NUTRITIONAL STRATEGIES TO CONTROL *CLOSTRIDIUM PERFRINGENS* IN GASTROINTESTINAL TRACT OF BROILER CHICKENS

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ABSTRACT

A series of experiments were conducted to examine the effect of chemical composition of the diet on intestinal *Clostridium perfringens* populations and necrotic enteritis (NE) in broiler chickens. In the first experiment, birds were fed high concentrations of dietary protein (fish meal or soy protein concentrate) and soluble fiber (guar gum). Clinical NE was not observed, however, there was a high level of *C. perfringens* colonization especially in guar gum fed birds. The next set of experiments examined the effect of various levels of DL-Met or MHA-FA on *C. perfringens* and other intestinal microbes. These experiments demonstrated a significant reduction ($P < 0.05$) in *C. perfringens* growth with methionine supplementation in ileum and cecum. The results suggest that both DL-Met and MHA-FA may reduce intestinal populations of *C. perfringens* in broiler chickens when used in high concentrations. The next three experiments were conducted to examine the effect of dietary glycine levels on gut *C. perfringens* populations, α-toxin production and NE lesion scores. Majority of birds showed clinical signs of disease with 4.16-8.33% mortality. There was a direct correlation between intestinal *C. perfringens* populations, NE lesions scores and mortality with dietary glycine level. However, due to the use of gelatin as the dietary source of glycine in these experiments, the diets also contained high proline levels which confounded our results. The last study was conducted to establish a direct causative relationship between dietary glycine concentration and *C. perfringens* growth and/or NE in broiler chickens using encapsulated amino acids. Birds fed diets containing high levels of encapsulated glycine had higher NE lesion scores than those fed encapsulated proline or no encapsulated amino acids, thus demonstrating a direct effect of glycine on intestinal

C. perfringens growth. It is concluded that amino acid composition of dietary protein is an important determinant of intestinal microbial growth, particularly *C. perfringens*, and could affect incidence of NE in broiler chickens.

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LIST OF ABBREVIATIONS

ADFI	Average daily feed intake
ADG	Average daily gain
AGPs	Antibiotic growth promoters
BHI	Brain heart infusion
CE	Competitive exclusion
CFU	Colony forming units
CMM	Cooked meat media
DFMs	Direct fed microbials
FC	Feed conversion (feed:gain ratio)
FOS	Fructo oligosaccharides
HBSS	Hanks' balanced salt solution
HEA	Hen egg antibody
ME	Metabolizable energy
MOS	Mannan oligosaccharides
NE	Necrotic enteritis
NSP	Non-starch polysaccharides
PLC	Phospholipase C enzyme
PMN	Polymorphonuclear cells
SCFA	Short chain fatty acids
SPC	Soy protein concentrates
TGB	Thioglycolate broth
VFA	Volatile fatty acids

1.0. INTRODUCTION

Enteric diseases are an important concern to the poultry industry because of lost productivity, increased mortality, reduced welfare of birds and the associated contamination of poultry products for human consumption. For the past four decades, antibiotics have been supplemented to animal and poultry feed to improve growth performance and efficiency and protect animals from adverse effects of pathogenic and non-pathogenic enteric microorganisms (Ferket *et al*., 2002). Currently, most antibiotics are used for therapeutic purposes in humans while medicated animal feeds are the second largest consumer of antibiotics. The Union of Concerned Scientists (2003) estimates that 11.4 million kg of antibiotics are used in medicated feeds in the US each year. In the EU, the European Federation of Animal Health reported that in 1999, farm animals consumed 4.7 million kg or 35% of all antibiotics administered in the EU (European Commission Directorate General XXIV Directorate B, 1999). The net effect of using antibiotic growth promoters in the poultry industry is estimated to be a 3-5 per cent increase in growth and feed conversion efficiency (Thomke and Elwinger, 1998). It has been argued that continued, excessive use of antibiotic growth promoters (AGPs) in animal feeds imposes a selection pressure for bacteria that are resistant to antibiotics (JETACAR, 1999). Hence, antibiotics have come under increasing scrutiny by some scientists, consumers and government regulators because of the potential development of antibiotic-resistant human pathogenic bacteria after prolonged use (Ratcliff, 2000). Various trade disputes among the European Union member countries, coupled with rising consumer concern about antibiotic resistance, have caused Scandinavian countries to severely curtail or prohibit the use of non-therapeutic antibiotics in poultry feed (Norton, 2000). With a ban

on sub-therapeutic antibiotic usage in Europe and the potential for a ban in North America, there is increasing interest in finding alternatives to antibiotics for poultry production. The Animal Health Institute of America (AHI, 1998) has estimated that, without the use of AGPs, the USA would require an additional 452 million chickens, 23 million more cattle and 12 million more pigs to reach the levels of production attained by the current practices. A total ban of prophylactic and therapeutic antibiotics would cost the US consumers some US$ 1.2-2.5 billion per year (Gill and Best, 1998).

The ban of AGPs in broiler feed is an important factor that will inevitably change the microbial ecology in the intestinal tract of broiler chickens (Dumonceaux *et al.*, 2006; Knarreborg *et al.,* 2002). In the poultry industry, the most significant threat is the sporadic outbreak of necrotic enteritis (NE) which, in sub clinical cases, affects growth and feed efficiency and in severe cases causes high mortality (Hofshagen & Kaldhusdal, 1992; Kaldhusdal *et al.,* 2001; Hofacre *et al.,* 2003). The EU ban on sub-therapeutic antibiotic usage has had a profound effect on the incidence of NE in broiler chickens throughout Europe. For example, the incidence of NE in France increased from 4.0% in 1995 to 12.4% of reported diseases in 1999 (Drouin, 1999) and similar increases have been reported in other countries in Europe (Kaldhusdal and Lovland, 2000). Broiler producers in USA who have stopped using in-feed AGPs have seen increases in NE, cholangiohepatitis, gangrenous dermatitis and botulism (Shane, 2004). Currently, the use of coccidiostats of ionophore type is probably one of the main tool to control NE in the absence of AGPs because ionophores not only exert an effect against coccidia, but also against several intestinal bacteria including *Clostridium perfringens* (Elwinger *et al.,* 1994; Elwinger *et al.,* 1998; Engberg *et al.,* 2000). However, the use of ionophore

anticoccidials is currently discussed in EU and will probably also be forbidden by the year 2012. The big question for poultry researchers, broiler producers and feed manufactures is how to control this disease in the absence of antibiotic growth promoters.

