



TGE = TG plus pancreatin

1. Area within the boxes represent the middle half of the data. Horizontal line within the box represent the median value. "*" indicates possible outlier.
2. Median values with different letters (A,B,C) indicate significant differences ($p<0.05$)

Table 3.3 Alpha Toxin Production Of *C. perfringens* When Grown In Unsupplemented Media Or Media Supplemented With Digested Wheat Or Corn Supernatant

	Corn (n = 12)	Wheat (n = 12)	TGE (n = 4)	TG (n = 4)
Median (Log10 U/CFU)	-6.69^{A}	-7.21^{B}	-6.98^{C}	-6.90^{A,B,C}
First quartile (Log10 U/CFU)	-7.88	-7.36	-7.06	-7.20
Third quartile (Log10 U/CFU)	-6.34	-7.10	-6.90	-6.73

^{A,B,C} median values with different superscript letters are statistically different ($P<0.05$).
TGE = TG plus pancreatin and pepsin.

3.5 Discussion

The pathogenesis behind the increased incidence of NE in broilers fed a wheat-based diet compared to a corn-based diet remains elusive. In our study, we confirmed that CPA proliferation is greater when grown in wheat supernatant compared to corn. However, we discovered that CPA alpha toxin production is less per CFU when proliferation occurred in wheat versus corn supernatant. It is possible that the differences in proliferation and alpha toxin production could be associated with a stressor intrinsic to the corn. However, if lower CFU were the only answer to increased alpha toxin production,

then in the current study we would expect to see equivalent to, or more, U of alpha toxin per CFU when CPA was grown in TGE. In this sample, proliferation, although not significantly different, is numerically less than corn, whereas in TGE there is significantly less (-6.98 U/CFU) alpha toxin production compared to corn, implying that corn increases alpha toxin production of CPA. CPA growth curves were performed for each sample (not conducted on TGE only, data not shown). The growth curve suggested that the log and stationary phase were relatively consistent between the various samples examined. Therefore, the possibility that CPA had reached its stationary phase sooner when grown in corn compared to when it was grown in wheat is not likely the explanation for the increased alpha toxin (U/CFU) production observed here.

Numerous bacterial species will produce toxins in response to environmental stress and/or in an effort to improve their environment to gain access to nutrients. Approximately 7.3% of CPA also contains the enterotoxin gene (Bueschel et al., 1998) that will produce this enterotoxin during sporulation, when the environment becomes unfavorable (Rood, 1998). CPA do not attach to their host, but rather rely on host ingesta for nutrients, and when nutrients are not available or inhibitory to growth, more alpha toxin may be produced whereby the bacterium can then utilize host tissue through inducing necrosis to the mucosa. The findings that CPA

proliferation was enhanced, but alpha toxin production decreased when grown in wheat supernatant in this study suggest that proliferation, or total numbers of CPA alone, may not be a sufficient indicator for the development of NE in domestic poultry. It may be possible that CPA in a growth-inhibitory environment, become primed to produce an increased quantity of alpha toxin. As a consequence, when the alpha-toxin primed CPA is suddenly placed in a favourable environment, the bacterium flourish, and along with that, produce, correspondingly, copious amounts of toxin that could result in NE in the host. In this study, alpha toxin activity was measured. Di-cation minerals such as zinc, calcium (Moreau et al., 1988) and magnesium (Ananthanarayan & Paniker, 1981) are important minerals for the activity of CPA alpha toxin. Therefore, it would seem likely that availability of these ions may influence the results obtained. Our analysis of the wheat and corn used in this study indicate that there is a higher content of all these di-cations in wheat compared to corn (Table 1). This suggests that since Zn^{2+}, Ca^{2+} and Mg^{2+} influences alpha toxin activity, we should have greater activity of alpha toxin when CPA was grown in wheat compared to corn. Because alpha toxin activity was greater in corn supernatant, it implies that in the assay used to evaluate enzymatic activity, there was sufficient di-cation availability. Alternatively, some factor in the wheat supernatant could have inhibited the availability of these ions, thereby reducing alpha toxin activity. Total mineral was evaluated from our cereals, and was not determined from the supernatant, which could influence the amount of ions in the assay. As well, activity of alpha toxin derived from CPA grown in wheat and corn supernatant should be evaluated in a biological system to better understand the implications of these results.

Protein concentration was higher in wheat compared to corn in both the whole cereal and supernatant (Table 3.1). It is possible that the protein content difference between corn and wheat

may have influenced the proliferation of CPA in our study. However, the proliferation data obtained here has a similar pattern to previous work where CPA growth was measured in complete broiler diets, in which protein content was similar (Annett et al., 2002), thereby suggesting that protein content was not a limiting factor for CPA proliferation.

The reduction in proliferation of CPA when grown in corn-supernatant is consistent with previous studies showing that NE is reduced in broilers fed a corn-based diet compared to wheat- or barley-based diets (Branton et al., 1987; Riddell & Kong, 1991; Kaldhusdal & Hofshagen, 1992; Takeda et al., 1995; Kaldhusdal & Skjerve, 1996; Branton et al., 1997). However, the information gained from this study showing the increase in alpha toxin production when CPA is grown in corn supernatant compared to wheat supernatant suggests that simply measuring CFU or U of alpha toxin does not reflect issues occurring in the field. De Cesare et al (2009) showed that a vast variation of ribotypes of CP that can be isolated from either the same or different birds within or among varying flocks. This information may help to explain the differences in disease occurrence and severity when CP is isolated from either sick or apparently healthy birds in the field. Differing ribotype could very well explain the variation in other toxin production (eg: theta toxin) as well. The importance of theta toxin activity has been demonstrated in a gas gangrene model, where mice were infected with either lethal or sub-lethal doses of CPA (O'Brien & Melville, 2004). In this study, the researchers found that if lethal doses of bacterium were administered to the host, then only alpha toxin was necessary for the onset of myonecrosis. However, if sub-lethal doses of the bacterium were given, then both theta toxin and alpha toxin were necessary to maintain CPA survival in the tissue. So, it may be possible that if certain ribotypes of CPA were lacking theta toxin activity (or perhaps other toxin of influence), then the ability of CPA to cause disease in the host may be altered. The influence of other intestinal

microbes on the proliferation of CPA and their effect on activity or degradation of alpha toxin should be explored. As well, investigating the effect of other known NE-causing diets, such as fishmeal, on the *in vitro* proliferation and alpha toxin production of CPA may provide insight into the pathogenesis of this disease. Further, it is warranted that specific components, such as protein, starch and lipids, of both corn and wheat should be examined to establish if these components affect the parameters studied here.

4. *IN VITRO* DIGESTED CORN GLUTEN MEAL INHIBITS *CLOSTRIDIUM PERFRINGENS* TYPE A PROLIFERATION

4.1 Abstract

This study was carried out to determine if there were differences in proliferation when *Clostridium perfringens* type A (CPA) was grown in different components of wheat and corn. Further, work was completed that demonstrated that the dipeptide Alanine-Glutamine could be one of the bioactive molecules in corn gluten meal responsible for the altered proliferation. *In vitro* proliferation of CPA was reduced when this bacterium was grown in digested corn gluten meal (CGM) compared to non-digested CGM, as well as digested or non-digested wheat gluten (WG) and the thioglycollate (TG). Digestion of wheat and corn oil and starch reduced proliferation significantly, when compared to the non-digested components of these cereals. Alpha toxin production was increased when proliferation was inhibited in all cases. This study suggests that, due to the inhibition of proliferation of CPA observed in our *in vitro* model, the component that could reduce the incidence of necrotic enteritis in broiler chickens is due to the hydrolyzed gluten found in corn meal.

4.2 Introduction

Clostridium perfringens type A (CPA) is the bacterium responsible for necrotic enteritis (NE) in poultry (Parish, 1961; Wages & Opengart, 2003). There are numerous reports suggesting that necrotic enteritis is less prevalent in broilers fed a corn-based diet compared to a wheat based diet (Branton et al., 1987; Riddell & Kong, 1992; Kaldhusdal & Skjerve, 1996; Branton et al., 1997). *In vitro* studies have shown that CPA proliferation was significantly less when grown in digested supernatants from corn- compared to barley- or wheat-based broiler grower diets (Annett et al., 2002). In addition, CPA appeared to be inhibited when grown in supernatants derived from corn cereal compared to wheat (Chapter 3). However, in this study, alpha toxin production was significantly greater when CPA was grown *in vitro* in the corn cereal supernatant, suggesting that the bacterium was stressed in the corn supernatant environment.

Studies have been performed in which high numbers of CPA have been retrieved from the intestine of experimentally or naturally infected broilers, but often these specimens lack clinical or pathological evidence of necrotic enteritis (Timms, 1968; Shane et al., 1984; Drew et al., 2004). Studies have also shown that CPA produces alpha toxin believed to be the major factor involved in the pathogenesis of this disease (Al-Sheikhly & Truscott, 1977). It has been shown that different strains of CPA can produce varying amounts of alpha toxin (Rood, 1998; Shimizu et al., 2002), and this could be one explanation for the variable incidence of necrotic enteritis when CPA is isolated from infected broilers. Alternatively, there could be some factor within the digesta of the host, directly related to diet, that affects CPA proliferation and subsequent alpha toxin production.

The factors that influence the proliferation and alpha toxin production of CPA when grown in supernatants derived from corn or wheat remains elusive. This study was performed to

determine if *in vitro* digested or non-digested components of wheat and corn, such as oil, starch or gluten specifically affect the proliferation and alpha toxin production of CPA.

4.3 Methods and Materials

4.3.1 Cereal components used for proliferation of CPA

Wheat germ oil, corn oil, wheat starch, corn starch and wheat gluten were purchased from Sigma (St. Louis MO). Corn gluten meal was obtained from a local feed mill.

4.3.2 *In vitro* digestion of the cereal components

All incubations were carried out in a Forma Scientific Orbital Shaker (model 4520, Forma Scientific Inc. Morietta OH). Digestions were incubated at 40^{o}C with agitation as specified below. Samples were described as digested or non-digested and were treated similarly except that no pepsin or pancreatin was added to the non-digested samples.

4.3.2.1 Oil

Titration of bile salts (50:50 sodium cholate & sodium deoxycholate, B-8756, Sigma Chemical Co. St. Louis, MO, 63178) in fresh, sterile, thioglycollate media (TG) determined that 0.0053% bile salts provided moderate emulsification but was not inhibitory to CPA growth. Oil (3.75 g), 3 ml of 20% (w/v) bile salts in ddH2O, and 4.59 ml ddH2O were combined in a 175 ml conical centrifuge container. Pepsin (128 mg, Sigma, St. Louis, MO) and pancreatin (8.3 mg, Sigma, St. Louis MO) were added, and then the mixture was incubated at 200 rpm for 2 h. Treated oil samples were shaken vigorously, then diluted to 1:1000 in TG. The TG control contained enzymes (0.128 mg pepsin, 0.0083 mg pancreatin) and bile salts (0.0053% w/v) equivalent to the final concentration of oil plus TG samples.

4.3.2.2 Starch

Forty g of starch plus 50 ml of 0.03 M HCl were combined into 175 ml conical centrifuge containers and incubated for 30 min at 200 rpm. Twenty ml of 1.5 M HCl and 120 000 U pepsin were added and then incubated for a further 45 min at 200 rpm. Finally, 30.5 ml NaHCO3 and 118.2 mg pancreatin were added and incubated for a further 2 h at 120 rpm. When the final incubation was complete, the mixture was centrifuged at 12 000 rpm for 12 min and then the supernatant was filtered through gauze. The supernatant was mixed 2:1 with sterile TG, pH adjusted with 1.0 M HCl (range 7.03 to 7.09) and then frozen at –20^{o}C until used.

4.3.2.3 Gluten

Twenty g of gluten plus 50 ml 0.03 M HCl were combined in a 175 ml conical centrifuge container and incubated for 30 min at 200 rpm. 10 ml of 1.5 M HCl and 60 000 U pepsin were added and the mixture incubated for a further 45 min at 200 rpm. pH was adjusted)(6.35 to 6.40) with 16 to 26 ml of 1M NaHCO3 and then each sample was volume-equalized by the addition of ddH2O. Pancreatin (59.1 mg) was then added and the mixture further incubated for 2 h at 120 rpm. When the final incubation was complete, the mixture was centrifuged at 12 000 rpm for 12 min and the supernatant filtered through gauze, then frozen at –20^{o}C until used.

4.3.3 Proliferation of CPA

An isolate of *Clostridium perfringens* from a broiler chicken with necrotic enteritis was confirmed type A using multiplex polymerase chain reaction as described by Songer & Bueschel (1998). The isolate was inoculated into fresh sterile TG and incubated for 16 h at 37^{o}C and 90 rpm, then dispensed into 800 µl ultracentrifuge containers and frozen at –80^{o}C until used. In preparation for the proliferation experiments, one sample was thawed and 100 µl inoculated into 100 ml TG then incubated for 13 h at 90 rpm and 37^{o}C. For each of the three trials, eight tubes

were inoculated (n=24) with 100 µl of the 13 h culture. In the tables with the results, where the n does not equal 24, the samples were out of our range (<30 or >300) of CFUs/ml deemed representative.

4.3.3.1 Oil

The 13 h culture (100 µl) of was placed into either 6 ml of the treated corn or wheat germ oil plus TG or TG plus pepsin, pancreatin and bile salts then incubated for 4 h at 40^{o}C and 120 rpm.

4.3.3.2 Starch

The 13 h culture (100 µl) was inoculated into each of 6 ml digested or non-digested corn or wheat starch plus TG or 6 ml TG plus pepsin (1194 U/ml) and pancreatin (1.18 mg/ml).

4.3.3.3 Gluten

The 13 h culture (100 µl) was inoculated into each of 4 ml TG plus 2 ml of either digested or non-digested wheat gluten or corn meal gluten as well as 6 ml TG containing enzymes (698 U/ml pepsin and 0.69 mg/ml pancreatin).

All proliferations were incubated anaerobically in the Forma Scientific Orbital Shaker described above. To determine colony forming units (CFU) each 4 h incubated replicate was serial diluted in sterile 0.85% NaCl then plated on blood agar containing neomycin (1 µg/ml). CFUs were counted following an overnight anaerobic incubation of blood agar plates at 37^{o}C. Each proliferation trial was performed in triplicate.

4.3.3.4 Dipeptides

To examine the possibility that bioactive dipeptides might be responsible for the affect that corn gluten meal had on CPA proliferation, three dipeptides (Alanine-Alanine, Glycine-D-Alanine and Alanine-Glutamine) were purchased from a commercial supplier (Sigma-Aldrich Inc, St. Louis, MO, 63103). These dipeptides were added to thioglycollate media at various

concentrations (wt/vol), and CPA was inoculated into the differing dipeptide media concentrations (0.125, 0.25, 0.5, 1.0 and 2.0%), incubated, plated and enumerated as described above.

4.3.4 Alpha toxin analysis

Alpha toxin was measured from remaining culture as described in Chapter 3 using an Amplex red phosphotidylcholine-specific Phospholipase C assay kit (A-12218, Molecular Probes, Eugene OR) and read on a Fluoroskan Ascent plate reader (Thermo LabSystems, Finland).

4.3.5 Protein analysis

Protein was analyzed on the supernatants derived from digested and non-digested wheat gluten and corn gluten meal using a LECO FP-528 Protein/Nitrogen analyzer (LECO Corporation, St. Joseph, MI, 49085-2396).

4.3.6 Osmolality

Osmolality on digested and non-digested starch supernatants was analyzed using the Advanced Micro-Osmometer (Advanced Instruments, Inc., Norwood, MA, 02062-9813).

4.3.7 Statistics

The Kruskal-Wallis non-parametric analysis of variance and the Mann-Whitney rank-sum tests for analysis between two samples were used to evaluate statistical significance of *C. perfringens* proliferation and alpha toxin production between like components.

4.4 Results

4.4.1 Proliferation of CPA

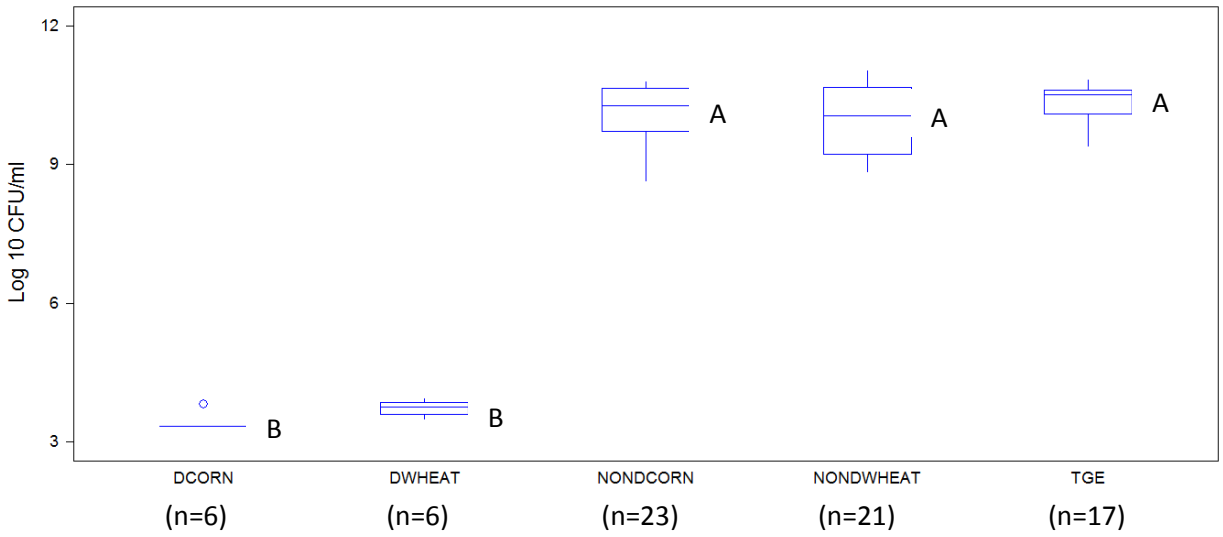
Proliferation results for CPA grown in digested and non-digested corn and wheat germ oil are shown in Table 4.1 and Figure 4.1, and corn and wheat starch are shown in Table 4.2 and Figure 4.2.