2.0. LITERATURE REVIEW

2.1. Etiology and Pathogenesis of Necrotic Enteritis

Necrotic enteritis of domestic broiler chickens was first described by Parish in 1961 and now it is a common disease found in all poultry-growing areas of the world (Ficken and Wages, 1997). It is a potentially fatal disease and flock mortality rates may reach up to 1% per day with total mortalities attaining 30% (Helmboldt and Bryant, 1971). The causative agent of NE, *Clostridium perfringens*, types A (Al-Sheikhly and Truscott, 1977b; Long *et al.,* 1974; Long and Truscott, 1976; Niilo, 1978; Songer and Meer, 1996; Truscott and Al-Sheikhly, 1977) or C (Engstrom *et al.,* 2003; Nairn and Bamford, 1967; Shane *et al.,* 1984), is a nearly ubiquitous gram positive, spore forming, extremely prolific, toxigenic anaerobic bacteria found in soil, dust, feces, feed, poultry litter, and intestinal contents. The disease usually occurs in broiler chickens 2 to 6 weeks after hatching and is characterized by sudden onset of diarrhea and mucosal necrosis caused by the overgrowth of *C. perfringens* in the small intestine (Fukata *et al.,* 1991). Normally the number of *C. perfringens* in the intestine is low (about 10^4 CFU/g of digesta) but disturbances in normal intestinal microflora may cause rapid proliferation of *C. perfringens*, increasing bacterial numbers to 10^7-10^9 CFU/g of digesta resulting in the development of clinical NE (Kondo, 1988). Also, the presence of *C. perfringens* in poultry meat posses an important threat to public health (Immerseel *et al.,* 2004). Over 800 serotypes of *C. perfringens* are known and 17 different toxic fractions have been

isolated (Hatheway, 1990; McDonel, 1980). However, one toxic fraction, the chromosomal-encoded α toxin is considered the main virulence factor for this disease because birds are about 200 times more susceptible to α toxin than to beta or epsilon toxin. Alpha toxin is a zinc-metalloenzyme phospholipase C spingomyelinase that hydrolyzes phospholipids and promotes membrane disorganization (Naylor *et al.,* 1998; Rood, 1998; Titball *et al.,* 2000). Alpha toxin has direct effects on host metabolism including inhibition of neutrophil chemotaxis, vasoconstriction and platelet aggregation, hemolysis of erythrocytes and necrosis of other body cells; and modulation of cell metabolism by activating the arachidonic acid cascade and protein kinase C (Titball *et al.,* 1999). More α toxin may be produced by isolates from birds with NE than by isolates from normal birds (Hofshagen and Stenwig, 1992). Alpha toxin destruction of mucosal tissue manifests as macroscopic lesions that are usually seen in jejunum and ileum but can also appear in duodenum (Al-Sheikhly and Truscott, 1977a; Fukata *et al.,* 1988). A mixture of sloughed-off epithelial tissue trapped in fibrin will form a diphtheritic membrane that is characteristic of NE. Outbreaks of NE are sporadic and may result in high mortality and severe economic losses. It has been estimated that the cost of sub-clinical NE can be as much as US $ 0.05 per bird with total global loss of almost US$ 2 billion (Van der Sluis, 2000a, 2000b). According to a rough estimate the NE (either clinical or sub clinical) is causing a US$ 2 billion loss to the world poultry industry per annum. The sub-clinical form of NE may be most economically important because of impaired feed conversion, reduced live weight at slaughter and increased condemnation percentage associated with *C. perfringens* infection (Lovland and Kaldhusdal, 2001; Stutz and Lawton, 1984).

2.2. Experimental Models of Necrotic Enteritis

Although *C. perfringens* is recognized as the etiological agent of NE, other contributing factors are usually required which predispose the flocks to disease. The presence of *C. perfringens* in the intestinal tract of broiler chickens or inoculation of animals with high doses of *C. perfringens*, however, does not always lead to the development of NE (Craven *et al.,* 2001; Drew *et al.,* 2004; Kaldhusdal *et al.,* 1999; Shane *et al.,* 1984). Due to lack of knowledge of predisposing factors that precipitate the disease, a reliable and reproducible *C. perfringens* infection model has not been published. However, some researchers have been able to induce disease and mortality in chickens using cultures of unwashed vegetative cells (Al-Sheikhly and Truscott, 1977a; Bernier *et al.,* 1977; Branton *et al.,* 1997; Cowen *et al.,* 1987; Long and Truscott, 1976; Prescott, 1979). Long and Truscott (1976) used a pure culture challenge model to reproduce NE in broiler chickens. Total NE mortality ranged from 1 to 28%. This model was later modified to include elevated dietary concentration of fish meal prior to initiation of inoculum administration and intra-duodenal administration of inoculum (Al-Sheikhly and Truscott, 1977a). Infection of broilers with *Eimeria acervulina* prior to administration of a *C. perfringens* challenge, markedly increased (from 28 to 53%) mortality due to NE. *E. acervulina* or *E. necatrix* infection alone or feeding *C. perfringens*-contaminated feed without *Eimeria* infection, did not result in mortality (Al-Sheikhly and Al-Saieg, 1980).

Successful induction of disease usually requires previous intestinal damage, particularly sloughing of intestinal epithelial cells and leakage of plasma protein into the intestinal lumen. Since the minimum requirements for growth of *C. perfringens* include

more than 11 amino acids, besides various growth factors and vitamins (Fuchs and Bonde, 1957; Muhammed *et al.,* 1975; Petit *et al.,* 1999), the leaking of plasma proteins to the intestinal lumen may provide a necessary growth substrate for over proliferation of *C. perfringens* and/or for up regulation of α toxin gene. Shane *et al.* (1985) reported that infection of broiler chickens with *Eimeria acervulina* prior to inoculation with *C. perfringens* increased mortality from 8% in Eimeria-free control to 35% in Eimeria-infected birds. This was associated with destruction and sloughing of intestinal epithelium, a 39% increase in intestinal passage time, reduced intestinal pH and depressed serum protein concentration.

Hamdy *et al.* (1983) used litter from a source that had experienced an outbreak of NE and were able to induce the disease. Cowen *et al.* (1987) tried three different methods: a broth culture and feed mixtures, infected litter, and a combination of these. The combination was the most effective. Kaldhusdal *et al.* (1999) experimented with a spontaneous disease model in some of their studies. Williams *et al.* (2003) demonstrated that a combination of diet (high inclusion of wheat and fish meal), infection with *Eimeria maxima* followed by per cloacal *C. perfringens* inoculation 6 days later resulted in NE lesions but no mortality. Jansman *et al.* (2003) presented a sub-clinical model of NE based on a combined challenge with *E. acervulina* on day 10 of age and a *C. perfringens* challenge on day 14-16 of age. The combined challenge was shown to reduce feed intake and body weight gain by 15 and 23%, respectively, and increase feed conversion ratio by 10% compared to control birds. Necrotic enteritis lesions were observed in almost 50% of the challenged birds. Recently, McReynolds *et al.* (2004) described the disease production in broiler chickens using immunosuppressive agents, commercial coccidial

and bursal disease vaccines. Despite numerous attempts, there is no generally accepted disease model for the experimental production of NE in broiler chickens because consistent reproduction of the disease is difficult to achieve and so more work on disease models is necessary. This lack of reliable experimental models hampers efforts to evaluate the predisposing effects of putative risk factors and efficacy of preventive measures (Pedersen *et al.,* 2003).