Table 4.1 *Clostridium perfringens* Type A Proliferation In Thioglycollate Media Or Thioglycollate Media Supplemented With Digested And Non-Digested Corn Or Wheat-Germ Oil

	Digested corn oil (n = 6)	Non-digested corn oil (n = 23)	Digested wheat-germ oil (n = 6)	Non-digested wheat-germ oil (n = 21)	Thioglycollate plus enzyme and bile salts (n = 17)
Median (Log10 CFU/ml)	3.34^{B}	10.27^{A}	3.75^{B}	10.05^{A}	10.51^{A}
First quartile (Log10 CFU/ml)	3.34	9.63	3.56	9.20	10.07
Third quartile (Log10 CFU/ml)	3.46	10.68	3.89	10.73	10.64

^{A,B} indicate significant differences ($P < 0.05$).
N/A = not assessed

Figure 4.1 *Clostridium Perfringens* Type A Proliferation In Thioglycollate Media Or Thioglycollate Media Supplemented With Digested And Non-Digested Corn Or Wheat-Germ Oil



DCORN = digested corn oil
DWHEAT = digested wheat oil
NONDCORN = non-digested corn oil
NONDWHEAT = non-digested wheat oil
TGE = thioglycollate plus bile acid, pancreatin and pepsin

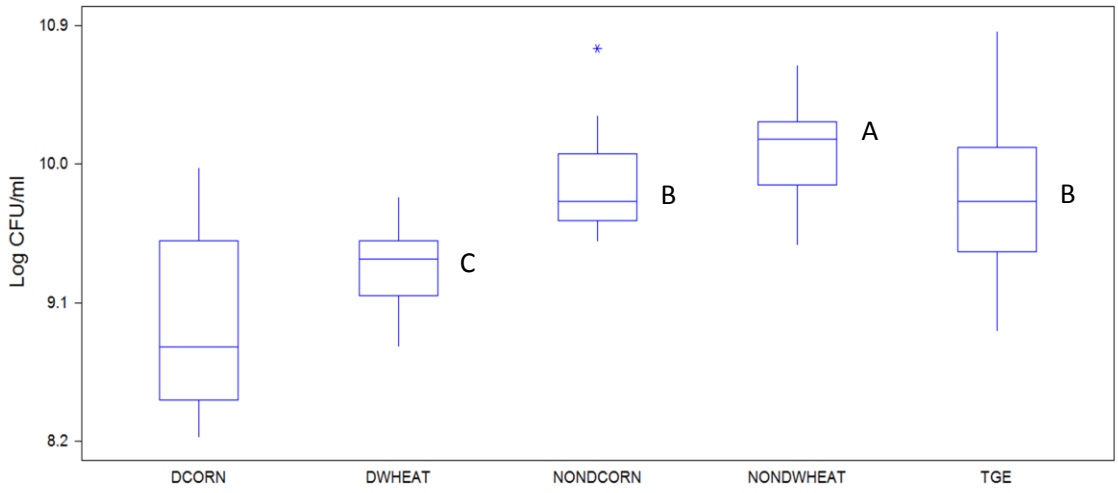
1. Area within the boxes represent the middle half of the data. Horizontal line within the box represents the median value. "o" indicates probable outlier.
2. Median values with different letters (A,B) indicate significant differences ($p<0.05$)

Table 4.2 *Clostridium perfringens* Type A Proliferation In Thioglycollate Media Or Thioglycollate Media Supplemented With Digested And Non-Digested Corn Or Wheat Starch

	Digested corn starch (n =22)	Non-digested corn starch (n = 24)	Digested wheat starch (n = 24)	Non-digested wheat starch (n = 24)	Thioglycollate Plus enzyme (n = 23)
Median (Log10 CFU/ml)	8.81^{C}	9.76^{B}	9.37^{C}	10.16^{A}	9.76^{B}
First quartile (Log10 CFU/ml)	8.46	9.62	9.11	9.86	9.35
Third quartile (Log10 CFU/ml)	9.53	10.09	9.50	10.30	10.15

^{A,B,C} indicate significant differences ($P < 0.05$).

Figure 4.2. *Clostridium perfringens* type A proliferation in thioglycollate media or thioglycollate media supplemented with digested and non-digested corn or wheat starch



DCORN = digested corn starch
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C

(n=22) (n=24) (n=24) (n=24) (n=23)

Proliferation was significantly reduced when CPA was grown in digested compared to non-digested supernatants in both oil and starch components and the TG controls.

In the first two oil proliferation trials, dilutions greater than 10^{-4} were plated, and no CFUs were observed on blood agar plates following incubation. In this trial, only samples with dilutions less than 10^{-4} CFUs had growth.

Proliferation of CPA in digested and non-digested gluten is shown in Table 4.3.

There are no significant differences between non-digested gluten and TG, nor is there a difference between the digested wheat gluten and the TG control. CPA CFUs were significantly lower than all other groups when grown in digested corn meal gluten.

When compared to our TG control group, the dipeptide Glycine-Alanine had no impact on CPA proliferation (data not shown). As well, the Alanine-Alanine dipeptide had only minor effects on the proliferation of CPA with a minor, non-significant reduction in proliferation of CP at 0.125%. However, CP proliferation was significantly reduced when grown in TG media containing 0.5, 1.0 and 2.0% Alanine-Glutamine (Ala-Gln) (Figure 4.7 and Table 4.7).

4.4.2 Alpha toxin analysis

Units of alpha toxin production per colony forming unit of CPA grown in digested and non-digested corn and wheat germ oil, corn and wheat starch and corn and wheat gluten are shown in Figures 4.4, 4.5, and 4.6 as well as in Tables 4.4, 4.5 and 4.6 respectively. The units are presented as Log10 U/CFU, and because the numbers are very small are displayed as negative. Digestion of oil and starch enhanced the production of alpha toxin units in both wheat and corn oil. Whereas, the non-digested samples produced alpha toxin not significantly different than the TG control. However, when alpha toxin production was examined in the gluten fractions of wheat and corn (Table 4.6), there was a significantly higher units per CFU production in the digested corn fraction compared to the other samples.

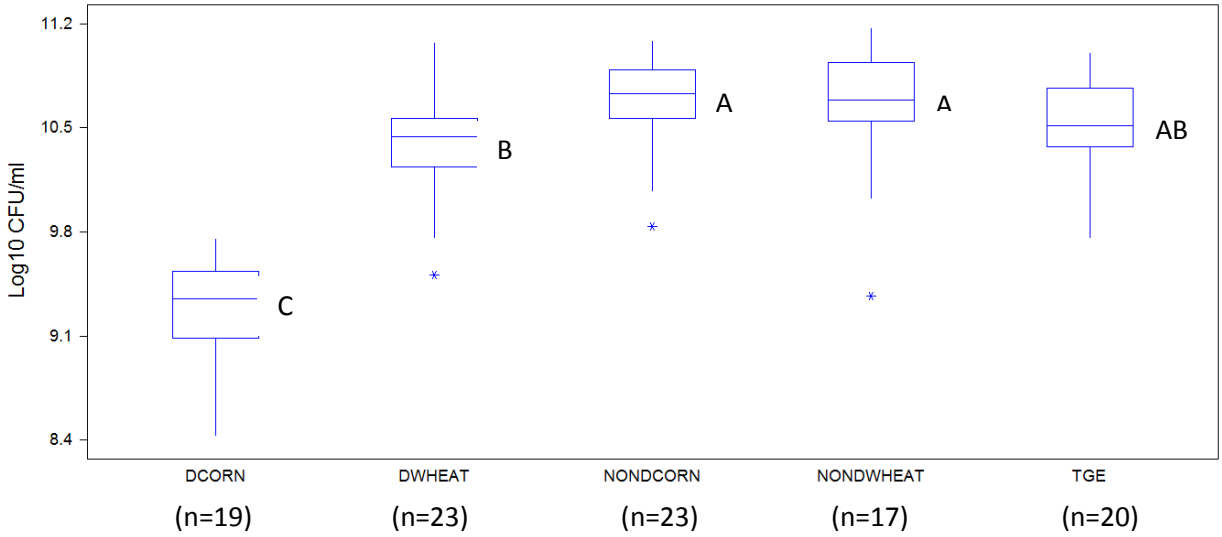
Non-digested fractions of corn gluten and wheat gluten were not significantly different than digested wheat gluten. There was no significant difference between non-digested wheat gluten and the TG control.

Table 4.3 *Clostridium perfringens* Type A Proliferation In Thioglycollate Media Or Thioglycollate Media Supplemented With Digested And Non-Digested Corn Or Wheat Gluten

	Digested corn gluten (n = 19)	Non-digested corn gluten (n = 23)	Digested wheat gluten (n = 23)	Non-digested wheat gluten (n = 17)	Thioglycollate plus enzyme (n = 20)
Median (Log10 CFU/ml)	9.35^{C}	10.73^{A}	10.44^{B}	10.69^{A}	10.52^{AB}
First quartile (Log10 CFU/ml)	9.08	10.57	10.22	10.52	10.35
Third quartile (Log10 CFU/ml)	9.55	10.90	10.61	10.98	10.78

^{A,B,C} indicate significant differences ($P < 0.05$).

Figure 4.3 *Clostridium Perfringens* Type A Proliferation In Thioglycollate Media Or Thioglycollate Media Supplemented With Digested And Non-Digested Corn Or Wheat Gluten



DCorn = digested corn gluten meal
DWheat = digested wheat gluten
NonDCorn = non-digested corn gluten meal
Nondwheat = non-digested wheat gluten
TGE = thioglycollate plus pancreatin and

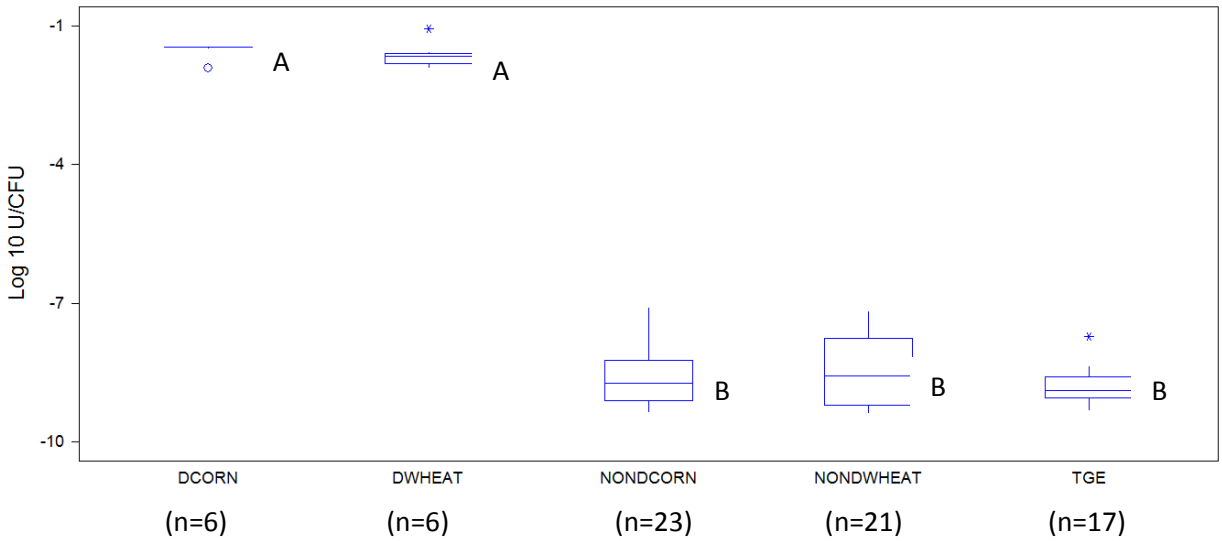
1. Area within the boxes represent the middle half of the data. Horizontal line within the box represents the median value. "*" indicates possible outlier.
2. Median values with different letters (A,B,C) indicate significant differences ($p<0.05$)

Table 4.4 Alpha Toxin Production Of *Clostridiump perfringens* Type A Grown In Media Containing Digested And Non-Digested Corn Or Wheat-Germ Oil

	Digested corn oil (n = 6)	Non-digested corn oil (n = 23)	Digested wheat-germ oil (n = 6)	Non-digested wheat-germ oil (n = 21)	Thioglycollate (n = 17)
Median (Log10 U/CFU)	-1.45[A]	-8.70[B]	-1.63[A]	-8.51[B]	-8.98[B]
First quartile (Log10 U/CFU)	-1.59	-9.08	-1.78	-9.20	-9.14
Third quartile (Log10 U/CFU)	-1.45	-8.03	-1.41	-7.72	-8.43

[A,B] indicate significant differences ($P < 0.05$).

Figure 4.4 Alpha Toxin Production Of *Clostridium Perfringens* Type A Grown In Media Containing Digested And Non-Digested Corn Or Wheat-Germ Oil



DCORN = digested corn oil
DWHEAT = digested wheat germ oil
NONDCORN = non-digested corn oil
NONDWHEAT = non-digested wheat germ oil
TGE = thioglycollate plus bile acid, pancreatin and pepsin

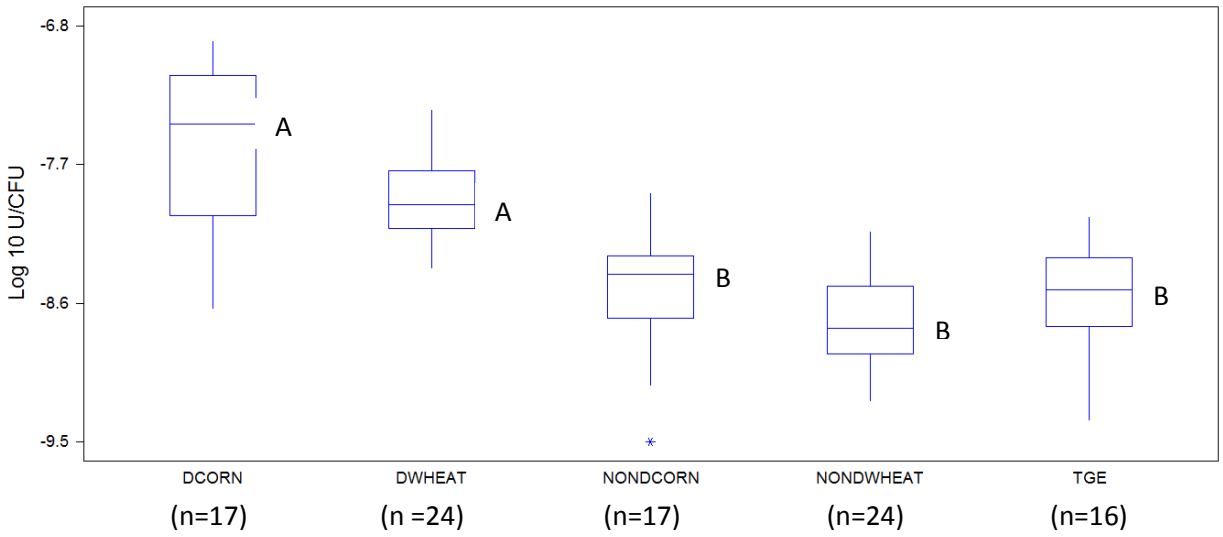
1. Area within the boxes represent the middle half of the data. Horizontal line within the box represents the median value. "*" indicates possible outlier, "o" indicates probable outlier.
2. Median values with different letters (A,B) indicate significant differences ($p<0.05$)

Table 4.5 Alpha Toxin Production Of *Clostridium perfringens* Type A Grown In Media Containing Digested And Non-Digested Corn Or Wheat Starch

	Digested corn starch (n = 17)	Non-digested corn starch (n = 17)	Digested wheat starch (n = 24)	Non-digested wheat starch (n = 24)	Thioglycollate (n = 16)
Median (Log10 U/CFU)	-7.53[A]	-8.50[B]	-7.84[A]	-8.76[B]	-8.53[B]
First quartile (Log10 U/CFU)	-8.40	-8.65	-8.05	-8.90	-8.79
Third quartile (Log10 U/CFU)	-7.30	-8.41	-7.53	-8.42	-8.22

[A,B] indicate significant differences ($P < 0.05$).

Figure 4.5 Alpha Toxin Production Of *Clostridium Perfringens* Type A Grown In Media Containing Digested And Non-Digested Corn Or Wheat Starch



DCORN = digested corn starch
DWHEAT = digested wheat starch
NONDCORN = non-digested corn starch
NONDWHEAT = non-digested wheat starch
TGE = thioglycollate plus pancreatin and pepsin

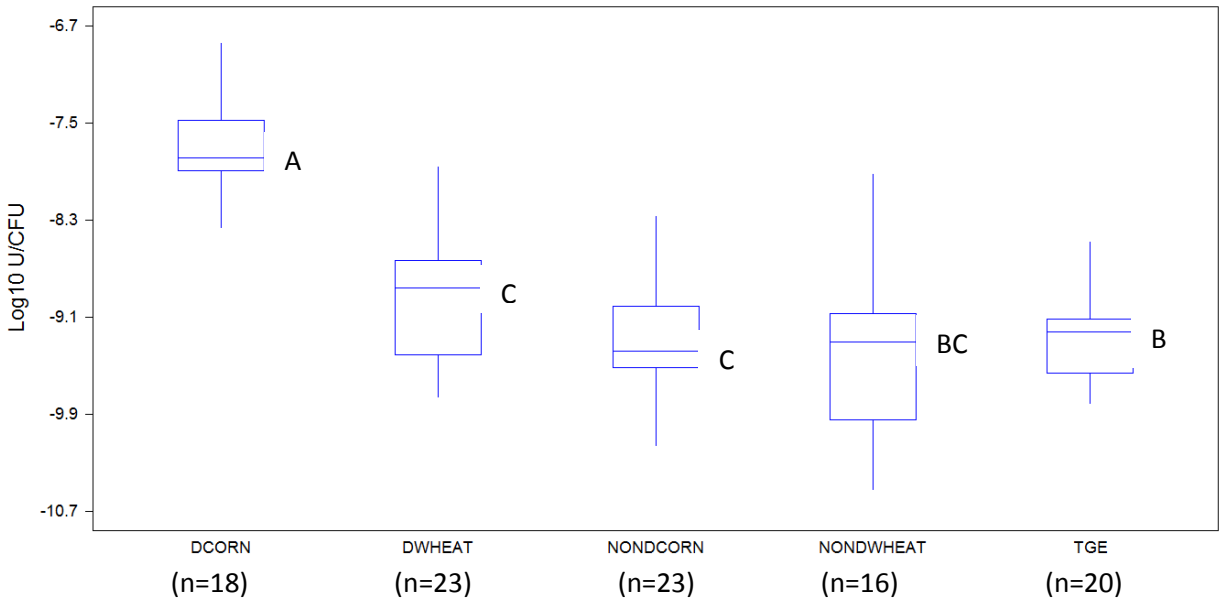
1. Area within the boxes represent the middle half of the data. Horizontal line within the box represents the median value. "*" indicates possible outlier.
2. Median values with different letters (A,B) indicate significant differences ($p<0.05$)

Table 4.6 Alpha Toxin Production Of *Clostridium perfringens* Type A Grown In Media Containing Digested And Non-Digested Corn Or Wheat Gluten

	Digested corn gluten (n = 18)	Non-digested corn gluten (n = 23)	Digested wheat gluten (n = 23)	Non-digested wheat gluten (n = 16)	Thioglycollate (n = 20)
Median (Log10 U/CFU)	-7.78[A]	-9.38[C]	-8.86[C]	-9.30BC	-9.55B
First quartile (Log10 U/CFU)	-7.92	-9.52	-9.47	-9.93	-10.06
Third quartile (Log10 U/CFU)	-7.47	-8.90	-8.63	-8.91	-9.37

[A,B,C] indicate significant differences ($P < 0.05$).

Figure 4.6 Alpha Toxin Production Of *Clostridium Perfringens* Type A Grown In Media Containing Digested And Non-Digested Corn Or Wheat Gluten



DCORN = digested corn gluten meal
DWHEAT = digested wheat gluten
NONDCORN = non-digested corn gluten meal
NONDWHEAT = non-digested wheat gluten
TGE = thioglycollate plus pancreatin and pepsin

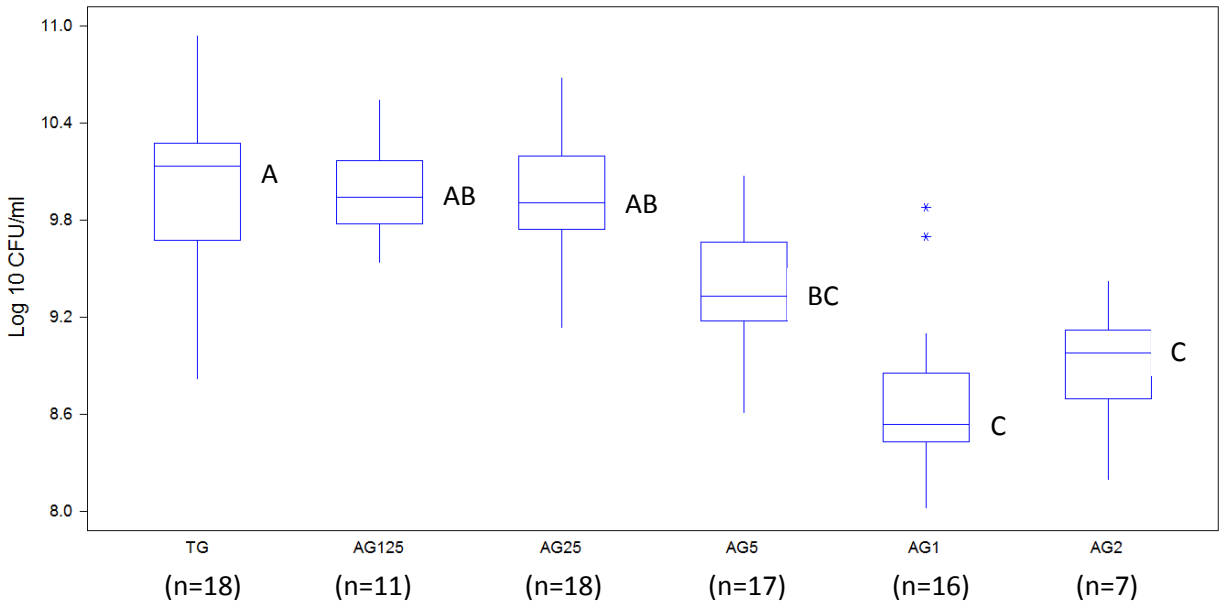
1. Area within the boxes represent the middle half of the data. Horizontal line within the box represents the median value.
2. Median values with different letters (A,B,C) indicate significant differences ($p<0.05$)

Table 4.7 Clostridium perfringens Type A Proliferation In Thioglycollate Media Or Thioglycollate Media Supplemented With Various Concentrations of the Alanine-Glutamine Dipeptide

	Ala-Gln 0.125% (n=11)	Ala-Gln 0.25% (n=18)	Ala-Gln 0.5% (n=17)	Ala-Gln 1.0% (n=16)	Ala-Gln 2.0% (n=7)	Thioglycollate (n=18)
Median (Log10 CFU/ml)	9.94^{AB}	9.91^{AB}	9.33^{BC}	8.54^{C}	8.98^{C}	10.14^{A}
First quartile (Log10 CFU/ml)	9.76	9.73	9.17	8.42	8.58	9.64
Third quartile (Log10 CFU/ml)	10.23	10.24	9.73	8.91	9.13	10.33

^{A,B,C} indicate significant differences ($P < 0.05$).

Figure 4.7 *Clostridium Perfringens* Type A Proliferation In Thioglycollate Media Or Thioglycollate Media Supplemented With Various Concentrations Of The Alanine-Glutamine Dipeptide



TG = thioglycollate media
AG125 = dipeptide at 0.125%
AG25 = dipeptide at 0.25%
AG5 = dipeptide at 0.5%
AG1 = dipeptide at 1%

1. Area within the boxes represent the middle half of the data. Horizontal line within the box represent the median value. "*" indicates possible outlier.
2. Median values with different letters (A,B,C) indicate significant differences ($p<0.05$)

4.4.3 Protein analysis

Protein evaluated from the supernatants of digested and non-digested corn meal gluten and wheat gluten is shown in Table 4.8. Following digestion, crude protein was extracted at a higher rate from both the wheat (98.38%) and the corn (55.76%) compared to wheat gluten (80.59%) and corn gluten (47.53%) samples not exposed to digestive enzymes. Mean crude protein level of the wheat gluten was higher (89.48%) compared to corn gluten (51.65%).

Table 4.8 Protein Content In Corn Meal Gluten Supernatant And Wheat Gluten Supernatant

Gluten source	Crude protein (% DM)
Digested corn meal	55.76
Non-digest corn meal	47.53
Mean corn meal	**51.65**
Digested wheat	98.38
Non-digested wheat	80.59
Mean wheat	**89.48**

4.4.4 Osmolality

Osmolality for digested and non-digested starch supernatants is presented in Table 4.9. Digestion of the corn starch fraction increased osmolality from 683 mmol/Kg (non-digested) to 704 mmol/Kg (digested). However, digestion of the wheat starch fraction lowered the osmolality slightly to 613 mmol/Kg (digested) from 627 mmol/Kg (non-digested). Overall, the mean osmolality of corn starch was higher (694 mmol/Kg) compared to wheat starch (620 mmol/Kg).

Table 4.9 Osmolality Of Digested And Non-Digested Corn Starch Supernatant And Wheat Starch Supernatant

Starch Source	Osmolality (mmol/kg)
Digested corn starch	704
Digested wheat starch	683
Mean digested	**694**
Non-digested corn starch	613
Non-digested wheat starch	627
Mean non-digested	**620**

4.5 Discussion

The objective of this study was to determine if a major component of corn or wheat affects proliferation and alpha toxin production in *Clostridium perfringens* type A. Previous *in vitro* research suggested that digested corn may inhibit CPA proliferation compared to non-digested corn (Annett et al., 2002, Chapter 3). In the current study we found that proliferation of CPA was inhibited when grown in digested compared to non-digested corn gluten meal (CGM), as well as digested or non-digested wheat gluten and the thioglycollate control. This suggests that the mechanism by

which broilers have less reported incidence of NE in the field when fed corn-based diet compared to wheat-based diet (Branton et al., 1987; Riddell & Kong, 1992; Kaldhusdal & Skjerve, 1996; Branton et al., 1997) could be due to the gluten component in corn.

Protein content of the digested and non-digested supernatants was measured, on a dry matter basis, to rule out the possibility that there was a nitrogen deficiency in the supernatant, leading to limitation of nutrients, and thereby growth, of the CPA. Protein content was lower in

the non-digested (47.53%) compared to the digested corn-gluten supernatant (55.76%). However, because proliferation was significantly higher when CPA was grown in the non-digested corn-gluten supernatant (Table 4.3), the likelihood of protein being a limiting component in these growth media was ruled out. If protein were the factor limiting growth of CPA, one would expect that there would be more proliferation of this bacterium once digestion had occurred because it is likely that there is less energy required to metabolize amino acids compared to whole proteins. In addition, a previous study showed that there was lower CPA proliferation when this bacterium was grown in a complete corn-based broiler grower diet compared to complete wheat-based or barley-based grower diet (Annett et al., 2002).

The effect of digested corn gluten meal on CPA proliferation may be due to the end products of digestion. This study found that proliferation of CPA was inhibited when grown in TG containing 0.5% of the Alanine-Glutamine (Ala-Gln) dipeptide. It could be that this Ala-Gln dipeptide is one of the contributing factors to the reduced proliferation of CPA when grown in digested CGM). CGM has been approved as an environmentally friendly herbicide for lawns (Pest Management Regulatory Agency, Canada, (2003). The herbicidal mechanism is believed to be due to the inhibition of new root growth (Christians, 1993). In addition, when Mediterranean fruit fly larva were provided diets consisting of various protein sources, they pupated when fed, among other proteins, wheat gluten but failed to pupate when fed corn hydrolysate, (Chan et al., 1989). CGM hydroylsate has been analyzed by high pressure liquid chromatography (Liu & Christians, 1994). These authors identified certain dipeptides (gln-gln, ala-asn, ala-gln, gly-ala, and ala-ala) present in the hydrolysate, and suggested that these dipeptides are the effective agents that provide CGM with its root-inhibiting activity. Ala-Gln, one of the aforementioned dipeptides, has been used as parenteral therapy to human patients. Fuentes et al. (2004) report

that incorporation of Ala-gln into total parenteral nutrition (TPN) of patients suffering from secondary peritonitis, significantly reduced infectious morbidity, compared to patients that did not have the dipeptide added to their TPN. It is possible that, in addition to the nutrient support that glutamine offers intestinal cells, the presence of the Ala-Gln dipeptide found in CGM hydrolysate, could also inhibit bacterial growth. Digested corn gluten meal supernatant was not evaluated for the presence and concentration of di-peptides in this study, but it is possible that these compounds limit bacterial proliferation in a similar fashion to the inhibition of new-root and larval formation mentioned previously. This is supported by the fact than the reduced proliferation only occurred when the CGM was exposed to digestive enzymes. This discovery, therefore, can at least in part, explain the reduction of NE in broilers fed a corn-based diet.

Proliferation in digested oil resulted in very low CFUs. Digestion of oil with the lipase found in pancreatin would break down the lipids and produce free fatty acids which are potentially toxic to CPA and therefore, could explain the reduced growth when exposed to digested compared to non-digested corn or wheat-germ oil. Non-digested oil had no effect on proliferation when compared to CPA grown in the TG control. Therefore, it seems likely that oil is not the component which differentiates the response observed when CPA is grown in corn diet supernatant compared to wheat diet supernatant.

There is more proliferation in non-digested corn and wheat starch compared to when these starches were exposed to digestive enzymes. The pH was balanced in all samples, so the potential for digested samples to be more acidic, and therefore, inhibitory to bacterial growth was eliminated. Osmolality was measured on the starch supernatants to determine if the treatment of digestion versus non-digestion affected osmotic pressure. There was greater osmolality in the digested compared to the non-digested starch supernatants (Table 4.8). This could explain the

reduced proliferation of CPA when grown in media containing digested starch supernatants, as the high osmolality in that solution could have possibly crenated the cells by virtue of osmosis. Examining the bacteria for conformational changes using electron microscopy may be useful to confirm potential physical changes when grown in high osmole solutions. However, the greater proliferation was observed in non-digested wheat, not in non-digested corn, which was the supernatant with the lowest osmolality. Therefore, there may be some other factor in corn starch supernatant, that was not analyzed, that could be affecting proliferation. There are at least two possible explanations for the increase in osmolality in the digested compared to non-digested supernatants. First of all, the level of glucose molecules could have been higher in the digested due to the starch exposure to amylase contained in the digestive enzymes. In certain areas of medicine, granulated sugar applied directly to wounds, is becoming a treatment for prevention of infection (Mathews & Binnington, 2002). Alternatively, the addition of the enzymes themselves (both pepsin and pancreatin) could have increased the osmolality of the solution. This explanation is less likely, as this alteration in effect of proliferation was not observed in the gluten supernatants. Equilibrating osmolatily prior to performing the experiment would have eliminated this variable and thereby perhaps eliminated the differences in proliferation between digested and non-digested supernatants.

Osmolality of the starch fractions was measured. The higher osmolality observed in corn, compared to wheat, may explain the differences in proliferation when CPA is grown in these two mediums. The difference in osmolality is particularly apparent when comparing the digested fractions of the starch component. There is the possibility that CPA has receptors that accommodate both glucose and amino acids. Thus glucose excess in the corn starch fraction, produced through exposure to amylase, could have competitively inhibited more amino acid

receptors, thereby reducing the potential for nitrogen uptake by the cell, and consequently, reducing proliferation.

Units of alpha toxin per CFU were calculated from CPA grown in all digested and non-digested oil, starch and gluten supernatants. In all instances, there is a pattern of greater alpha toxin production per CFU, when CPA proliferation is reduced (Tables 4.4, 4.5 and 4.6). This work confirms observations in a previous study that examined the effect of proliferation and alpha toxin production by CPA when it was grown in ground corn or wheat (Chapter 3). Here, it was suggested that the increase in alpha toxin production could be due to the bacterium finding itself in an unfavorable environment and that the perceived stress stimulates toxin production from the bacterium.

Overall, this work shows that digesting specific components of corn or wheat, such as starch and oil will reduce proliferation of CPA *in vitro*. However, digesting gluten derived from wheat did not reduce proliferation compared to the thioglycollate control. There was, however, significantly reduced CPA proliferation only when this bacterium was grown in digested corn gluten. This finding requires further investigation into the effect of corn gluten meal in broilers infected with *Clostridium perfringens*. Further work needs to be performed to examine the mechanism behind the reduction of CPA proliferation when grown in digested corn gluten meal hydrolysate. As well, the occurrence and concentration of dipeptides found in corn gluten meal hydrolysate that could have inhibitory effects on bacterial proliferation needs to be examined.