2.3. Predisposing Factors for Necrotic Enteritis in Poultry

Despite our present understanding of this disease, and the identification of *C. perfringens* as the etiological agent, the predisposing factors that lead to over proliferation of *C. perfringens* and the subsequent progression to disease are poorly understood. It is well accepted that NE is a multi-factorial disease process in which a number of co-factors are usually required to precipitate an outbreak of the disease. These predisposing factors are numerous, but many are ill-defined and experimental results have been contradictory. We have grouped the various risk factors for NE into 5 broad categories **(Table 2.1)**. This short list of well-documented predisposing factors is probably far from complete. Even when taking into account the known precipitating factors; it is still difficult to reproduce the symptoms and lesions of NE experimentally. Despite the identification of various risk factors, no study has been conducted that correctly calculates the relative risk of the different dietary factors and no study on the relative importance of different environmental management strategies has been performed.

2.4. Potential Strategies to Control Necrotic Enteritis

There are three basic strategies that can be employed to cope with the loss of AGPs and control NE in broiler chickens. These are: 1) pathogen reduction; 2) augentation of immune response; and 3) nutritional strategies and/or feed additives (Revington, 2002). Pathogen reduction strategies typically involve establishing effective farm-site biosecurity and poultry house sanitation protocols and will not be addressed here. Vaccination strategies have been investigated to augent specific immunity against *C. perfringens* and there are a number of diet-related strategies designed to control NE which rely on several proposed mechanisms of action, including direct antimicrobial activity, competitive exclusion as well as augentation of immunity. We will review a number of strategies for control of *C. perfringens* including: 1) Direct-fed microbials including competitive exclusion products and probiotics, 2) Prebiotics, 3) Organic acids, 4) Plant extracts or essential oils, 5) Feed enzymes, 6) Hen egg antibodies, 7) Vaccination against *C. perfringens,* 8) Anticoccidial vaccination, 9) Bacteriophages, and 10) Diet formulation and ingredient selection.

Table 2.1. List of various predisposing factors of NE in broiler chickens.

Risk factors	References
***Eimeria* infection**	(Al-Sheikhly and Al-Saieg, 1980; Baba *et al.,* 1992; Baba *et al.,* 1997; Balauca, 1976; Broussard *et al.,* 1986; Droual *et al.,* 1995; Elwinger *et al.,* 1992; Frame and Bickford, 1986; Helmboldt and Bryant, 1971; Hofacre *et al.,* 2003; Hofacre *et al.,* 1998; Jackson *et al.,* 2003; Jansman *et al.,* 2003; Kwatra and Chaudhury, 1976; Nairn and Bamford, 1967; Shane *et al.,* 1985)
Removal of coccidiostats or AGPs from poultry feed	(Arakawa and Oe, 1975; Brennan *et al.,* 2001; Brennan *et al.,* 2001; Brennan *et al.,* 2003; Broussard *et al.,* 1986; Collier *et al.,* 2003; Elwinger *et al.,* 1998; Elwinger *et al.,* 1994; Elwinger *et al.,* 1992; Emborg *et al.,* 2001; Engberg *et al.,* 2000; George *et al.,* 1982; Prescott *et al.,* 1978; Stutz and Lawton, 1984; Vissiennon *et al.,* 2000; Watkins *et al.,* 1997; Williams *et al.,* 2003)
Environmental and managemental conditions	(Cowen *et al.,* 1987; Craven *et al.,* 2003; Craven *et al.,* 2001; Craven *et al.,* 2001; Kaldhusdal *et al.,* 1999)
Physiological stress and immunosuppression	(Craven, 2000; Dykstra and Reid, 1978a; Estrada and Wilkie, 2000; Fukata *et al.,* 1991; McReynolds *et al.,* 2004)
Nature and form of diet	(Annett *et al.,* 2002; Branton *et al.,* 1997; Branton *et al.,* 1987; Dahiya *et al.,* 2004; Drew *et al.,* 2004; Engberg *et al.,* 2002; Kaldhusdal and Hofshagen, 1992; Kaldhusdal *et al.,* 1999; Kaldhusdal and Skjerve, 1996; Kaldhusdal, 2000; Knarreborg *et al.,* 2002; Nairn and Bamford, 1967; Riddell and Kong, 1992; Truscott and Al-Sheikhly, 1977; Wilkie *et al.,* 2005)

2.4.1. Direct-fed Microbials

The term direct-fed microbial (DFM) or probiotic has been defined as a "live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller, 1999). DFMs are intended to modify the gastrointestinal microflora in such a way that bacterial activities advantageous to the host are stimulated and those adverse to the host health are suppressed (Netherwood *et al.,* 1999; Simmering and Blaut, 2001). The modes of action of DFM include: 1) maintaining "normal" intestinal microflora by competitive exclusion and antagonism of pathogens 2) altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production, 3) improving feed intake and digestion and 4) neutralizing enterotoxins and 5) stimulating the immune system (Collins and Gibson, 1999; Simmering and Blaut, 2001; Walker and Duffy, 1998). Competitive exclusion of pathogens may be accomplished by a number of possible mechanisms including competition for mucosal binding sites, competition for luminal nutrients or production of inhibitory substances such volatile fatty acids, low pH and bacteroicins which are bacteriostatic or bacteriocidal for pathogenic bacteria (Tannock, 1997).

DFM can broadly be categorized as undefined preparations of "normal gut flora" or defined products composed of a single or small number of characterized bacterial strains. In relation to the avian intestinal tract, "normal gut flora" is a preparation of live obligate and facultative anaerobic bacteria, originating from normal, healthy adult individuals of an avian species, which is free from specific pathogenic microorganisms and is quality controlled (WHO, 1994). Such preparations can be provided orally in newly hatched chicks to promote the rapid establishment of an adult-type intestinal

microflora and produce almost immediate resistance to colonization by pathogens that gain access to the rearing environment (Mead, 2000; Nisbet, 1998; Nurmi *et al.,* 1992).