5. PREVALENCE OF *CLOSTRIDIUM PERFRINGENS* IN BROILER CHICKENS IN SASKATCHEWAN

5.1 Abstract

The objective of this study was to determine the prevalence of *Clostridium perfringens* (CP) in broiler chickens in the province of Saskatchewan was conducted over a one year period. There were two sampling periods involving up to 41 barns per period. Prevalence of CP and anti-alpha toxin antibodies were evaluated in the birds at hatch and at slaughter. CP prevalence in the flock was also determined at mid-production and in starter feed samples. CP was present in 12.4% of birds (7.7% for winter/spring and 17.2% summer/fall) between 18 – 28 days of age and occurred in 17.8% of birds (13.7% for winter/spring and 22% for summer/fall) at slaughter. CP was not isolated from chicks at hatch. CP could be isolated from 76% of feed samples. Although not all birds had titres, the number of hatched chicks and broilers tested at slaughter peaked at an anti-alpha toxin antibody titre of 1:64. This suggests that broiler chicks acquire maternal antibody to CP alpha toxin and that they could be exposed to alpha toxin at various periods throughout their lifecycle.

5.2 Introduction

Clostridium perfringens type A (CP) is the cause of necrotic enteritis (NE) in poultry (Hofshagen & Stenwig, 1992; Engstrom et al., 2003; Wages & Opengart, 2003). This bacterium, in addition to causing clinical NE (diarrhea, sudden death, huddling, decreased feed intake) can also cause subclinical NE (reduced feed conversion and reduced growth rate) and has been attributed to causing cholangiohepatitis in the host, resulting in increased condemnations at slaughter (Onderka et al., 1990; Hutchison & Riddell, 1990; Lovland & Kaldhusdal, 1999; Sasaki et al., 2000; Lovland & Kaldhusdal, 2001). CP is believed to be ubiquitous in the environment, and along with *Escherichia coli*, considered to be one of the first colonizers of the chick gut (Smith,

1965). The exact mechanism, therefore, of how CP infection develops into NE in the chicken remains unclear. However, NE has been shown to frequently occur in broilers experiencing mild to severe coccidiosis (Baba et al., 1997; Wages & Opengart, 2003).

Currently, in Saskatchewan, CP is controlled in commercial poultry operations by the incorporation of antimicrobials and anticoccidial medications in the feed. With increased consumer awareness and concern about antimicrobial resistance, and its perceived transfer from food animals to humans, there is enhanced motivation for poultry producers to reduce or eliminate the utilization of antimicrobial growth promoters (AGPs) in poultry feed. The prevalence of CP in broiler chickens with current management practices may provide a baseline to which CP-associated production parameters are based. If AGPs are reduced or eliminated from poultry feed in the future there is the potential for an increase in CP infection in broiler chickens, Therefore, knowing the current prevalence of CP in this commodity group, prior to the elimination of a substantial management tool, may assist the poultry industry when deciding which alternative management practices are to be carried out.

This work was performed to determine the prevalence of *Clostridium perfringens* (CP) in broiler starter feed, in broilers at hatch, when the birds were between 18 and 28 days of age and at slaughter. In addition, we wanted to determine if there was exposure of newly hatched chicks and chickens at slaughter to CP-alpha toxin in commercially raised broiler chickens in Saskatchewan. The prevalence of the antibodies to this toxin could help correlate, and perhaps predict, the occurrence of NE in broiler chickens.

5.3 Materials and Methods

5.3.1 Samples collected at barn

Twenty three Saskatchewan broiler chicken producers participated in the study. Two collection periods were established throughout the year. During the study each farm was visited once during both seasonal rotations. The first rotation included flocks that were placed in winter or spring (n=41 barns) and the second rotation if the flock was placed in summer or fall (n=39 barns). The farms were visited when the flock was between 18 - 28 days of age. A sample of broiler starter diet was collected from each producer at each visit. Starter diet was collected as a means to determine if the newly placed chicks had been exposed to CP through their feed. Fresh fecal samples were collected from 18 birds in each barn. Feces were swabbed with a sterile instrument and inoculated directly onto blood agar with 0.5% neomycin (BA) plates and placed in an anaerobic chamber. During the first rotation, a portion of each fecal sample, to examine for coccidial organisms, was placed into a 2 ml screw cap tub, mixed with 1% sulphuric acid and stored at 4^{o}C.

5.3.1.1 Fecal samples

CP evaluation: Fecal samples plated on BA at the barn were incubated overnight at 37^{o}C. Plates were examined for β-hemolysis around bacterial colonies. If β-hemoloysis was observed, colonies in the centre of the area were gram-stained. Those colonies, in which large gram positive rods were observed on the gram stain, were subsequently inoculated into 5 ml of Litmus milk and incubated anaerobically at 37^{o}C overnight. If clotting and gas formation in the Litmus milk was observed, the sample was considered positive for CP.

Coccidial evaluation: Fecal samples collected and stored in 1% sulphuric acid were examined for coccidial oocysts. Each fecal sample was mixed with a vortex then one drop of the mixture placed on a microscope slide and a cover slip mounted. Microscopic examination at 600x

magnification was performed on an 18 mm^2 slide. Samples were recorded as positive or negative.

5.3.1.2 Feed samples

Three grams of each feed sample collected was placed in 10 ml sterile TG containing 0.5% neomycin and incubated overnight at 37^{o}C. Samples were vortexed then 10 µl spread on BA and incubated anaerobically at 37^{o}C overnight. Plates were assessed for β-hemolysis, and those positive were gram-stained and if warranted, grown in Litmus milk as described above.

5.3.2 Samples collected at the slaughter plant

5.3.2.1 Tissues

Flocks that had been sampled during the collection period were also sampled at slaughter. Because of unpredictable changes in slaughter schedule, only 38/41 flocks were sampled during the first collection period, and 37/39 during the second rotation (total of 75 flocks sampled at plant). At slaughter, 40 birds from each flock were selected for sampling. Preference for sampling was given to birds which were removed from the evisceration line due to hepatitis. Birds that were not diagnosed with liver disease were randomly selected from the line. From the birds selected, liver, gall bladder and jejunum were swabbed with a sterile applicator and the sample plated directly on BA. When collection was complete, BA plates were transported back to the lab in an anaerobic chamber, placed in a 37^{o}C incubator overnight, and then assessed for presence of CP as described above.

5.3.2.2 Blood

In addition to sampling tissues from the evisceration line, blood samples were collected from 20 randomly selected birds at the time of euthanasia. Once at the lab, blood samples were centrifuged at 3,000 rpm for 10 minutes to separate cells from serum. Serum was retained and

frozen at -20^{o}C until evaluated for the presence of anti-alpha toxin antibodies. Anti-phospholipase C antibodies were examined using an ELISA technique. Serum collected from broiler chickens experimentally infected with CP was used as a high inter-assay control, and serum from broiler chickens raised in rearing cages was used as a low inter-assay control. Commercially available 96-well plates (Falcon 353910 Pro-bind U-bottom) were coated with 100 ul/well of 5 ug/ml phospholipase C (PLC) and incubated at 4^{o}C for 48 hours. Plates were decanted and blocked with 200 µl/well of PBS-T-1% bovine serum albumin (BSA, EM Science BSA, Fraction V, #2930) for 30 minutes at 37^{o}C under mild agitation, then washed with PBS-T. 100 µl of two-fold serial-diluted serum samples in PBS were added and incubated at 37^{o}C for l hr with mild shaking. Following a wash with PBS-T, 100 ul/well of goat anti-chicken IgG-alkaline phosphatase enzyme conjugate (KPL #15-24-06, Mandel) diluted 1:1000 in PBS-T was added and incubated, with mild agitation, for one hour at 37^{o}C. The plates were washed again with PBS-T before 50 µl/well p-nitropheyl phosphate (pNPP) in diethanolamine buffer was added. Plates were incubated under mild agitation for a further 20 minutes then absorbance was read at 405 nm using a Dynex Revelation 4.21 Microplate Spectrophometer. The optical density (OD) for the sample was reported as the titre providing the closest OD to the interassay control.

5.3.3 Hatchery

5.3.3.1 Presence of anti-alpha toxin antibody

The hatchery was visited on a weekly basis during both rotations. At each visit 20 chicks from each breeder flock that hatched that day were humanely euthanized and blood collected (n = 820). Blood was centrifuged at 3,000 rpm for 10 minutes, the serum decanted and frozen at -20^{o}C until used to evaluate anti-alpha toxin antibody titres (method described above).

5.3.4 Presence of CP

Six of the chicks selected for blood donation were examined for the presence of intestinal CP (n = 246). Using aseptic technique, intestines were swabbed with a sterile loop and the inoculum plated directly on BA. Plates were incubated overnight in an anaerobic chamber at 37^{o}C and examined for evidence of beta haemolysis the following day.

5.4 Results

5.4.1 *Clostridium perfringens* in tissues

The prevalence of CP in broilers at slaughter was 13.68% (winter/spring rotation) and 22.02% (summer/fall rotation) with a mean of 17.8% from all broilers sampled at the plant (Table 5.1).

Table 5.1. Prevalence Of *Clostridium perfringens* (CP) Isolated From Fecal Samples At The Barn And Visceral Samples From The Processing Plant

	# of birds sampled	# positive	Prevalence
First Rotation (Winter/Spring)			
Barn	738	57	7.7%
Plant	1520	208	13.7%
Second Rotation (Summer/Fall)			
Barn	702	121	17.2%
Plant	1480	326	22.0%
Mean for both rotations			
Barn	1440	178	12.4%
Plant	3000	534	17.8%

5.4.2 *Clostridium perfringens* in feces

The prevalence of CP, as determined by the culture of fresh fecal samples of broilers between 18 and 28 days of age, varied from 7.72% (winter/spring rotation) to 17.24% (summer/fall rotation) (Table 5.1). Mean prevalence of CP from all fresh fecal samples was 12.36%.

During the first rotation, CP was identified from both fresh fecal samples and from visceral samples in 18 of the 38 flocks (Table 5.2). In 4 flocks, CP was isolated from the barn, but could not be cultured from samples collected at the plant. There were 10 flocks in which fresh fecal samples were negative for CP, but visceral samples collected at the plant were positive. During this rotation, there were 6 flocks where CP was not cultured from fresh fecal samples nor from visceral samples at the plant. In the second rotation, CP was identified in both fresh fecal samples and visceral samples from 24 of 37 flocks. In 2 flocks CP was cultured from feces only, while in 11 flocks, CP was isolated from the plant samples only. Two barns were negative for CP when both fresh feces were sampled and when viscera were cultured at the plant.

Table 5.2. Number Of Barns *Clostridium perfringens* (CP) Positive

	CP + barn only	CP + plant only	CP + both barn and plant	CP - both barn and plant
First Rotation*	4/38	10/38	18/38	6/38
Second Rotation	2/37	11/37	24/37	2/37

*There were two barns in the first rotation and one barn in the second rotation in which CPA was found in the feces of birds. However, these flocks were not tested at the plant, so are not included in the data above.

5.4.3 Clostridium perfringens in feed

Results for prevalence of CP in broiler starter feed are shown in Table 5.3. The total number of feed samples tested were 42 out of possible 46 participants. Of these 42, 32 were positive for CP

(over the two rotational periods). From the producers in which we were able to collect serial samples, 11/19 were positive in both rotations for CP, whereas, only 2/19 were CP negative both rotations.

Table 5.3. Prevalence Of *Clostridium perfringens* (CP) Isolated From Feed Samples*

	# of feed samples received	# of feed samples CP +	Prevalence of CP in feed	# of feed samples CP + both rotations	# of feed samples CP - both rotations
First Rotation	20/23	16/20	80.0%		
Second Rotation	22/23	16/22	72.7%		
Both Rotations				11/19	2/19
Mean	42/46	32/42	76.2%		

* There were four farms in which feed samples were collected and tested only once during the study period.

5.4.4 Anti-alpha toxin antibodies collected from slaughter plant

All flocks examined at slaughter had birds with anti-alpha toxin antibodies (Table 5.4). Not all birds from all flocks had titres. In all, 70 flocks, and a total of 1,392 birds had serum antibody analysis performed. One hundred and twenty-three birds from thirty eight of these flocks had no titre. The median titre was 1:32; however, the most common titre level was 1:64.

5.4.5 Anti-alpha toxin antibodies collected from hatchery

Seven hundred and sixty-nine chicks from 40 breeder flocks had serum of sufficient quantity and quality to be analyzed. All batches of chicks had birds with anti-alpha toxin antibodies. There were 13 flocks from which at least one chick had no titre. The median and most commonly observed titre was 1:64 (Table 5.4).

Table 5.4. Anti-Alpha Toxin Antibody Levels In Chicks At Hatch And In Broilers At Slaughter

	Broilers at slaughter		Chicks at hatch	
Titre	(n=1392)	%	(n=769)	%
0	123	8.8	25	3.3
8	87	6.3	18	2.3
16	216	15.5	60	7.8
32	286	20.5	131	17.0
64	303	21.8	175	22.8
128	203	14.6	170	22.1
256	107	7.7	109	14.2
512	35	2.5	41	5.3
1024	1	0.1	2	0.3
2048	31	2.2	38	4.9

5.4.6 Prevalence of CP in chicks

CP was not isolated from any intestines of the chicks that were sampled.

5.4.7 Coccidial oocysts

At least 16/18 fecal samples examined from each barn were positive for coccidial oocysts. Based on this information, evaluation for this organism was not performed in the second rotation.

5.5 Discussion

Clostridium perfringens was found in 12.36% of broilers that were tested on farm, and in 17.8% of broilers at slaughter. The barn CP prevalence in this study is greater than reported in previous studies, where Craven et al (2003) found prevalence of 0 to 4% from fresh fecal samples, which was substantially lower than the number of positive samples detected in our samples. In this same study, these workers found 16% prevalence of CP at processing, which is more consistent

with the values obtained in the current study. However, Craven et al. (2003) examined washings developed to examine carcass contamination, suggesting that infection rate could have been higher (ie: contamination rate lower than infection rate). There was a mean increase of positive birds (8.93%) in the second rotation (summer/fall) compared to the first rotation (winter/spring). This increase corresponds to Long (1973) who found that there was an increase of NE in Ontario broilers in the summer months. Although no explanation was given in Long's (1973) paper, it may be possible that intermittent heat stress in the birds during the summer months, may contribute to increased CP infection. Although there was an increase in the occurrence of CP in broilers in the summer/fall rotation, the producers that participated in this study did not indicate an increase in the incidence of clinical NE in these same flocks. This study was conducted when there was a significant increase in local feed grain prices (barley, wheat etc) that likely led to the incorporation of more corn in broiler diets during the winter/spring rotation (24/41 barns) compared to the summer/fall rotation (13/40 barns). It has been shown in numerous studies that corn-fed broilers have a reduced incidence of NE compared to wheat- or barley-diets (Branton et al., 1987; Riddell & Kong, 1991; Kaldhusdal & Hofshagen, 1992; Takeda et al., 1995; Kaldhusdal & Skjerve, 1996; Branton et al., 1997). Therefore, it is possible that the incorporation of more corn in the broiler diet could have contributed to the numerical reduction of the number of CP infected flocks during the spring/winter rotation. However, since this study was carried out for only one year, it is difficult to conclude that the changes observed could be due to a seasonal effect; subsequent longer term studies may help to confirm seasonal differences.

There was higher prevalence of CP in broilers when viscera were examined at slaughter compared to when feces were cultured in mid production. This difference may be due

in part to the number of samples per bird, because three tissues were sampled (intestine, gall bladder and liver) at the plant, whereas, only one fecal sample per bird was examined at the barn. Craven (2000) reported a correlation between the isolation of CP from fresh fecal samples and isolation of CP from the ileum. Therefore, we anticipated that the fresh fecal samples that were collected at the barn were a true indication of small intestinal infection with CP. However, since we sampled the small intestine, liver and gall bladder at slaughter, our isolation rate may have been higher yet if we had cultured the ceca. In healthy poultry, it may have been better to sample ceca to look for carriers. Cox et al (2005) found that when they tested the ceca of roosters, 14/15 tested were positive for CP. Therefore, the data may be underestimating CP prevalence. This correlated with Lovland et al (2004) who observed the majority of CP-related lesions in the intestine. However, when single birds from a flock were examined in this study, there were instances where CP could be isolated from the gall bladder or the liver and not from the intestine. It is also possible that there is an increase in the presence of CP in birds as they age. Lovland et al., (2004) showed that there was an increase in cecal CP with increasing age. As a mechanism to reduce the potential of fecal contamination in the carcass, withdrawing feed from the flock prior to shipping is a common practice. We did not measure the length of time each flock was without feed prior to slaughter, however, the stress of feed withdrawal prior to slaughter could have contributed to the increase of CP seen at that time compared, to at the farm. Feed withdrawal has been associated with increased mortality in experimentally infected birds (Truscott & Al-Sheikhly, 1977). Fasting could lead to intestinal mucosal deterioration, thereby reducing essential protective barriers and provide a suitable environment for CP growth.

There were CP-positive birds found at the barn and at the plant in 42 out of 76 barns tested. We did not examine the subtypes of *Clostridium perfringens*, to determine if the same

type found in the barn would be isolated from the flock later, at slaughter, as it has been shown that there are numerous subtypes of CP (Engström et al., 2003). There were 6 barns in the first rotation and 1 barn in the second rotation in which CP could not be identified from birds either at the barn or at the plant. However, none of these same barns were negative in both rotations. The one flock that was negative for CP in both barn and plant in the second rotation was positive for both flocks in the first rotation. In 4 of 6 barns that were negative in both barn and plant in the first rotation were positive in both locations in the second rotation, the other 2 were positive at the plant only.

It is possible that we may have been unable to detect CP in some of our samples. That is, we sampled only a small portion of tissues at the plant, and only one fresh fecal sample per bird. There may be intermittent shedding of the bacterium which could have led to inconsistent detection, or the bacterium was present in the tissues, but in such low levels that our method of isolation unable to confirm its presence.

Starter samples were requested from the producer, as we wanted to determine if there was the possibility of early exposure to CP by this route. As we did find CP in 76.2% of the samples tested, we can conclude that starter feed can be source of early CP infection in the chick. In addition, finding CP in feed suggests that this could be a source of not only early infection, but a source of continued exposure of the bacterium to the chicken.

There was no CP isolated from the intestines of newly hatched chicks. The lack of identification of CP at hatch, but the prevalence of CP in feed, feces in mid production and organs at slaughter is consistent with Smith (1965) who showed that CP inhabits the intestine of chicks early in life. Although the intestine may be sterile at hatch, the potential for contamination shortly after hatch is possible. Craven et al., (2003) found that 30% of fluff and

egg shell samples tested were *Clostridium perfringens* positive at hatcheries examined in their study.

The finding of anti-alpha toxin antibodies in the day-old chicks suggests that maternal transfer of antibody is occurring. Lovland et al (2004) showed that hens that had been vaccinated for alpha toxin production passed IgG anti-alpha toxin antibodies to their progeny. However, this study evaluated natural exposure through antibody titre, and may closely reflect levels seen in progeny from non-vaccinated hens used as control in Loveland's paper. The fact that the titres were overall higher in the chicks compared to birds at slaughter could indicate that maternal antibody levels are sufficient to protect the chick against the effects of alpha toxin and necrotic enteritis until waning of antibody titres at approximately 2 weeks of age. This could perhaps explain why the incidence of NE is higher in broilers over 2 weeks of age. The lower level of antibody titres observed at slaughter could be a result of the birds active immune system not producing the level and concentration of antibody that it was endowed with through passive immunity at hatch. Cells can produce phospholipase C (alpha toxin). Alternatively, broilers may have a higher percentage of bodily water compared to chicks, thus resulting in a lower concentration of this antibody at slaughter. A decrease in anti-alpha toxin antibody from hatch to slaughter was also reported by Heier et al (2001), when they examined the effect of maternal transfer of this antibody on broiler health. In broiler breeders, Loveland et al (2004) saw an increase in antibody levels with increasing hen age. Broiler breeders are not typically supplemented with antibiotic growth promotants, and therefore, the hens may be continually getting exposed and thereby develop antibodies to the degree that CP overgrowth and NE does not occur. The birds could be continuously exposed to CP, through feed for example, and maintain antibody levels throughout their lifecycle.

The presence of anti-alpha toxin antibody does not necessarily imply that protection against NE. Lovland et al (2003) found a correlation between higher anti-alpha toxin antibody levels in broilers from flocks that had suffered from clinical necrotic enteritis (CNE) or CP-associated cholangiohepatitis (CPH) compared to broilers from flocks with low levels of these lesions. The number of birds per flock that were considered positive in Lovland's study varied from 18% for CNE to 79% for subclinical necrotic enteritis. NE occurs more frequently in birds that are over 3 weeks of age, and this may suggest that maternal antibody is sufficient to offer protection, but certain levels must be obtained to be protective. As well, Heier et al (2001) reported an inverse relationship between the level of maternal anti-alpha toxin antibodies and flock mortality. During the time period of the current study, participating producers did not report CNE in any of the flocks examined. As a result, unlike Lovland et al (2003) who could make a correlation, our data does not allow the same interpretation, as there were no overt CNE to make a comparison.

That all chicks at hatch did not have antibody suggests that transfer of material anti-alpha toxin antibody is inconsistent. In addition, the finding that not all broilers at slaughter had antibody suggests that production of this antibody may be relative to recent exposure, or that some birds do not produce anti-alpha toxin antibody following exposure. In comparison, McCourt et al (2005) had a different curve for alpha toxin antibody titres when they looked at 310 broilers over a 5 to 6 week grow out period. McCourt et al (2005) found a higher proportion of birds to have no CP alpha toxin antibody, this is compared to the current study where the highest proportion of broilers had a 1:32 titre. The difference between the two studies could possibly be explained by sample type that was used to evaluate the titres. McCourt et al (2005) tested antibody level from intestinal contents compared to serum antibody levels in the current

work. That there may be difference in the levels between the two studies suggests that more work should be done to compare the two methods to enhance the ability to interpret alpha toxin antibody titres. Lovland et al (2004) in their examination used a cut-off point of 1:250 dilution, and they stated that only titres greater than that dilution were considered positive. As a correlation between antibody titre and protection against NE was not made in this study, our data for all levels of antibody titres are provided in Table 5.4. Therefore, any titre greater than 0 was considered positive.

To our knowledge, this is the first study to look at alpha antibody titres at hatch and at slaughter and CP infection rates during the broiler cycle (hatch, mid production and slaughter). The low titres observed in broilers at slaughter could be due to the impact of anticoccidial and antimicrobial medications used in poultry feed in Saskatchewan. These medications help to reduce CP infection and overt CP-associated disease which could have contributed to the low titres observed in this study.

All flocks tested in the first rotation were infected with coccidial organisms. All flocks examined in this trial were provided with a combination of anticoccidial and antimicrobial growth promotants in their feed. It is possible that the inclusion of anticoccidial and antimicrobial medications in the feed prevented overt coccidiosis and NE during this time period. However, this current work shows that these medications did not prevent CP carriage.

There is no withdrawal period required for these licensed medications in Canada; therefore, these can be utilized in the feed until slaughter. The increase in the number of CP-carrier birds at slaughter, however, may suggest that some producers did in fact, limit the usage of these medications prior to shipping the flock. This work could serve as a baseline of CP prevalence in broiler chickens while producers in Saskatchewan are utilizing preventative

medications and the difference that may be experienced if medications are no longer routinely incorporated into broiler feed in the future.

6. RISK FACTORS AND OUTCOMES ASSOCIATED WITH THE ISOLATION OF *CLOSTRIDIUM PERFRINGENS* FROM BROILER CHICKENS IN SASKATCHEWAN

6.1 Abstract

A study of the influence of management practices on the occurrence of *Clostridium perfringens* (CP) in broiler chicken production in the province of Saskatchewan was conducted over a full year. There were two sampling periods involving up to 41 barns per period. Factors associated with the isolation of CP included anti-alpha toxin antibody (AATA) titres at hatch and at slaughter. Barn factors that influenced the frequency of CP isolation included relative humidity, CO2, floor type and barn type. Feed form, feed supplier, contamination of starter feed and in feed antimicrobials also affected CP isolation. In addition there was a difference in CP isolation when bird strain was compared. There was a positive correlation between isolating CP from feces during mid-cycle production and isolating CP at slaughter. The effects of CP infection included an increase in lesion score with decreasing isolation of CP. There were no significant effect of CP infection on whole carcass or liver condemnations, total flock mortality or birds arriving dead at the plant, nor was there an effect on total weight gain or feed conversion.

6.2 Introduction

Since 1961, *Clostridium perfringens* (CP) has been designated as the bacterium responsible for causing necrotic enteritis (NE) in poultry (Parish, 1961a; Parish, 1961b; Parish 1961c; Opengart, 2008). NE is considered to be an economically significant disease in poultry (Van der Sluis, 2000) with manifestations of acute mortality (Nairn & Bamford, 1967; Long, 1973), poor performance (Stutz & Lawton, 1984; Kaldhusdal & Hofshagen, 1992; Lovland & Kaldhusdal, 2001) and increased condemnations at slaughter (Lovland & Kaldhusdal, 1999; Lovland and Kaldhusdal, 2001). NE in Canada is primarily controlled by the incorporation of prophylactic antimicrobials in poultry feed (George, et al., 1982, Prescott et al., 1978). If prophylactic

antimicrobials are withdrawn from use in poultry feed in Canada, it is anticipated that the occurrence of both clinical and subclinical NE will increase, as it did in the European Union following the ban of these compounds in poultry feed (Grave et al., 2004). In light of this, focus on NE has been on executing various management practices that reduce the colonization of CP and subsequently, the incidence of NE in broiler flocks. Management to manipulate predisposing factors to NE, such as coccidiosis (Al-Sheikhly & Al-Saieg, 1980; Shane et al., 1985), diet (Annett et al., 2002; Kaldhusdal & Skjerve, 1996; Truscott & Al-Sheikhly, 1977; Branton et al., 1987; Riddell & Kong, 1991; Kaldhusdal & Hofshagen, 1992; Takeda et al., 1995; Branton et al., 1997), seasonal effects (Kaldhusdal & Skjerve, 1996; Long, 1973), feed form (Branton, et al., 1987) and prevention through vaccination (Heier et al., 2001; Lovland et al., 2004) have all been reported to affect the incidence of CP carriage and NE. However, because of the lack of a suitable NE model, the reproducibility of several of these predisposing factors has been difficult. In light of that, the current study was performed in an attempt to further characterize CP carriage in broilers in Saskatchewan and to examine what factors would increase or decrease the incidence of the bacterium and what the consequences of the infection were.

6.3 Materials and Methods

6.3.1 Barn sampling

Broiler chicken producers in Saskatchewan were invited to participate in this study, of which 23 agreed. Our goal was to visit each farm once during both seasonal collection periods. The first collection period included flocks that were placed in winter or spring and the second flocks that were placed in summer or fall. A total of 41 and 39 barns were visited in the first and second collection periods, respectively, and flocks were between 18 - 28 days of age at the time of the visit. At the first visit to each farm, a questionnaire requiring information on housing (flooring,

size, waterer and feeder types, heat source, ventilation type), lighting (type and program), cleaning (days barn left empty, disinfectant usage), feed type (cereal, feed form, incorporation of feed additives (eg: antibiotic growth promotors), feed source, stocking density and bird strain was completed. Data on bird age and barn environmental parameters (eg: relative humidity, temperature, CO_2) were recorded at time of visit. Feed conversion, total weight gain, mortality, condemnations, and dead on arrival at the processing plant were calculated at the end of the growing cycle. To establish if there was a correlation between management techniques and CP carriage and then to establish how the subsequent infection with CP affected flocks, the following samples were collected. A sample of broiler starter diet that was fed to the current flock was collected from each producer at each visit. Freshly released fecal samples were collected from 18 birds in each barn and analyzed for CP infection. Feces were swabbed with a sterile instrument and inoculated directly onto blood agar with 0.5% neomycin (BA) plates and placed in an anaerobic chamber. During the first rotation a portion of each fecal sample, to examine for coccidial organisms, was placed into a 2 ml screw cap tub, mixed with 1% sulphuric acid and stored at 4^oC.

6.3.1.1 Fecal samples

CP evaluation: In the barn, broilers were observed so that fresh fecal samples could be collected and plated direction onto BA at the barn level. The plates were placed in an anaerobic chamber and transported to the lab where they were then placed in a 37^oC incubator overnight. The next day, the BA plates were examined for bacterial growth and β-hemoloysis, typical of *Clostridium perfringens*. Bacterial colonies in the centre of the β-hemoloytic zone were gram-strained. If large, gram positive rods were observed on gram stain, then 5 ml of Litmus milk was inoculated with the remaining colony, and the tube incubated in a 37^oC anaerobic chamber overnight. The

following day, tubes were examined for gas formation and clotting. If these were evident, the sample was considered positive for CP.

Coccidial evaluation: A portion of the fresh fecal samples collected at the barn were placed in 1% sulphuric acid. Samples were stored until they could be examined for coccidial oocysts. In preparation for examination, each preserved fecal sample was vortexed so to evenly distribute the contents, then one drop was placed on an 18 mm2 microscope slide and a cover slip mounted. Microscopic examination was performed at 600x magnification and samples were reported as positive or negative.

6.3.1.2 Feed samples

Starter feed samples provided by the producer were tested for the presence of CP. To achieve this, 10 ml of sterile, 0.5% neomycin TG was inoculated with 3 gm of each feed sample. The samples were then incubated overnight at 37^{o}C. The following day, the tubes were vortexed and 10 µl spread on BA. Following an overnight, anaerobic incubation at 37^{o}C, the plates were examined for β-hemolysis. Those plates with β-hemolysis were gram-stained and if large gram-positive rods were observed, the samples grown in Litmus milk as described above.

6.3.2 Plant sampling

6.3.2.1 Tissues

Presence of CP: Tissue samples were collected at the processing plant, from the flocks that had fresh fecal samples collected previously (between days 18 – 28). A total of 75 flocks were sampled at the plant. The unpredictable nature of the slaughter plant schedule resulted in five flocks (total of 80 tested at the barn level) not being tested at slaughter. From each flock, 40 carcasses were sampled. There was a preference of sampling the viscera from those birds that had been moved from the evisceration line because of hepatitis. If carcasses were not removed because of hepatitis, the samples were selected at random from the line. Liver, gallbladder and

jejunum were swabbed with a sterile applicator and the sample plated directly on BA plates that were placed in anaerobic chambers. When the samples arrived at the lab, the chambers were placed in a 37^{o}C incubator overnight, then examined for the presence of CP as described above.

Histology: Tissue samples, gall bladder, liver and jejunum, from the same 40 birds that were tested for CP were collected at the plant as well. Approximately 1 cm^2 samples from the gall bladder and liver were collected and approximately 3 cm length of jejunum was collected. Tissue samples were immediately placed in 10% buffered formalin. Formalin fixed tissues were embedded and 5 – 8 µm thick sections placed on slides, stained with haematoxylin and eosin. Tissues were viewed using 400 magnification on an Nikon microscope and evaluated using a graded scale. Lesions consistent with inflammation, necrosis and fibrosis were recorded as: 0 if there were no lesions; 1+ mild; 2+ moderate and 3+ if lesions were severe.

6.3.2.2 Blood

To establish any effect that anti-alpha toxin antibodies may have on CP carriage or flock performance, blood samples were collected from 20 randomly selected birds at the time of processing. Once at the lab, blood samples were centrifuged at 3,000 rpm for 10 minutes to separate cells from serum. Serum was retained and frozen at -20^{o}C until evaluated for the presence of anti-alpha toxin antibodies. Anti-phospholipase C antibodies were examined using an ELISA technique. Serum collected from broiler chickens experimentally infected with CP was used as a high inter-assay control, and serum from broiler chickens raised in rearing cages was used as a low inter-assay control. Commercially available 96-well plates (Falcon 353910 Pro-bind U-bottom) were coated with 100 ul/well of 5 ug/ml phospholipase C (PLC) and incubated at 4^{o}C for 48 hours. Plates were decanted and blocked with 200 ul/well of PBS-T-1% bovine serum albumin (BSA, EM Science BSA, Fraction V, #2930) for 30 minutes at 37oC under mild agitation, then washed with PBS-T. 100 ul of two-fold serial-diluted serum samples

in PBS were added and incubated at 37^{o}C for l hr with mild shaking. Following a wash with PBS-T, 100 ul/well of goat anti-chicken IgG-alkaline phosphatase enzyme conjugate (KPL #15-24-06, Mandel) diluted 1:1000 in PBS-T was added and incubated, with mild agitation, for one hour at 37^{o}C. The plates were washed again with PBS-T before 50 ul/well p-nitropheyl phosphate (pNPP) in diethanolamine buffer was added. Plates were incubated under mild agitation for a further 20 minutes then absorbance read at 405 nm.

6.3.3 Hatchery

6.3.3.1 Presence of anti-alpha toxin antibody

To examine the potential protection that maternal antibodies may have on flocks infected with CP, the hatchery was visited on a weekly basis during both rotations. At each visit 20 chicks from each breeder flock that hatched that day were humanely euthanized and blood collected (n = 820). Blood was centrifuged at 3,000 rpm for 10 minutes, the serum decanted and frozen at -20^{o}C until used to evaluate anti-alpha toxin antibody titres (method described above). Percentage of chicks from a breeder flock contributing to flock tested was multiplied by log dilution of antibody titre. There were chicks from more than one breeder supplier contributing to each broiler flock monitored. Log dilutions (base 10) were multiplied by percent of breeder contribution to flock.

6.3.3.2 Presence of CP

To examine the potential of CP infection at hatch, six of the chicks selected for blood collection were examined for the presence of intestinal CP (n = 246). Using aseptic technique, intestines were swabbed with a sterile loop and the inoculum plated directly on BA plates which were anaerobically incubated at 37^{o}C overnight. The following day, the plates were assessed for the presence of β-haemolysis.

6.3.4 Statistical analysis

All statistical models were developed in a commercial software program (SAS for Windows ver 9.1). A multilevel mixed model was used to account for unmeasured factors associated with the breeder flock, the broiler producer, and the barn where the flock was raised.