The chick exits the egg with a sterile intestinal tract which is rapidly colonized by bacteria present in the local environment (Hume *et al.,* 2003). In commercial broiler hatcheries this environment is far removed from the hen and thus delays the normal succession of intestinal colonization characterized by increasing complexity and dominance by anaerobic bacteria over aerobes (Barnes *et al.,* 1980; van der Wielen *et al.,* 2002). Normal gut flora preparations have shown efficacy against food borne pathogens such as *Salmonella spp.*, *Campylobacter jejuni, C. botulinum, C. perfringens,* pathogenic strains of *Escherichia coli* and *Yersinia enterocolitica* (Mead, 2000). Morishita *et al.* (1997) observed a 70% reduction in frequency of *Campylobacter jejuni* shedding and 27% reduction in jejunal colonization when day old chicks were given a *Lactobacillus acidphilus* and *Streptococcus faecium* probiotic. In a comprehensive study of 296 strains of lactic acid bacteria from 50 chicks, 77 of the strains were found to inhibit the growth of enteropathogenic *S. enteritidis* and *E. coli* (Garriga *et al.,* 1998).

A number of studies have reported a potential benefit of "normal gut flora" on NE in broiler chickens including reduced NE mortality and caecal colonization of *C. perfringens* (Barnes *et al.,* 1980; Elwinger *et al.,* 1992; Snoeyenbos *et al.,* 1983). Hofacre *et al.* (1998) challenged broiler chickens experimentally with *C. perfringens*, and observed that normal gut flora products reduced gross intestinal lesions and improved feed efficiency in their disease model. Craven *et al.* (1999) reported a reduction in *C. perfringens* colonization and decreased incidence of NE in chickens treated with normal gut flora, together with increased slaughter yield. In a field study, Kaldhusdal *et al.*

(2001) found that post-hatch use of flora prepared from adult birds was associated with delayed intestinal proliferation of *C. perfringens*, delayed appearance of NE gross lesions and better production performance at slaughter. Taken together, these studies suggest that the use of DFMs designed to promote an adult-type intestinal flora have promise for control of NE. For widespread commercial use in this application, however, it is essential that these preparations be free of all known human and avian pathogens, can be reproducibly produced and efficacy against *C. perfringens* clearly established.

Much work has been done to establish defined bacterial cultures that are equally effective as undefined cultures, but so far with limited success (Mead, 2000). Many strains of organisms have been used commercially, including *Lactobacillus spp.*, *Bacillus spp.*, *Enterococcus spp.*, and *Saccharomyces spp.* (Gusils *et al.,* 1999; Jin *et al.,* 2000, 1998; Lan *et al.,* 2003; Patterson and Burkholder, 2003). Most of the studies concerning effects of probiotics on *C. perfringens* were conducted in humans and other mammals. Rinkinen *et al.* (2003) documented that lactobacilli reduced the adhesion of *C. perfringens* to immobilized canine mucus *in vitro.* Although some studies are inconclusive, oral uptake of lactobacilli and bifidobacteria have been shown to decrease the number of intestinal *C. perfringens* bacteria and spores in humans and mice (Gallaher *et al.,* 1996; O'Mahony *et al.,* 2001; Romond *et al.,* 1998). Studies are limited regarding the protective effects of probiotic strains against *C. perfringens* in chickens. Fukata et al (1991) and Fukata et al (1988) reported a very low mortality in chicks inoculated with *Lactobacillus acidophilus* or *Streptococcus faecalis* and then challenged with *C. perfringens* compared with a 50% mortality rate in germ free chicks and there was no mortality in conventional birds. They also observed a suppression of α toxin production

when chick intestinal contents were cocultured with *C. perfringens*. Hofacre *et al.* (1998) observed that a commercial probiotic product reduced gross lesions of NE in chickens, but the protection was far less than that conferred by a normal gut flora preparation. Mortality due to NE was significantly reduced from 60 to 30% in an experimentally challenged broilers when they were treated with a defined lactic acid bacterial culture at day 1 of age (Hofacre *et al.,* 2003). In this experiment, feed to gain ratio was decreased in the group that was given lactobacilli, but weight gain was not affected. La Ragione and Woodward (2003) documented that when 1-day-old and 20-day-old chickens were challenged with 10^9 spores of *Bacillus subtilis* strain and infected 24 h later with 10^5 CFU of *C. perfringens*, colonization and persistence of *C. perfringens* was suppressed. Thus, some promising results have been reported for control of *C. perfringens* with probiotics. However, most of the proposed mechanisms of action remain hypothetical and much work remains to be done to confirm the efficacy of this approach.

2.4.2. Prebiotics

Another approach which may be employed to manipulate the broiler gut ecosystem is the supplementation of diet with prebiotics. A prebiotic is an indigestible feed ingredient that beneficially affects the host by selectively stimulating the growth or activity of beneficial bacterial species already resident in the intestinal tract (Gibson and Roberfroid, 1995). For a feed ingredient to be classified as a prebiotic, it must not be hydrolyzed or absorbed in the small intestine, must be a selective substrate for one or a limited number of potentially beneficial commensal bacteria in the large intestine, thus stimulating the bacteria to grow, become metabolically activated and thus alter the intestinal microflora toward a healthier composition (Collins and Gibson, 1999).

Prebiotics are predominantly polysaccharides including fructooligosaccharides (FOS) inulin, trans-galactooligosaccharides, glucooligosaccharides, glycooligosacchriades, lactulose, lactitol, maltooligosaccharides, xylo-oligosaccharides, stachyose, raffinose, and sucrose thermal oligosaccharides (Collins and Gibson, 1999; Orban *et al.,* 1997; Patterson *et al.,* 1997). Some oligosaccharides, such as mannan oligosaccharides (MOS) have been described as prebiotics; however, their mode of action may be different. MOS are thought to block pathogen binding to mannan receptors on the mucosal surface and stimulate the immune system (Spring *et al.,* 2000).

Various studies have been conducted on the effects of prebiotics on *C. perfringens* but most were performed in mammals and *in vitro* systems. McBain and MacFarlane (2001) and Bello *et al.* (2001) documented that when FOS were added to batch cultures inoculated with human faeces *in vitro* or a continous culture model of human intestine, growth of *C. perfringens* was enhanced. However, a significant reduction in the *C. perfringens* populations was reported in the intestinal tract of rats, cats and dogs due to addition of FOS to their diets compared to those fed unsupplemented diets (Gallaher *et al.,* 1996; Sparkes *et al.,* 1998; Swanson *et al.,* 2002).

The prebiotic effect of oligofructose has been studied in a quail model of the human disease neonatal necrotizing enterocolitis. *C. perfringens* is one of the several bacterial species assumed to be associated with the disease. In this case, the passage of an indigestible nutrient into the caeca apparently does not exert any prebiotic effect (Butel *et al.,* 2002). There is a strong possibility that the prebiotic products may also be effective in reducing NE lesions in broiler chickens. Takeda *et al.* (1995) reported that the number of *C. perfringens* organisms were significantly lower in the cecal contents of chickens on

2% and 10% lactose-supplemented feed than in chickens on unsupplemented feed. However, there has been no work to determine if including competitive exclusion and complex carbohydrates will more effectively prevent clinical NE, sub-clinical NE, or both. The lactic acid producing bacterial culture alone or combined with the mannan-oligosaccharide, was effective in reducing *C. perfringens*-associated mortality and sub-clinical effects on feed efficiency in experimentally challenged broiler chickens but neither addition of FOS or MOS to the diet had a significant effect on mortality caused by NE (Hofacre *et al.,* 2003).