The association between the presence of anti-alpha toxin antibodies (AATA) in newly hatched chicks and numerous parameters were examined to determine if these antibodies were predictive for presence of CP in the flock at either the barn or at slaughter, for flock performance (weight gain, mortality or condemnations at slaughter) and for AATA at slaughter. Prior to analysis the AATA dilution was log transformed and multiplied by the percent of chicks from each breeder flock that contributed to the proportion of chicks in the broiler flock. In addition to collecting blood and analyzing titers at hatch, it was also of interest to determine if there was an influence of various production parameters on the level of AATA at slaughter.

Effects of various parameters were evaluated to determine if there was an association between CP exposure and various outcomes in the broiler flock. Also, the effects of the breeder flock, producer and the barn on the broiler were examined. Further, how these, and other factors may influence infection with CP, and finally, the consequences of CP infection

6.4 Results

Twenty-three producers of broiler chickens (80 flocks) and 10 producers of broiler breeder chickens (16 flocks) participated in this study. The descriptive statistics for the broiler flocks are outlined in Table 6.1. In the cases where the number (n) does not equal 80, it is because the flocks were not tested on a certain rotation or there was more than one shipment from the barn (thinning loads) to the processing plant. The broiler breeder flocks from which the antibody levels of chicks tested at the hatchery, ranged from 30 to 59 weeks of age.

6.4.1 Associations between hatch CP exposure (anti-alpha toxin antibodies) and various outcomes

There was no association between AATA at hatch and AATA at slaughter (p=0.96) or total weight gain of birds in the flock (p=0.94) after accounting for grouping at the hatchery (breeder flock) and at slaughter (producer, barn, and rotation) (Table 6.2). There were, however, associations between AATA concentrations at hatch and the probability of isolating CP on farm and at the slaughtering plant. As AATA titres increased the number of isolates of CP from feces (p=0.0001) and total CP isolates at slaughter (p=0.0001) decreased. Also, as AATA increased, total flock mortality (p=0.0001) and condemnations at the slaughter plant (p=0.002) increased.

6.4.2 Associations between Slaughter CP exposure (anti-alpha toxin antibodies) and various outcomes

The concentration of AATA at slaughter was associated with an increased frequency of CP isolated from feces (p=0.04), but was not significantly associated with an increase in the total frequency of CP isolation at slaughter (p=0.19) after accounting for grouping by producer, barn and rotation (Table 6.3). The presence of CP isolated from starter feed was negatively associated with the ability to detect AATA at slaughter (p=0.005). One other negative correlation existed between the presence of cholangiohepatitis (p= 0.0006) and the ability to detect AATA at slaughter. Positive correlations were found between slaughter AATA and the degree of intestinal necrosis (p=0.04), enteritis (p=0.0001) and multifocal hepatitis (p=0.0001) when these tissues were examined histologically. There was no correlation between AATA at slaughter and liver necrosis (p=0.95), cholitis (p=0.72) and gallbladder necrosis (p=0.63) at slaughter.

Table 6.1. Descriptive Statistics For Various Measured Parameters For Rotation 1 And Rotation 2

Parameter	Mean	Standard Deviation	Min.	Median	Max.	Sum Total
Birds housed per flock (n=80)	19582	9844.4	4800	16870	55488	1566563
Birds/sq. meter (n=78)	16.05	4.21	7.5	15.45	30.5	
Age at slaughter, d (n=86)	40.14	7.67	35	37	63	
Mortality per flock (n=69)	757.52	491.25	84	669	2809	52269
% Mortality (total mortality/birds housed)	4.21	2.34	0.37	3.86	11.75	
Feed conversion (n=55)	1.96	0.21	1.57	1.91	2.73	
CO_2 (n=79)	2198.1	1234.5	37.1	2132	7088	
Barn temperature (°C; n=80)	26.8	2.7	21.1	26.7	32.2	
Relative humidity (%; n=80)	57.49	11.18	33	58	100	
Birds processed per flock (n=68)	18330	9083.3	4509	16536	51762	1246430
% birds processed (processed/housed)	94.5	2.7	85.9	95.1	98.9	
Dead on arrival per flock (n=86)	34.4	30.3	0	23.5	158	839
% Dead on arrival (DOA/DOA + processed)	0.2	0.2	0	0.15	1.44	
Birds condemned per flock (n=86)	228.9	242.96	34	143	1292	19684
% Birds condemned (condemned/processed)	1.1	0.84	0.26	0.84	4	
Livers condemned per flock (n=86)	2.05	2.67	1	1	12	176
% Livers condemned (livers condemned/processed)	0.013	0.023	0	0.005	0.144	
CP in feces (n=82)	2.05	2.67	0	1	11	168
% CP in feces (# positive per barn/18)	11.37	14.79	0	5.56	61.11	
CP in gallbladder (40/flock; n=81)	1.49	3.18	0	0	22	121
% in gallbladder (#positive/40)	3.86	7.89	0	0	55	
% CP in intestine (40/flock; n=81)	4.2	4.73	0	2	25	340
% CP in intestine (#positive/40)	10.77	11.84	0	7.5	62.5	
CP liver (40/flock; n=81)	2.54	3.52	0	1	16	206
% CP in liver (# positive/40)	6.48	8.82	0	2.5	40	

Table 6.2. Correlation Between Chick Anti-Alpha Toxin Antibodies And Various Factors

Factor	βb	95% CI (low)	95% CI (high)	P-value
Total weight gain	0.000002	-0.00006	0.000064	0.94
CP isolated in feces	-0.00265	-0.00337	-0.00194	0.0001
CP isolated at slaughter	-0.01043	-0.01276	-0.0081	0.0001
% condemnations at slaughter	0.000003	0.000001	0.000005	0.002
% flock mortalitya	0.00002	0.000017	0.000024	0.0001
CP antibody at slaughter	-0.000004	-0.00018	0.00017	0.96

a Flock mortality is calculated based on total birds placed, less birds shipped. This does not include birds that were dead on arrival or that were condemned during processing.

b β=regression coefficient.

Table 6.3. Correlation Between Anti-Alpha Toxin Antibodies Detected At Slaughter And Various Parameters

Factor	βa	95% CI (low)	95% CI (high)	P-value
CP isolated from starter feed	-0.04433	-0.07539	-0.01327	0.005
CP isolated from feces	0.04374	0.00185	0.08564	0.04
CP isolated at slaughter	0.00779	-0.00395	0.01953	0.19
Intestinal necrosis	0.05096	0.00283	0.0991	0.04
Enteritis	0.04746	0.02425	0.07006	0.0001
Liver necrosis	-0.00033	-0.01013	0.00948	0.96
Cholangiohepatitis	-0.02267	-0.03567	-0.00968	0.0006
Multifocal hepatitis	0.01963	0.01013	0.02912	0.0001
Cholitis	0.00574	-0.02618	0.03765	0.72
Gallbladder necrosis	0.02604	-0.07858	0.1307	0.63

a β=regression coefficient.

6.4.3 Association with breeder flock and offspring infection/exposure/performance

Differences among breeder flocks explained 10.8% of the variability in chick AATA (p=0.004) and a 32.4% variability in chick weight (p=0.03) (Table 6.4). In contrast, variability in the concentration of AATA at slaughter was best explained by differences among barns in which the flock was raised (p=0.004); 23.4% of variability of AATA could be attributed to the barn in a model accounting for breeder flock, producer and rotation. The producer of the broiler flock had a significant influence on total weight gain (p=0.001) and the frequency of CP isolation from feces (p=0.02). Differences among producers explained 94% of the variability in the weight gain of the flock and 30% of the variation in the frequency of CP isolation from fresh fecal samples collected at the barn. Factors that differed across both producers and the individual barns in which flocks were raised explained variation in flock mortality and the total frequency of CP isolation. Differences among producers accounted for 52% of the variation in flock mortality (p=0.02) and differences among barns accounted for 76% (p=0.02). Of the total variation in CP isolated from feces and at slaughter, 31% was explained by differences among producers (p=0.03) and 55% by the differences among barns (p=0.03).

6.4.3.1 Factors that may influence carriage with CP

The associations between flock management and the frequency of isolating CP from feces or at slaughter were summarized in Tables 6.5 and 6.6. The unconditional differences in the number of CP isolations from feces (p=0.001) or at slaughter (p=0.04) between summer and winter were not significant when CO_2 was accounted for in the model (p>0.46). There was a reduction in CP isolated from both the feces collected at the barn (p<0.005) and from tissues at slaughter

Table 6.4. Correlation Between Breeder Flock And Various Outcomes

Outcome variable	Factors	Variance	ICC[e]	P-value
CPA antibody in chick	Flock[a]	0.04376	10.80%	0.004
			0.40336	
CPA antibody at slaughter	Flock	0.02143	0.72%	0.10
	Producer[b]	0.1026	3.40%	0.29
	Barn[c]	0.593	23.38%	0.004
	Rotation[d]	0.06502	25.57%	0.08
			2.97485	
Average chick weight	Flock	0.00000254	32.40%	0.03
			0.00000785	
Total weight gain	Producer	0.2477	94.20%	0.001
			0.26307	
Percent mortality	Producer	0.000355	52%	0.02
	Barn	0.000167	76.4%	0.03
			0.000683	
CP in feces	Producer	1.9244	30%	0.02
	Barn	0.5114	37.90%	0.22
			6.4199	
Total CP	Producer	36.7984	31.20%	0.03
	Barn	28.434	55.25%	0.03
			118.0661	

[a] Broiler breeder flock.
[b] Producer that raised the broiler flock.
[c] Barn in which the broiler flock was raised.
[d] Rotation samples were collected in.
[e] ICC = Intraclass correlation coefficient.

Table 6.5. Factors That Influence *Clostridium perfringens* Infection In Broilers Isolated From Feces

Risk Factors	β[a]	95% CI (low)	95% CI (high)	*P*-value
Season				
Fall (n=9)	1.8429	-0.090301	3.7762	0.0613
Summer (n=30)	2.4212	1.0201	3.82223	0.001
Spring (n=23)	1.521	-0.09994	3.1419	0.0654
Winter (n=18)	Reference	Category		
Season with CO_2 effect				
Fall	0.773	-1.3734	2.9194	0.4739
Summer	0.7572	-1.2974	2.8118	0.4637
Spring	0.7444	-1.009	2.4977	0.3989
Winter	Reference	Category		
Relative humidity	-0.07284	-0.1234	-0.02225	0.0055
Carbon dioxide	-0.00091	-0.00135	-0.00047	0.0001
Ventilation type	Reference	Category		
No roof exhaust (n=17)	1.0096	-0.4752	2.4943	0.179
Roof exhaust (n=63)	Reference	Category		
Feed supplier				
Small off-premises	5.7955	3.6757	7.9152	<0.0001
Commercial	0.4972	-2.974	3.9684	0.7755
Farm milled	1.657	0.3691	2.9449	0.0126
Commercial	Reference	Category		
Feed supplier				
Commercial	-2.4162	-3.5453	-1.287	<0.0001
Farm milled	Reference	Category		
Major cereal type				
Corn (n=24)	0.162	-1.3173	1.6413	0.8274
Partial corn (n=11)	-0.7871	-2.5587	0.9845	0.3778
No corn (n=44)	Reference	Category		
Feed form				
Crumble (n=54)	-1.7915	-3.1153	-0.4676	0.0088
Mash (n=26)	Reference	Category		
Antimicrobials				
Bacitracin (n=61)	-1.2423	-2.8372	0.3526	0.1246
Bambermycin (n=5)	-2.1548	-5.5028	1.1932	0.203
Virginiamycin (n=14)	Reference	Category		
Anticoccidial				
Salinomycin	0.6568	-1.1619	2.4754	0.4729
Monensin	0.5159	-1.6626	2.6944	0.6374
Narasin/Nicarbazine	-1.5657	-3.873	0.7416	0.1797
Narasin	Reference	Category		
Floor type				
Earth (n=35)	-0.7429	-2.1233	0.6375	0.2862
No earth (n=45)	Reference	Category		
Barn type				
Level 1 (n=78)	-3.0145	-5.1252	-1.0839	0.0032
Not level 1 (n=7)	Reference	Category		
Bird strain – feces only				
Hubbard	1.6828	-0.6452	4.0108	0.1535
Ross	2.0569	0.5821	3.5317	0.007
Ross and Hubbard	Reference	Category		
CPA in starter feed	-2.9431	-4.6687	-1.2175	0.0021

[a] β=regression coefficient.

Table 6.6. Factors That Influence *Clostridium perfringens* Infection In Broilers Isolated At Slaughter

Risk Factors	β[a]	95% CI (low)	95% CI (high)	*P*-value
Season				
Fall	4.7119	-2.5316	11.9555	0.1979
Summer	5.407	0.1272	10.6869	0.0449
Spring	1.6906	-4.5367	7.9178	0.5887
Winter	Reference	Category		
Season with CO_2 effect				
Fall	1.5151	-6.5836	9.6137	0.7091
Summer	0.1553	-7.8622	8.1727	0.9692
Spring	-0.9789	-7.921	5.9632	0.7785
Winter	Reference	Category		
Relative humidity	-0.2739	-0.4576	-0.903	0.0041
Carbon dioxide	-0.0024	-0.00409	-0.00072	0.0061
Ventilation type	Reference	Category		
No roof exhaust	3.6641	-1.7315	9.0597	0.1793
Roof exhaust	Reference	Category		
Feed supplier				
Hillside	-1.2076	-10.3829	7.9676	0.793
Masterfeeds/Hill	3.8912	-10.9442	18.7266	0.6014
Farm milled	-2.5193	-8.2977	3.2591	0.3862
Commercial	Reference	Category		
Feed supplier				
Commercial	1.8988	-3.4068	7.2045	0.4766
Farm milled	Reference	Category		
Major cereal type				
Corn	-1.2094	-6.3531	3.9342	0.6396
Partial corn	-6.1273	-12.4758	0.2212	0.0582
No corn	Reference	Category		
Feed form				
Crumble	1.4803	-3.8287	6.7894	0.5789
Mash	Reference	Category		
Antimicrobials				
Bacitracin	-4.6462	-10.3716	1.0792	0.1097
Bambermycin	10.3139	0.2604	20.3673	0.0445
Virginiamycin	Reference	Category		
Anticoccidial				
Salinomycin	-1.8108	-9.236	5.6144	0.6271
Monensin	3.588	-4.6751	11.8511	0.3881
Narasin/Nicarbazine	2.0165	-6.6648	10.6978	0.6435
Narasin	Reference	Category		
Floor type				
Earth (n=35)	5.9049	1.4348	10.3751	0.0105
No earth (n=45)	Reference	Category		
Barn type				
Level 1 (n=78)	1.5476	-6.4958	9.591	0.7015
Not level 1 (n=7)	Reference	Category		

[a] β=regression coefficient.

($p<0.006$) when either relative humidity or CO_2 increased. Ventilation type was not associated with the frequency of isolation of CP from feces or at slaughter ($p=0.18$). There was a greater chance of isolating CP from feces if producers obtained their broiler feed from small, off-premises-milled suppliers ($p<0.0001$) or had on farm-milled broiler feed ($p=0.01$) compared to obtaining feed from a commercial mill (Table 6.5). Feed source was not associated with the frequency of CP isolation from slaughter samples. There were 13% less CP positive samples (2.4/18) from feces if broilers were fed a ration obtained from a commercial supplier compared to farm-milled feed ($p=0.0001$), but there was no difference for samples collected at slaughter. The extent of corn in the diet was not associated with the frequency of CP isolation ($p>0.05$). There were 10% (1.79/18) less CP positive samples from feces if the flock was fed a crumble diet compared to mash ($p=0.008$), but there no differences at slaughter associated with feeding crumble.

The frequency of CP isolation from feces was 16% (2.9/18) if CP was isolated from starter feed ($p=0.0021$).

Reported antibiotic use was not associated with the frequency of CP isolation from fecal samples ($p>0.12$). There were 26% (10.3/40) more CP positive samples at slaughter if the flock was given Bambermycin compared to Virginamycin ($p=0.04$). The use of anticoccidial medication was not associated with the frequency of CP isolation from either feces or at slaughter ($p>0.18$).

There were 15% (5.9/40) more CP positive samples at slaughter if the flock had been housed on an earth floor as compared to another floor type ($p=0.01$), but this difference was not seen for fecal samples at the barn . However, there were 17% (3.1/18) less CP positive fecal

samples if the birds had been housed on level 1 compared to levels 2 or 3(p=0.003) of the barn. This difference was not seen at slaughter. The frequency of CP isolation from feces was 11% (2.1/18) higher if the flock being tested were Ross strain, compared to Ross-Hubbard cross strain (p=0.007).

6.4.4 Consequences of CP infection

It was of interest to examine the predictability of CP infection when CP was isolated from feces (Table 6.7). We found that if CP were isolated from feces, it could also be isolated at slaughter (p=0.005). Specific tissue isolations of CP correlated with previous fecal isolation also included liver (p=0.006) and intestine (p=0.005).