2.4.3. Organic Acids

Organic acids are widely used both in raw materials and finished feed in Europe to inhibit enteric pathogens like Salmonella. For microorganisms, organic acids can act either as a source of carbon and energy, or as inhibitory agents depending upon the concentration of the acid, its ability to enter the cell and capacity of the organisms to metabolize the acid (Cherrington *et al.,* 1991). Despite their long history of use as antimicrobial agents in both food and feeds, the mode of action of organic acids against microorganisms still has not been satisfactorily explained (Adams, 2004). The key basic principle on the mode of action of organic acids on bacteria is that non-dissociated (non-ionised, more lipophilic) organic acids can penetrate the bacterial cell wall and disrupt the normal physiology of certain types of bacteria. As described by Lambert and Stratford (1999), after penetrating the bacterial cell wall, the un-dissociated organic acids will be exposed to the internal pH of bacteria and dissociate, releasing H^+ and anions. The internal pH will decrease and because pH sensitive bacteria do not tolerate a large spread between the internal and external pH, a specific mechanism (H^+-ATPase pump) will act

to bring the pH inside the bacteria to a normal level. This process consumes energy and eventually can stop the growth of the bacteria or even kill it (Gauthier, 2002). The anionic part of the acid is trapped inside the bacteria and becomes toxic to the bacteria by complex mechanisms leading to internal osmotic problems for the bacteria (Roe *et al.,* 1998). Organic acid-based products can be designed to inhibit the growth of undesirable microorganisms in different feed and poultry health situations.

The effect of organic acids on broiler performance is dependent upon dose, type of acid product, and whether the acids are included in the feed or drinking water (Patten and Waldroup, 1988). The performance enhancing effect of organic acids in poultry does not appear to be as pronounced as in pigs (Patten and Waldroup, 1988; Thompson and Hinton, 1997). Izat *et al.* (1990) noticed a significant reduction in the total number of coliforms and *E. coli* in the intestinal tract of broiler chickens fed buffered propionic acid in their diets at different concentrations. A propionic acid-based product was quite effective in alleviating turkey poult enteritis and mortality syndrome (Roy *et al.,* 2002). These workers also found that addition of organic acids at 1.25% to the feed decreased the bacterial load in the intestine and caeca. Chaveerach *et al.* (2004) demonstrated that organic acids in drinking water were able the keep the water free from *Campylobacter,* and acidified drinking water could have a potential effect on *Campylobacter* infection in young chicks. The promising results obtained from past research have resulted in the development of a large number of commercial products, however more comprehensive and thorough research is needed to improve their effectiveness. Of particular interest is the phenomenon of acid tolerance which may be considered as a problem and represents a challenge for future work.

2.4.4. Herbs, Spices and Various Plant Extracts/Essential Oils

For many years, herbs and spices and their essential oils have been used as pharmaceuticals in alternative medicine and as a natural therapy. Herbs have been found to enhance anti-microbial activity, known to have anti-viral and anti-oxidative properties and are said to stimulate the endocrine and immune system. They promote a higher metabolic and immune status within the animal, as well as enhancing welfare. Various botanical ingredients have been shown to facilitate beneficial effects on gut environment and microflora (Besra *et al.,* 2002; Rao and Nigam, 1970). Essential oils are known to stimulate digestive enzymes and to have an *in vitro* antimicrobial activity against many bacteria. Briozza *et al.* (1989) documented that various strains of *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, C. perfringens, and E. coli* inoculated at a level of 10^7 CFU/mL were killed (greater than 99.99%) after 2-7 min in a laboratory broth supplemented with 63% (v/w) of sugar, and containing 0.4% (v/w) of essential oil of clove, with its active principle eugenol. There are various reports in literature regarding the antibacterial effects of *Origanum vulgare*, *Piper nigrum*, *Syzygium aromaticum*, and *Thymus vulgaris*, and the essential oil components thymol, carvacrol, curcumin, piperin and eugenol against various strains of clostridia including *C. perfringnes* (Briozzo *et al.,* 1989; Dorman and Deans, 2000) and other bacteria such as *E. coli*, *Staphylococcus aureus*, *Salmonella typhimurium, Listeria monocytogenes and Yersinia enterocolotica.* (Cosentino *et al.,* 1999; Fabio *et al.,* 2003). Lutomski *et al.* (1974) reported growth inhibition in *Clostridium septicum*, *Clostridium novyi*, and *Clostridium sporogenes* with alcohol extract and the essential oil from *Curcuma longa*. However, majority of these tests were performed *in vitro* with only a few tests performed

in animal models. To be effective on a practical scale, it is likely that these compounds will need to be provided in more concentrated form than they are found in their natural source. Wilkinson *et al.* (2003) demonstrated *in vitro* activity against *C. perfringens* of several plant products.

Giving a supplementation of a commercial preparation of essential oil components in the feed (Crina® poultry; Crina SA, Gland, Switzerland), Losa and Kohler (2001) found a reduced concentration of *C. perfringens* per g of intestinal contents and a lower rate of detection of *C. perfringnes* in the ileum, rectum and colon on day 5, 18 and 32. The use of Crina® poultry (Crina SA, Gland, Switzerland) was found to decrease the intestinal *C. perfringens* counts of broilers in a field study on days 14, 21 and 30 (Mitsch *et al.,* 2002). Lee *et al.* (2003) did not find any significant effect of thymol, cinnmaldehyde and Crina® poultry on growth performance in female broiler chickens, but they did not rule out the possibility that positive effects would have been observed under less hygienic environmental conditions or when using a less digestible diet. Mitsch *et al.* (2004) reported that specific blends of essential oil components containing thymol or carvacrol and eugenol, curcumin and piperin can control the proliferation of *C. perfringens* in the broiler intestine and therefore, may reduce the risk of NE. They suggested that essential oil antibacterial effect *in vitro* and effect of stimulation of digestive enzymes, stabilization of intestinal microflora, and inactivation of *C. perfringens* toxins may reduce *C. perfringerns* colonization in the broiler gut.