Table 6.7. Correlations Between Isolating *Clostridium perfringens* From Fecal Samples And Various Factors

Factor	β[a]	95% CI (low)	95% CI (high)	P-value
CP isolated at slaughter (Total)	0.08865	0.02862	0.1487	0.0045
CP isolated from liver	0.289	0.129	0.449	0.006
CP isolated from intestine	0.1797	0.05625	0.3031	0.0051
CP isolated from gallbladder	0.06317	-0.1264	0.2527	0.5076

[a] β=regression coefficient.

However, there was no correlation between fecal isolation of CP and isolating this bacterium from the gall bladder at slaughter (p=0.5)

When feed type was accounted for in the analysis (Table 6.8), there was no significant association between total frequency of CP isolation in the feces and at slaughter and production factors such as either whole carcass or liver condemnations, dead on arrival at slaughter plant, total flock mortality, weight gain or feed conversion regardless of the level of CP positive birds in that flock (p>0.08).

Table 6.8 Consequential Effects On Production Parameters Of The Broiler Chicken Infected With *Clostridium perfringens*

Outcome	Factors	β[a]	95% CI (low)	95% CI (high)	P-value
% Birds condemned	Total CP	0.000011	-0.00017	0.000188	0.8983
	Crumble	0.002933	-0.00245	0.008312	0.278
	Mash	Reference	Category		
% Livers condemned	Total CP	0.00000428	-0.000000557	0.00000911	0.0815
	Crumble	0.000118	-0.00002	0.000254	0.0887
	Mash	Reference	Category		
Birds arriving D.O.A	Total CP	0.4818	-0.129	1.0926	0.1196
	Crumble	6.1123	-9.9546	22.1792	0.4497
	Mash	Reference	Category		
Total mortality	Total CP	9.0876	-0.7089	18.8841	0.0682
	Crumble	149.23	-182.98	481.44	0.3706
	Mash	Reference	Category		
Total weight gain	Total CP	-0.00004	-0.00013	0.000041	0.3101
	Crumble	0.001306	-0.0013	0.003913	0.3192
	Mash	Reference	Category		
Feed conversion	Total CP	0.005069	-0.00344	0.01358	0.2345
	Crumble	-0.1327	-0.3451	0.07959	0.2125
	Mash	Reference	Category		

[a] β=regression coefficient.

However, when inflammatory processes were studied, we found that there was a significant inverse correlation between the isolation of CP from tissues at slaughter and the level of inflammation, fibrosis or necrosis in the liver ($p<0.03$) and inflammation of the gallbladder ($p=0.04$) (Table 6.9). Interestingly, there was no correlation between CP infection and inflammation of the intestine ($p=0.09$) or necrosis of the gallbladder ($p=0.34$). Age at slaughter

had no significant effect on the ability to isolate CP (p=0.36). At the same time, we found that there was a significant positive correlation between isolating CP and observing coccidial organisms in intestinal tissues (p=0.001). No correlation was made between the presence of CP and identifying Long Segmented Filamentous Organisms (LSFO) in tissue (p=0.17).

Table 6.9. Consequential Effects Of *Clostridium perfringens* On The Pathology Of Liver, Gall Bladder And Intestine

Factor	β[a]	95% CI (low)	95% CI (high)	*P*-value
Enteritis	0.7416	-0.1269	1.6101	0.09
Liver necrosis	-0.7247	-1.0917	-0.3576	0.0001
Cholangiohepatitis	0.5493	-1.0097	-0.08887	0.02
Multifocal hepatitis	-0.6167	-0.9771	-0.2563	0.0008
Cholitis	-1.2022	-2.3711	-0.03334	0.04
Gallbladder necrosis	-1.9335	-5.8896	2.0227	0.34
Liver fibrosis	-0.7631	-1.458	-0.06817	0.03
Coccidia in tissue	0.8012	0.3213	1.2812	0.001
LSFO in intestinal sections	-1.4307	-3.4705	0.609	0.17
Age at slaughter	-0.158	-0.5061	0.1901	0.36

[a] β=regression coefficient.

6.5 Discussion

The goal of this research was to determine if there were factors in the production of commercial broilers that would affect the detection of *Clostridium perfringens* in the flock. In addition, the effect certain parameters that were related to the detection of CP in the flock (eg: serum antibodies to alpha toxin, CP isolation etc) might have on the productivity of the flock were examined.

The correlation between higher AATA titres at hatch and the decreased isolation of CP in feces and at slaughter, suggests that these maternally-transferred antibodies may be associated

with reducing the level of infection by this bacterium in broilers during their production cycle. However, the data also show that increased levels of AATA were positively correlated with condemnations at slaughter and with increased flock mortality (Table 6.2). This is in contrast to Heier et al. (2001) who demonstrated that flocks with high AATA at hatch had lower mortality during the production period compared to flocks that had low hatch AATA levels. A greater percentage of chicks tested also had higher AATA titres (46.8% with titres > 1:128) than birds tested at slaughter (27.1% with titres >1:128). This trend in lower levels of AATA with age is similar to that reported by Heier et al (2001). The higher level of AATA at hatch suggests that there is efficient maternal transfer of AATA to the offspring, but because there were no overt breaks of NE during the sampling period, its ability to protect the flock from NE could not be effectively assessed. However, Lovland et al. (2004) looked at AATA levels in broilers from CP type A and type C toxoid vaccinated hens and provided promising results for the protection against subclinical necrotic enteritis. The present study did not find a relationship between hatch AATA and weight gain, a production parameter that is affected by subclinical NE (Stutz & Lawton, 1984; Lovland & Kaldhusdal, 2001). The breeder flocks in our study were not vaccinated, and therefore, antibody transfer to the offspring would be due to direct exposure of the hen to alpha toxin. Because maternal antibody typically wanes at approximately 2 weeks of age, the presence of AATA at slaughter, suggests that these birds are producing primary antibodies to alpha toxin through exposure to alpha toxin during the production period. Also, there was no correlation between flock antibody titres at hatch and antibody titres at slaughter, further supporting the possibility that maternal antibodies were no longer present at slaughter, and that the titres measured were due to re-exposure during grow out. AATA at hatch could, however, be attributed to the breeder flock in 10.8% of the cases (Table 6.4). Unlike Heier et al

(2002), this study did not find an association between the age of the breeder flock and hatch AATA.

Examination of the correlation between AATA at slaughter and disease detected at slaughter demonstrated an increase in multifocal hepatitis, enteritis and intestinal necrosis with an increase in AATA titres (Table 6.3). These findings are interesting because one would expect that with increasing AATA titres, the potential for enteritis and intestinal necrosis would decrease, based on the assumption that AATA would offer some protective effect against these inflammatory conditions (Lovland et al, 2004), if caused by CP. Feed withdrawal prior to slaughter can also increase the potential for necrosis and inflammation in the small intestine mucosa, thereby potentially confounding our results in that there are often variable times between feed withdrawal and actual shackle time. A more accurate measure of antibodies and their effect on subclinical NE might be to examine the lesions in the liver specifically described to be a result of CP infection (Onderka et al, 1990; Hutchison & Riddell, 1990; Lovland & Kaldhusdal, 1999; Sasaki et al, 2000; Lovland and Kaldhusdal, 2001). In the present work, there was a higher incidence of cholangiohepatitis in flocks with lower AATA at slaughter. This suggests that AATA could offer protection against the subclinical manifestations of NE. There was no correlation between cholitis, liver necrosis and gallbladder necrosis and AATA.

The increased AATA titres at slaughter correlated positively with isolation of CP from feces during the production period (Table 6.3). These data suggest that with increased exposure to CP, there is an increased exposure to alpha toxin to which the host subsequently produces antibodies. This correlation was lost when we took into account isolation of CP from the intestinal contents at slaughter. This may not be surprising because the birds could very well have passed the peak of CP infection, which may have been during the 3 to 4 week period – a time often associated with a feed change in the flock, and changes in feed sources have been

attributed to increased incidence of NE (McDevitt et al., 2006), and hence an increased potential for isolating CP during that same period.

A positive correlation between AATA at slaughter and the isolation of CP from starter feed was expected. However, there was a negative correlation (Table 6.3) in that the more CP that could be isolated from feed, the lower the corresponding AATA that was detected. This may relate to the fact that starter feed was tested, and any antibody production stimulation from that feed sample could have been diminished by the time antibody levels were checked several weeks later.

When AATA were examined at slaughter, the titres were not correlated to the breeder flock from which the broilers were derived, or to the producer (Table 6.4). However, when the variables or parent flock and producer were taken into consideration, it was found that the barn in which the broilers were raised significantly affected the level of AATA at slaughter. In other words, there are differences between barns that producers own that can influence antibody levels at slaughter. This is not surprising, as some producers in Saskatchewan have very different barn types on the same farm. The differences in barn could affect humidity, ventilation performance and cleaning post harvest, thereby affecting the potential for proliferation and presence of CP as well as concurrent pathogens.

Not surprisingly, variable chick weight could be attributed to the breeder flock; variability of chick weight could be attributed to the breeder flock in 32.4% of the cases (Table 6.4). This corresponds with several papers that have examined the effect of breeder age on broiler weight at hatch (Peebles, et al., 2001; Shalev & Pasternak 1993; Shalev & Pasternak, 1995; Suarez et al., 1997), where they have demonstrated that the average weight of the chick increased with increasing age of the breeder flock. Another production parameter, total weight gain, could be attributed to the producer in 94.2% of the flocks (Table 6.4), and was not a result

of the breeder flock from which the chicks were derived nor was total weight gain affected by the level of CP detection at slaughter (Table 6.7). This shows that the producer raising the flock has the greatest influence on the gain of the birds. In addition, 52% of the variability in mortality rate in the broilers could be attributed to the producer, and 76.43% of this variability could be further attributed to the barn in which the birds were raised. This information indicates that the parent flock has little influence on the mortality of the flock. Since there was only one hatchery that was employed to hatch all the chicks in this study, the effect of hatchery was eliminated. In addition, the effect that various hatchers within the hatchery have on chick livability was not explored, so it is not possible to completely rule out the effect that individual hatchers may have had on flock mortality. This information may be of value to the commercial hatcheries because of the supposition that first week mortality, in particular, is often deemed to be the result of hatchery malpractice. Interestingly, total mortality as a production parameter did not appear to be affected by the level of CP isolated at the end of the production cycle (Table 6.7). This is not necessarily surprising because of the numerous reports of CP infection without concurrent necrotic enteritis, the cause of CP-associated mortality in broilers. During the data collection period, there were no reports of necrotic enteritis in any of the flocks surveyed.

The isolation of CP from broiler feces at the farm could be attributed to the producer, but not to a specific barn on the farm (Table 6.4). This correlation extended to include isolation of CP from slaughter samples as well. However, when fecal and slaughter samples were examined together, 55.25% of the CP isolation could be attributed to the barn in which the birds were reared and may be directly related to the management differences observed. The quantitative management records of various barns can be noted from the descriptive statistics (Table 6.1) where density (birds/sq meter), number of birds housed, CO_2 level, barn temperature and relative humidity are all had high variability. These factors did influence the ability to isolate CP from

feces and at slaughter (Table 6.5) where there was a reduction in the isolation of CP with increased humidity and CO_2. This may contrast with previous reports which have reported that increased litter moisture enhances the potential for flocks to acquire NE (McDevitt et al., 2006). Humidity and CO_2 levels in barns are typically manipulated by altering the ventilation practiced in individual barns. In the current study, although amount of ventilation may have affected CP isolation, the type of ventilation employed did not affect the incident of isolation of CP from either feces or at slaughter (Table 6.5). The amount and frequency of ventilation is dependent upon the ambient temperature and humidity in the environment, which typically varies from one season to another. When CO_2 was included as a factor in our analysis, season did not have an influence on CP isolation either at the barn level or at slaughter (Table 6.5). There are conflicting reports on the ability to isolate CP in various seasons; where Long (1973) reported higher levels of NE in the late summer and early fall, compared to Kaldhusdal and Skjerve (1996) who observed NE in broilers more often during the winter months.

The decreased isolation of CP (17% less) from fecal samples when the flocks were raised on the first level of the barn compared to other levels (Table 6.5) suggests that there may be some protective effect from NE when the broilers are raised at ground level. These factors could also relate back to the decrease in incidence CP from feces attributed to CO_2 and relative humidity (RH), suggesting that the first level of the barn may have higher CO_2 and RH compared to other levels.

Alternatively, if the barn had an earth floor, there were 15% more CP positive broiler samples at slaughter. Interestingly, the difference due to floor type that influenced isolation of CP at slaughter was not observed in fresh fecal samples that were collected during mid-cycle. The higher rate of isolation at slaughter could be due to the fact that birds are not able to overcome the excessively high load of CP that they are exposed to throughout the production

period. The lack of difference in isolation of CP at mid cycle could possibly be confounded due to other factors, such as the time at which the samples were collected. Samples were collected when the flock was between 18 and 28 days of age. It has been demonstrated that during the third week of age, there is a change of microflora in the gut, which could be associated with feed changes (McDevitt et al., 2006). This change is often seen as an increase in opportunistic bacteria, such as Clostridial spp. (Loveland, 2004). Therefore, no change in the levels of CP during this time period could be detected, relative to floor type, because all birds at that age were universally experiencing high levels of CP in their GIT. However, the differences were detected at slaughter because with time, CP isolation typically decreases, except for the flocks that were raised on earth floors, where exposure would continue to be higher up to to slaughter.

The ability to isolate CP from feces was affected by both feed supplier and feed form. There were 13% fewer positive fecal samples when broilers were fed a diet produced at a commercial milling operation compared to diets produced on farm (Table 6.5). This reduction in isolation from feces could also attributed to broilers being fed a crumble diet compared to a mash. These effects may be confounding because farm-milled diet are typically mash and commercial diets typically crumble. The difference is logically associated with the application of heat during pelleting that precedes the crumbling process, and its impact on the survivability of the Clostridial spores and in turn exposure of the bacterium to the chicken. Regardless of where the feed was obtained, or in what form it was provided to the flock, these variables showed no difference in CP isolation at slaughter. Again this may relate to the CP isolation in starter feed.

Anticoccidial medications are incorporated into feed to reduce the effect of coccidial organisms (*Eimeria spp*) on the flock. Examination of the different types and brands of anticoccidial medications incorporated into the broiler feed made no difference in the ability to

isolate CP at any time during this study (Table 6.5). The incorporation of different antimicrobials into broiler feed is to provide protection against the development of NE. In this study, no cases of NE were reported by any of the producers, so all antimicrobials, therefore, could have offered protection against the disease. However, when we look at the ability to isolate CP from the flocks, we found that there were 26% more CP positive samples at slaughter when the flocks were provided with Bambermycin as a protective antibiotic compared to Virginiamycin (Table 6.4). This is not surprising as anecdotal reports from the field concur with this finding. It could be possible that the mechanism of action for these two prophylactic antimicrobials is different and that CP in these flocks has developed resistance to the Bambermycin.

If CP was found in the starter feed, there was a 16.1% less chance of finding this bacterium in the feces (Table 6.5). This finding seems counterintuitive, but it may be possible that the early exposure to CP could have provided some immunity against future infection, thereby reducing the amount of CP found in those birds.

One final factor that we examined that affected the isolation of CP from the flock was strain of bird. We found that there was 11.4% more CP positive feces if the flock tested were Ross, compared to Ross-Hubbard cross (Table 6.5). This could be due to genetic susceptibility by the Ross strain, allowing easier colonization by CP or alternatively, the Hubbard strain could have a higher potential for resisting CP infection.

We wanted to determine if there was a correlation between isolating CP during mid cycle and isolating it at slaughter. We found that there was a correlation between fecal CP isolated from feces and CP isolated from liver, intestine or both (Table 6.6), but there was no correlation between fecal CP and CP isolated from the gallbladder at slaughter. This suggests that fecal CP could be used as a predictor for the amount of infection a flock is experiencing at slaughter.

The final part of this study dealt with the effect or consequences of CP infection on the flock. Previous reports have sited that CP infection can affect production parameters such as condemnation (whole bird and liver), total mortality, weight gain and feed conversion (Elwinger et al., 1992; Hofshagen & Kaldhusdal, 1992; Kaldhusdal et al., 2001; Hofacre et al., 2003). However, the present study found no correlation between these issues and birds arriving to the plant dead on the truck, and the isolation of CP at slaughter, when feed type was taken into account (Table 6.7). The discrepancy between our results and those previously reported before us are likely because we took into account the effect that feed type (mash versus crumble) has on these production parameters (Leeson et al., 2000), which shows that broilers will achieve a heavier body weight at 70 days of age, when fed pelleted diet compared to mash, but when corrected for morality (because mortality was lower in the mash-fed flock compared to the flock fed pelleted feed), there was a greater feed efficiency in the flocks fed the mash diet.