2.4.5. Feed Enzymes

The effect of antibiotics and disease outcomes is gut-microflora related and therefore investigation of any diet-induced changes in the gut ecosystem is the starting point for the quest of finding suitable alternatives to antibiotics. Feed is a complex mixture containing many chemical species. In addition to the major nutrients like starch, protein and fats, there are many other components such as β-glucans, pentosans, mannans, cellulose, lignin, and phytic acid, which cannot be digested by poultry. These non-digestible feed ingredients generate digestive stress in poultry causing reduction in nutrient utilization which in turn allows pathogenic bacteria to grow in the gastrointestinal tract (Adams, 2004). Excessive growth of bacteria in the gastrointestinal tract requires energy and protein which reduces the nutrients available to support the growth of the bird. Recently, in the absence of AGPs, these problems have been overcome by using various feed enzymes. The utilization of diets containing high levels of xylans and β-glucans from wheat and barley, respectively, can be significantly improved through supplementation of xylanase and β-glucanase enzymes (Bedford and Classen, 1992; Jackson *et al.,* 1999; Mathlouthi *et al.,* 2002; Mathlouthi *et al.,* 2003; Wu and Ravindran, 2004). The mechanism responsible for this effect is still unclear. It has been suggested that these complex carbohydrates might influence gastrointestinal bacteria. Choct *et al.* (1999) reported that important effects of supplementary enzymes included improved nutrient digestibility, reduced small intestinal fermentation and increased cecal fermentation in chickens. The increased microbial activity in the caeca is a likely result of poorly absorbed products of enzymatic degradation entering the caeca, where they stimulate bacterial fermentation (Bedford, 2000). Increase in VFA production

and changes in VFA profile may serve to favor the beneficial organisms (for example *Bifidobacteria*) and suppress populations of deleterious bacteria (*Salmonella, Campylobacter* and *Clostridium*).

Published studies have produced contradictory results regarding the effects of enzyme supplementation on various bacterial populations including *C. perfringens* in the intestinal tract of broiler chickens (Hubener *et al.,* 2002; Riddell and Kong, 1992; Sinlae and Choct, 2000). Riddel and Kong (1992) could not find any beneficial effect of dietary pentosanase supplementation on the susceptibility of NE in broilers fed wheat-based diets. Sinlae and Choct (2000) demonstrated that the number of *C .perfringens* may be manipulated by diet or use of dietary enzymes. However, Jackson *et al.* (2003) reported that the feed enzyme β-mannanase significantly reduced the severity of challenge by *Eimeria sp*. and *C. perfringens* in broiler chickens. They reported a significant improvement in body weight gain and a reduction in intestine lesion score with β-mannanase supplementation in corn-based diets. A number of experiments have demonstrated that β-mannans crossing the intestinal mucosa are potent stimulators of innate immune system, resulting in increased proliferation of macrophages and monocytes and resultant cytokine production (Duncan *et al.,* 2002; Zhang and Tizard, 1996). However, further research in this area is required to elucidate the effect of feed enzymes on the incidence of NE.

2.4.6. Hen Egg Antibodies

One promising alternative to antibiotics in controlling NE in poultry involves **t**he feeding of anti-clostridial antibodies to neutralize pathogenic organisms in the gastrointestinal tract. The advantage of feeding antibodies with the diet is that they

provide a continuous control of potential pathogens and do not result in the development of antibiotic resistant strains of microorganisms. In simple terms, hens are exposed to inactivated clostridia antigens and their immune system is stimulated to produce immunoglobulins. The immunoglobulins are harvested from eggs and mixed in feed. Immunized hens transport approximately 100-150 mg of immunoglobulin to each egg (Rose *et al.,* 1974). Thus, an immunized hen can produce in excess of 40 g of immunoglobulin each year making this a cost-effective measure for the immunotherapeutic control of enteric pathogens. The use of pathogen-specific antibodies obtained from the egg yolks of hyperimmunized hens has been examined as a means for the control of gastrointestinal disease in various animal and avian species. Various reports have documented that hen egg antibodies can be used effectively to reduce morbidity and mortality in piglets (Imberechts *et al.,* 1997; Marquardt *et al.,* 1999; Yokoyama *et al.,* 1997), calves (Kuroki *et al.,* 1997; Yokoyama *et al.,* 1998), mice (Peralta *et al.,* 1994; Yokoyama *et al.,* 1998), fish (Lee *et al.,* 2000) and humans (Sugita-Konishi *et al.,* 2000). Although the literature is scant regarding the immunoprophylactic effect of hen egg antibody (HEA) in the avian model, they have been shown to be efficacious in the reduction of *Salmonella enteritidis* in Pekin ducklings when administered with probiotics (Fulton *et al.,* 2002). More recently, Kassaify and Mine (2004) demonstrated that HEA from normal or salmonella hyper-immunized hens may act to reduce intestinal colonization by *Salmonella enteritidis* in laying hens.

To the best of my knowledge their use for the control of *C. perfringens* has not been previously investigated. Earlier, Wilkie *et al.* (2005) observed that HEA antigen binding activity was present throughout gastrointestinal tract of birds when they were fed

HEA-supplemented feed, however, HEA from hens immunized against a *C. perfringens* bacterin did not provide a protective affect against *C. perfringens* colonization. As in all vaccination strategies, the selection of target antigen has a major influence on efficacy. For example, it is possible that feeding HEA harvested from hens immunized against the *C. perfringens* α toxin, which is the most accepted cause of NE, may significantly reduce the incidence of NE by blocking the ability of α toxin to interact with the intestinal mucosa.

2.4.7. Vaccination against *Clostridium perfringens*

Vaccination is a widely used preventive measure against many infectious diseases in mammals and poultry. *C. perfringens* may cause necrotizing enteritis in several mammalian species including humans, and some of these conditions have been successfully prevented by vaccination (Lawrence *et al.,* 1990; Springer and Selbitz, 1999; Uzal and Kelly, 1998). There are several studies in the literature to show that vaccination can induce antibody titers against clostridia and clostridial toxins in calves, lambs, goats, piglets and mice (Clarkson *et al.,* 1985; Schoepe *et al.,* 2001; Springer and Selbitz, 1999; Troxel *et al.,* 1997; Uzal *et al.,* 1998). Antibodies to α toxin inhibited the development of gas gangrene in an experimental mouse model (Williamson and Titball, 1993). Recently, Belyi and Varfolomeeva (2003) constructed a chimeric peptide carrying antigenic determinants of enteric clostridial toxins viz. α toxin, β toxin and enterotoxin and demonstrated that this fusion molecule was effective in eliciting antibody responses against each component when injected in mice. At present no vaccine against *C. perfringens*-associated diseases in poultry is available in the market.

It is well known that necrotizing α toxin released by *C. perfringens* type A or C is the main pathogenic factor responsible for intestinal mucosal necrosis (Al-Sheikhly and Truscott, 1977b, 1977c), so it is reasonable to assume that specific antibodies against α toxin might protect the birds from the disease. Fukata *et al.* (1988) demonstrated that specific antibodies against *C. perfringens* α toxin neutralized the effect of a *C. perfringens* culture supernatant in a challenge study using germ free chickens. Based on this observation, there is a possibility that antibodies against *C. perfringens* toxins may protect the birds against NE. Heier *et al.* (2001) conducted a field study to determine the level of naturally occurring specific humoral immunity against phospholipase-C in Norwegian flocks and observed that flocks with high titres of maternal antibodies against α toxin had lower mortality during the production period than flocks with low titres. They also noticed that progeny from older parent flocks had significantly higher levels of specific maternal antibodies against α toxin than did chicks from younger flocks. In support of this, vaccination of broiler breeder hens with *C. perfringens* type A and type C toxoids adjuvanted with aluminium hydroxide resulted in a strong serum IgG response to *C. perfringens* α toxin in parent hens, and specific antibodies were transferred to their progeny (Lovland *et al.,* 2004). The authors suggested that maternal vaccination may protect against the toxins without any clear effect on numbers of intestinal *C. perfringens*.

2.4.8. Anticoccidial Vaccination

We have already discussed the controversial relationship between coccidia and clostridioses. Various laboratory experiments and natural field cases suggest that coccidiasis or coccidiosis especially *Eimeria* species may predispose birds to clostridial enteritis (Al-Sheikhly and Al-Saieg, 1980; Baba *et al.,* 1997; Hofacre *et al.,* 1998;

Jackson *et al.,* 2003; Shane *et al.,* 1985) but other observations have not necessarily corroborated such evidence (Helmboldt and Bryant, 1971; Kaldhusdal and Hofshagen, 1992; Long *et al.,* 1974). Recently this relationship was nicely reviewed by Williams (2005). It has been proven in some studies that coccidial vaccines were able to prevent *C. perfringens*-associated NE (Engberg *et al.,* 2000; Jackson *et al.,* 2003; Williams *et al.,* 2003). Use of live anticoccidial vaccines is on the rise in the poultry industry around the world. Keeping this in mind it is very important to know the effects, whether beneficial or harmful, of vaccination against coccidiosis on clostridial enteritis. Williams *et al.* (1999) reported that birds receiving a live attenuated anticoccidial vaccine in drinking water consisting of seven species of *Eimeria* were protected against NE, whereas birds fed ionophores with AGPs suffered from clostridiosis. Again Williams *et al.* (2003) examined the relationships between coccidiosis, anticoccidial vaccines and NE in broiler chickens and observed that immunization against virulent coccidial challenge reduced the severity of the later clostridial challenge. It might be due to the fact that any coccidial lesions that might have predisposed birds to NE were prevented because of vaccination.

In contrast, Williams (1994) and Williams and Andrews (2001) documented that vaccination itself may cause mild coccidial lesions in some birds, but those lesions were not severe enough to predispose immunized birds to NE, as suggested by Williams *et al.* (2003). Further research experiments involving simultaneous vaccination and *C. perfringens* challenge is needed to reach a definite conclusion.

2.4.9. Use of Bacteriophages

Bacteriophages are viruses which infect bacterial cells, utilize the bacterial metabolic machinery to replicate and then destroy the bacterial host by lysis in order to release new phage into the environment. Bacteriophages have received significant attention as potential alternative to antibiotic control of bacterial replication. Indeed, Immerseel *et al.* (2004) suggested the use of bacteriophages, which are active against *C. perfringens* strains, for controlling NE in poultry. Bacteriophages were successfully used to combat experimentally induced vancomycin-resistant *Enterococcus faecium* infection in mice (Biswas *et al.,* 2002). Zimmer *et al.* (2002) investigated the murein hydrolase from bacteriophage Φ3626, which lysed all 48 strains of *C. perfringens* tested, whereas other clostridia and bacteria belonging to other genera were generally unaffected. This highly specific activity towards *C. perfringens* might be useful for novel biocontrol measures in food, feed, and complex microbial communities.

2.4.10. Appropriate Diet Formulation and Ingredient Selection

Physical and chemical attributes of the diet can modify the populations of microorganisms in the gastrointestinal tract and the integrity of delicate intestinal epithelium in various mammals and birds (Apajalahti *et al.,* 2001; Hill *et al.,* 2005; Jansman *et al.,* 2003; Jensen and Jorgensen, 1994; Langhout *et al.,* 1999; Smith and Macfarlane, 1998; Wagner and Thomas, 1978). Apajalahti (2004) documented that feeding corn and sorghum-based diets increased numbers of *Enterococcus*, barley increased *Lactobacillus* populations, oat promoted growth of *Escherichia* and *Lactococcus*, and rye increased *Streptococcus* population in broiler chickens. Supplementation of flaxseed or certain fractions of flaxseed may have benefits beyond its

nutritional value through modification of intestinal microbial colonization. Linolenic acid, which represents 59% of the fatty acids present in flaxseed, may inhibit bacterial growth by preventing the adhesion of bacteria to intestinal mucus and epithelial cells (Kankaanpaa *et al.,* 2001). An increase in lactobacilli was observed in poultry fed flaxseed (Alzueta *et al.,* 2003). Dibner *et al.* (1996) reported an increase in numbers of *E. coli* and decrease in lactobacilli with unstabilized rancid fat in the diet. Dietary ingredients have a great influence on the incidence of NE in broiler chickens (Annett *et al.,* 2002; Baba *et al.,* 1992; Drew *et al.,* 2004; Elwinger *et al.,* 1992; Kaldhusdal *et al.,* 1999; Kaldhusdal and Skjerve, 1996; Knarreborg *et al.,* 2002; Riddell and Kong, 1992). There appear to be two important dietary factors which predispose broiler chickens to NE. The first factor is cereal grain type, such as wheat, rye and barley that increases the viscosity of digesta, prolong intestinal transit time and increase incidence of NE. The second dietary factor is protein level and source, where high levels of protein from animal sources predispose to NE. Oxidized fats and mycotoxins have also been shown associated with NE (Ferket, 1996). More recently, the dietary fat source was also found to be an important factor affecting *C. perfringens* populations in broiler chickens (Knarreborg *et al.,* 2002). These workers reported that there was a significant elevation in the *C. perfringens* population in ileum of broiler chickens which were fed animal fat (mixture of lard and tallow) compared to those fed soy oil.