A research objective was to examine the effect that isolation of CP at slaughter might have on inflammation or inflammatory derivatives observed in portions of the liver, intestine and gallbladder collected at the slaughter plant. Except for gall bladder necrosis and enteritis, the more often CP that was isolated, the less severe were the tissue pathological lesions. In effect then, CP isolation was inversely correlated to lesion severity observed in the liver and inflammation in the gallbladder. Cholangiohepatitis has been associated with CP infection (Onderka et al, 1990; Hutchison & Riddell, 1990; Sasaki et al, 2000) and this liver lesion is therefore considered a common sequelea to CP infection in the broiler (Lovland & Kaldhusdal, 1999). The negative correlation could be explained by a residual effect of the disease caused by a previous CP infection. However, the data may be skewed because samples were prioritarily examined if the liver was condemned by the on duty inspector. As no other bacterial isolation was attempted on these samples, the lesions could very well have been caused by another

pathogen such as *E. coli* (multifocal hepatitis) or *Salmonella* spp (cholitis) for example. Therefore the assumption that the lesions were caused by by a previous CP infection is far reaching.

There was a positive correlation between intestinal coccidial identification when examining the tissue histologically and the isolation of CP from slaughter samples. Since coccidiosis is considered to be a predisposing factor that can lead to outbreaks of NE (Baba et al., 1997; Opengart, 2008) the positive correlation is not surprising.

The current work leaves numerous questions as to the causes and effect of CP infection, and it remains unclear as to the reason why there were a number of CP isolates from the flocks, but with no reported concurrent NE. The unpredictability of the development of NE has been a continual problem for the poultry producer. The ability to predict NE would be especially helpful, considering that there are jurisdictions that have banned the prophylactic use of CP-controlling antimicrobials. Several explanations have been developed to help address the erratic nature of CP and its inconsistent production of NE. Since alpha toxin has in the past, been considered a major contributor to the development of NE, several studies have been conducted to determine how this enzyme may be involved, and subsequently controlled. It has been proposed that the alpha toxin may have differing configuration at the carboxy-chain, thereby issuing variable toxicity in the enzyme (Titball et al., 2000). Others have suggested that the toxin activity of some strains with identical alpha toxin sequences may be due to differences in strain growth or expression rather than to differences in specific activity (Sheedy et al., 2004). Keyburn et al (2006) suggested that alpha toxin was not necessary to the development of NE in chickens. While development of NE seems difficult to reproduce in the lab, it could be due to the immune status of the host that has been artificially inoculated. That is, immunity tends to develop if the host has been exposed to a virulent strain of CP but no immunity develops when

the strain is avirulent (Kulkarni et al, 2006). The current study examined the effect of antibodies to alpha toxin in the broiler. Since none of the flocks were reported to have developed NE, the presence or absence of AATA cannot be attributed to prevention of NE. A conclusion drawn in this study is that AATA that were present in newly hatched chicks are maternally derived and that AATA at slaughter are likely due to continual exposure of CP during the production cycle.

Major factors that affected the isolation of CP from broilers included the feed supplier and feed form. Antibiotic type affected CP isolation, but differing anticoccidials did not. Floor type, barn level, bird strain and contamination of starter feed also influenced the ability to isolate CP at various stages of the growth cycle. It seems that it may be possible to predict slaughter CP infection based on the ability to isolate CP from mid-cycle fecal samples, however, a model to fully describe this predictability was not developed. Contrary to other's reports, an effect of CP infection on condemnations, birds arriving dead at the plant, total mortality, weight gain or feed conversion was not found. This is interesting because CP infection was basically inversely correlated to lesion severity in various intestinal, liver and gall bladder tissues.

Further work is necessary to fully understand the causes and consequences of CP infection in broilers and other poultry species.

7. GENERAL DISCUSSION AND CONCLUSIONS

Necrotic enteritis (NE) continues to be a serious disease of poultry worldwide. The causative agent, CP can be isolated from affected as well as apparently healthy birds. The conundrum, then, is what causes some flocks of broiler chickens to succumb to disease caused by CP, and why are do some flocks remain relatively unscathed? In Canada, NE is still controlled by the incorporation of antimicrobial growth promotants (AGP) and coccidiostats. However, with the suggestion that antimicrobial resistance may be transferred from animal to human pathogens, there is the increased pressure from human health advocacy groups for withdrawal of these products from poultry feed. Should that come to pass, a re-emergence of NE could afflict Canadian poultry flocks, as happened in the European Union following their ban on AGP from poultry feed. As such, the necessity to explore other, non-medicated options, to control CP is of utmost urgency so that poultry producers can be prepared for a potential change in management method. Overall, NE is considered to be a multifactorial disease, manifest when certain conditions favor the proliferation of the CP and the subsequent production of its toxins in the host's small intestine. Intrinsically, diet is considered one of the numerous factors that can influence the proliferation of CP, and lead to development of NE. The reason for the alteration in proliferation of CP, and occurrence of NE using various diets in broiler chickens has been investigated. The utilization of *in vitro* methods to mimic the passage of food through the gut of the chicken was utilized to explore the effects of digested versus non-digested cereals on the proliferation of CP. The demonstration of decreased proliferation of CP when grown in the supernatant of digested corn compared to digested wheat supports studies where broilers fed corn- based diets had less CP compared to broilers fed wheat-based diets. Also, the difference in CP proliferation between digested and non-digested supernatants provides some suggestion that

pancreatic enzymes can also influence the growth of CP. The effect that pancreatic enzymes have on alpha toxin may also influence the occurrence of the disease. However, the overall milieu of the gut, including other gut enzymes, pH, bacterial, viral and protozoal populations should be taken into consideration, along with other factors such as nutritional status of the host, genetic susceptibility and stress levels prior to making affirmed conclusions. To further characterize what might be specifically affecting the differential proliferation in CP when grown in corn versus wheat supernatant, a digestion process on isolated components of the corn and wheat was chosen. That CP proliferation was reduced when grown in digested corn gluten meal (protein fraction) compared to wheat gluten suggests that there may be some molecule or compounds released as a result of the digestion process that is the inhibitory factor. Both the *in vitro* and *in vivo* examination into the effect that various protein fractions, including various dipeptides derived from the digestion of corn gluten meal (CGM), may have on the proliferation of CP and its various toxins should be explored further. The incorporation of these ingredients (CGM and/or Ala-Gln) in poultry feed using a NE-challenge model would further characterize the effect that these have on CP proliferation and the occurrence of NE. In light of recent discoveries of the various strains of CP that can be found in one host, and that there appears to be a propensity for the ability of certain strains to illicit disease, it would be interesting to examine the effects of CGM on these specific strains. The poultry industry worldwide is tending towards an antimicrobial-free era, and mechanisms that can be used in the management to control NE need utmost, and immediate attention. If the inhibitory component or components within CGM could be identified, then there is the possibility to select for corn varieties that produce higher quantities of this component(s) selected for feeding poultry. In addition, the possibility that certain components could inhibit CP may also be effective at limiting the growth or toxin

production of other pathogenic bacteria such as *Salmonella* spp, *Campylobacter spp* or *Listeria monocytogenes* for example. The identification of the active component(s) would be paramount as a first step. Further to this, mechanisms to deliver this compound to the intestinal tract where the potentially pathogenic bacteria reside would be necessary. If the active component in CGM were a dipeptide, such as Ala-Gln, the molecule may be digested and/or absorbed in the upper digestive tract, and have little effect in the lower intestine. However, since NE typically occurs in the upper and mid intestinal tract, delivery of a dipeptide to this area may not be as challenging as trying to have it available in the lower small intestine and ceca. Extrapolating the findings from this lab data to the field is an important next step. As well, determining the true prevalence and effect that CP may have on local flocks, under current management practices is necessary so that specific issues discovered in the field can be addressed, at least in part, in a controlled setting.

The current research found that all flocks examined in the first part of the prevalence study confirmed that *Eimeria spp* was present at relatively high numbers (16/18 samples per barn were positive). The association between coccidiosis and NE has been well demonstrated, hence making all our flocks potentially susceptible for the development of NE. In none of the flocks examined did the producer of the broiler chickens confirm that clinical NE had developed in the flock. This suggests that the disease had been well controlled by the incorporation of AGPs and/or there was not a virulent strain of CP in any of the barns tested during this cycle. We found that approximately 12% of all flocks tested at the barn, between 18 and 28 days of age, were positive for CP. Correspondingly, the prevalence appeared to increase when these same flocks were tested at the plant. The increase in prevalence could very well correlate with the increased number of tissues sampled at the plant (liver, gallbladder and small intestine) versus

one sample (fresh fecal) at the barn. In addition, there is the possibility that there could be intermittent shedding of this bacterium in the feces, and that not all potentially positive birds had shed CP organisms in the fecal sample that was tested. Feed was also considered to be a source of CP infection, and indeed, 76% of samples tested were positive for CP. Isolates found in the feed, in feces or at slaughter were not compared to determine if there was a direct relationship between contaminated feed and the incidence of CP in broiler chickens. Considering the potential variety of isolates that have been documented, it can only be speculated that the isolates found in contaminated feed might have been the same isolates found in the birds tested. There are studies to indicate that various strains of CP can produce toxins other than alpha toxin that may not be responsible for the manifestation of NE. NetB is one of these toxins. However, NE has been demonstrated to be produced in chickens infected with netB negative and alpha toxin positive strains (Songer and Cooper, 2010). As a result of this relatively new addition to the library of information regarding CP, study data on alpha toxin, with respect to production parameters could perhaps be extrapolated to those birds infected with the netB strain as well. As we did not examine the genotype further than to confirm the presence of alpha toxin, it is very possible that some strains found within the barns or at slaughter could have been netB positive too. Chicks at hatch had detectable anti-alpha-toxin antibodies (AATA), but in none of the chicks tested, could CP be isolated. In 10.8% of the cases, AATA could be attributed to the breeder flock. The median AATA titre was higher in chicks (1:64) compared to the median titre of broilers at slaughter (1:32). This suggested then, that there is maternal transfer of AATA to the chick, and that AATA are present throughout the life of the chicken. Although AATA were present at slaughter, the likelihood that these titres were of maternal origin is highly speculative as maternal antibodies tend to wane at approximately two weeks post hatch. The presence of

AATA at slaughter then, suggests that these birds had been exposed to alpha toxin prior to slaughter.

There were associations between AATA concentrations at hatch, the probability of isolating CP, and many corresponding association with flock production parameters. As chick AATA titres increased the number of isolates of CP from feces ($p=0.0001$) and total CP isolates at slaughter ($p=0.0001$) decreased. Also as chick AATA increased total flock mortality ($p=0.0001$) and condemnations at the slaughter plant ($p=0.002$) also increased. An increase in specific disease conditions such as multifocal hepatitis, enteritis and intestinal necrosis was found with an increase in AATA titres at slaughter. One would expect that with increasing AATA titres, the potential for enteritis and intestinal necrosis would decrease, however, alpha toxin has been demonstrated to reduce the influx of inflammatory cells in experimental mouse models, and this phenomenon could explain the results observed in this study. A higher incidence of cholangiohepatitis was found in flocks with lower AATA at slaughter. As cholangiohepatitis has been documented as a manifestation of subclinical NE (SNE), these results suggest that AATA could then, offer protection against the subclinical manifestations of NE. Lesions that were examined that have not been repeatedly documented as lesions consistent with SNE such as cholitis, liver necrosis and gallbladder necrosis were not associated with AATA. The AATA titres at slaughter correlated positively with isolation of CP from feces collected at the barn when the birds were 18 to 28 days of age. This correlation was not observed when we compared AATA to the isolation of CP from the intestinal contents at slaughter. It is likely that the flocks had peak shedding at the time that we tested the flocks in the barn, and that this degree of shedding had decreased by the time the birds were sent for slaughter. Interestingly, the more CP that could be isolated from feed, the lower the

corresponding AATA that was detected. The feed that was tested for the presence of CP was provided to chicks at placement, so there are likely several factors to explain this observation that were not measured or examined. AATA at slaughter was correlated to the barn in which the broilers were produced. Since each barn has its own atmosphere, this could perhaps influence the increased or decreased presence of CP in each barn correspondingly resulting in differing levels of AATA detected at slaughter. Chick weight could be attributed to the breeder flock with variability of chick weight assigned to the breeder flock in 32.4% of the cases. This is agreement with previous research that demonstrates that the average weight of the chick increases with increasing age of the breeder flock. Total weight gain of the broiler chicken could be attributed to the producer in 94.2% of the flocks and it was not correlated to the breeder flock, nor was it correlated to the level of CP at slaughter. In addition, 52% of the variability in mortality rate in the broilers could be attributed to the producer, and 76.43% of this variability could be further attributed to the barn in which the birds were raised. This information indicates that the parent flock had little influence on the mortality of the progeny flock. Broiler producers were not asked if they had chicks replaced in their flocks if they suffered from high first week mortality. This information would have been useful in interpreting the overall health status as it might relate to broiler breeder age. Typically very young flocks produce smaller eggs, leading to smaller, often less viable chicks and could have influenced our assessment of flock mortality as it relates to the parent flock. When fecal and slaughter samples were examined together, 55.25% of the CP isolation could be attributed to the barn in which the birds were reared. The quantitative management records of various barns such as density (birds/sq meter), number of birds housed, CO_2 level, barn temperature and relative humidity are all had high degree of variability. These components did influence our ability to isolate CP from feces and at slaughter where there was a

reduction in the isolation of CP with increased humidity and CO_2. The amount of ventilation is dependent upon the ambient temperature and humidity in the environment, which typically varies from one season to another. When CO_2 was included as a factor in our analysis, we found that season did not have an influence on our ability to isolate CP either at the barn level or at slaughter. There was a decreased rate of isolation of fecal CP if the flocks were raised on the first level of the barn, but an increase in slaughter CP if the flock was raised on an earth floor. The ability to isolate CP from feces was affected by both feed supplier and feed form with a lower possibility of isolating CP from feces if the flocks were fed feed from a commercial supplier and/or were fed a crumble compared to a mash. These variables in feed source and type showed no difference in CP isolation at slaughter. Examination of the different types and brands of anticoccidial medications incorporated into the broiler feed made no difference in the ability to isolate CP at any time during the study. However, examination of antimicrobial inclusion in the broiler diet revealed 26% more CP positive samples at slaughter when the flocks were provided with Bambermycin as a protective antibiotic compared to Virginiamycin. This finding could indicate that some strains of CP have developed resistance to Bambermycin. Although the difference was small, bird genotype also affected CP isolation with 11.4% more CP positive feces if the flock was Ross compared to Ross-Hubbard cross. This suggests that some strains could be more susceptible to CP infection than other strains. The finding that fecal CP isolated from feces was correlated with CP isolation from the liver and intestine or both suggests that fecal CP could be used as a predictor for the amount of infection a flock is experiencing at slaughter if the appropriate tissues are chosen at slaughter. The final part of this study dealt with the effect or consequences of CP carriage on the flock. No correlation was found between the presence of CP and incidence of condemnation (whole bird and liver) or mortality, weight gain,

feed conversion, birds arriving to the plant dead on the truck, and the isolation of CP at slaughter, when feed type was taken into account. These results were surprising because there have been several reports suggesting that CP infection can affect these parameters of production. To say that CP would not affect these parameters would likely be misleading as well. Although we could isolate CP from approximately 17% of the samples tested, there were no reports of NE during the time course of our trial. In addition, the incorporation of antimicrobials into the broiler diet would have had a masking effect of some of the detrimental effects that CP can have on its host. It is also possible that the isolates that were detected in this study were non-NE-producing strains. Histologically, lesion severity with respect to liver and cholecystitis was inversely correlated to the isolation of CP. This may relate to priority selection of viscera from broilers that had been previously suspected by the line inspector to have liver lesions failure determine if other pathogens were involved in the development of liver lesions. Further to this, histological examination of intestinal sections for the presence of coccidial organisms revealed a positive correlation between the presence of these organisms in the tissue and the ability to isolate CP from samples collected at slaughter. Although some issues that further the understanding on the pathogenesis of NE have been addressed in this study, this disease remains a conundrum for determining when it can afflict a flock. During the course of this study, producers did not report outbreaks of NE in their flocks. This could be directly related to the CP that was identified as not being a highly pathogenic strain. Saying that, since CP can acquire virulence through in vivo passage, these same farms could very well have experienced NE in rotations subsequent to our testing.

The multifactorial nature of this disease allows its reproducibility in research situations to be elusive. However, with the discovery of high strain variability and the speculation that there

may be more than one toxin produced by CP that could be responsible for NE, the ability to produce this disease in a model will be welcome. This research suggests that there is in fact a diet-related component that could influence CP proliferation and toxin production. To examine cereal varieties further would be warranted, as this could be a mechanism to reduce the growth of CP. Our research was conducted shortly after Bovine Spongiform Encephalopathy had been discovered in a cow in Alberta. As a consequence, Canadian feedlots were becoming very crowded and barley was being consumed through this route. This resulted in a higher than normal proportion of corn available to market, to which, more was being fed to poultry at the time. It cannot be confirmed that higher corn in the diet may have reduced the incidence of NE in the flocks tested; however, it did not seem to have an effect on our ability to isolate CP. Finally, the ability to isolate CP from poultry samples may appear to be affected by bird strain. Taking this into account as well, the ability to predict the outcome of CP infection in a flock could be based on strain of CP, strain of host, immunization status of host, diet type, diet form, co-infection with other pathogenic organisms (eg: *Eimeria spp*) and barn environment . This study also confirmed several associations that had previously been made regarding CP and management factors. These factors need to be examined more closely in an attempt to determine the exact consequence of CP infection and ultimately how to control NE in broiler chickens.

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