2.4.10.1. Types of Cereal Grains

There are various studies in literature that have reported that corn-based diets reduce the incidence and severity of NE compared to diets based on wheat, barley, rye or oats. Diets based on rye caused a decline in chick growth and increase in the number of

bacteria adhering to the lower part of intestine, an effect that might be associated with the relatively high content of complex carbohydrates in rye (Untawale *et al.,* 1978). Branton *et al.* (1987) reported 2.9% mortality in broilers fed a corn-based diet, while mortalities were 28.9 and 18.1% when hammer mill or roller mill-ground wheat were used, respectively. When broiler chickens were challenged by feeding *C. perfringens*-inoculated feed for 3 consecutive days, mortality ranged from 0 to 12.5% in broilers fed a corn-based diet, however, it ranged from 26 to 35% in broilers fed a diet with high amounts of wheat, barley or rye (Riddell and Kong, 1992). They also reported that addition of pectin and guar gum to different rations reduced growth rate and eliminated NE from test birds. In a separate study, Kaldhusdal and Hofshagen (1992) indicated that a barley-based diet was associated with a lower number of lactobacilli and streptococci and higher frequencies of sub-clinical NE than a corn-based diet. Kaldhusdal and Skjerve (1996) studied the association of the cereal content of diets fed to broiler chickens in Norway from 1969-1989 and observed that two major outbreaks of NE were associated with increased use of barley and wheat in broiler diets. The inclusion levels of corn, barley, oats and wheat in broiler starter feed varied between 0 and 38%, 0 and 20%, 0 and 26% and, 6 and 38.6%, respectively. They demonstrated that the ratio of wheat plus barley to corn was positively correlated with the incidence of NE. Branton *et al.* (1996) conducted two experiments to determine the *in vitro* effect of water soluble wheat extracts on the growth of *C. perfringens* in thioglycolate broth medium and found that the growth was suppressed by inclusion of both autoclaved and non autoclaved wheat extracts. Branton *et al.* (1997) reported that NE lesion scores were lowest in chickens given the corn-based diets with no *C. perfringens* challenge and highest in chickens fed

the wheat diets with *C. perfringens* challenge. Chickens consuming a wheat diet with no added complex carbohydrates or added fiber exhibited the highest lesion score. Langhout *et al.* (1999) found increased deconjugation of bile salts and higher numbers of *Enterococci, Bacteriodaceae, Clostridia* and *E. coli* in the ileum of pectin-fed chickens and also reported that fat digestibility decreased when microbial activity increased.

Craven (2000) documented that intestinal numbers of *C. perfringens* in contaminated broiler chicks on the corn-based diet were $\leq \log_{10} 6.2$ and did not increase in any portion of the intestinal tract from day 2 to 21, however, numbers of *C. perfringens* during this time period increased 3.3 to 4.9 log units in the ileum, ceca, and feces of birds on the 50% rye diet to as high as $\log_{10} 7.1$ to 7.9 at 21 day. Annett *et al.* (2002) reported that *C. perfringens* growth was significantly lower in *in vitro* digested broiler diets based on corn than in corresponding diets based on wheat and barley. This difference was not found when undigested diets were compared. They suggested that digestive enzymes may generate or activate one or more components in the corn diet, thereby suppressing clostridial proliferation.

While many hypotheses have been postulated to explain the effect of cereal type on intestinal *C. perfringens* populations, the actual mechanism(s) behind this effect is still unknown. It has been suggested that complex carbohydrates (viscous polysaccharides) may influence intestinal bacterial populations and broiler growth performance (Choct *et al.,* 1996; Hubener *et al.,* 2002; Langhout *et al.,* 1999). Wheat, rye, barley, and oat groats contain different levels of complex carbohydrates, including arabinoxylans and β-glucans, which may elevate the digesta viscosity, increase intestinal transit time and thus interfere with digestion. Enzymes such as xylanase can be used to break down non-starch

polysaccharides and thus addition of enzymes to diets based on wheat/barley grains can reduce digesta viscosity, increase passage rate while reducing the bacterial population in small intestine. Riddle and Kong (1992) also suggested that carbohydrates in corn may be less available to microbial digestion than those in wheat, a hypothesis they supported by showing that there was an increase in mortality due to NE in a corn-based ration to which glucose had been added. Williams (1992) documented that pathogenicity of *E. tenella* was lower in corn-fed chickens than in wheat-fed chickens and it might be due to the protective effect of higher concentrations of vitamin A and E derived from corn diets or that the higher concentration of niacin and riboflavin in wheat than in corn might enhance coccidial pathogenicity. Since coccidial lesions are a predisposing factor for NE, this can also play a significant role in the protective effect of corn. Annett *et al.* (2002) postulated that proliferation of *C. perfringens* and production of α toxin may be elevated by various components present in wheat and barley, or wheat and barley diets may inactivate intestinal digestive enzymes, thereby decreasing the α toxin degradation. The release of soluble non-starch polysaccharides from certain cereals represents another possible explanation of the phenomenon. Corn alone among the commonly used cereals usually does not appear to release soluble non-starch polysaccharides in amounts sufficient to produce a detectable depression in performance (Chesson, 2001).

2.4.10.2. Feed Processing Methods

Feed processing methods also appear to be important precursors for NE. It has been observed that highly processed feeds can lead to atrophy and malfunction of gizzard, which then acts more like a transient organ rather than a grinding organ. Normal gastric reflux does not occur when birds consume finely ground feed and, as a consequence, more protein ends up in the hind gut where it is subjected to microbial fermentation. One of the consequences is the potential for finely ground material to trigger proliferation of clostridia and, as a result, NE. Branton *et al.* (1987) found that the mortality due to NE was increased in birds fed ground feed (hammer-mill) compared with coarsely ground feed (roller-mill). The influence of feed grinding (coarsely or finely ground feed) and feed form (mash or pellet) on the intestinal microenvironment was also investigated in a growth experiment by Engberg *et al.* (2002) with broiler chickens taking into consideration the intestinal microflora and intestinal viscosity. They found no effects of the feed grinding on *C. perfringens* counts in the intestine, however, counts of *C. perfringens* and lactobacilli were significantly higher in the distal end of the intestinal tract (caecum and rectum) of mash-fed birds. Microbial fermentation as assessed by VFA concentration was found to be significantly higher in the ceca of pellet-fed birds than in mash-fed birds. Grinding raw materials over 4 mm screen size has been found beneficial (Engberg *et al.* 2002). Many broiler companies in Europe have been diluting broiler feed by adding up to 30% whole wheat on top of the manufactured feed. Inclusion of whole wheat stimulates the function of the gizzard with a resulting benefit on gut motility. This, in turn, is felt to be beneficial in helping to inhibit the *C. perfringens* multiplication in the intestinal tract. Engberg *et al.* (2002) and Bjerrum *et al.* (2005) observed that whole

wheat feeding reduced the pH in gizzard contents and decreased intestinal counts of *C. perfringens* in broiler chickens.

2.4.10.3. Dietary Protein Source/Level

Numerous studies have reported that feeding high animal protein based-diets to pigs (Mansson & Smith, 1962; Mansson & Olhagen, 1967) and dogs (Zentek *et al.*, 1998; Zentek, 2000; Zentek *et al.,* 2003; Zentek *et al.,* 2004) resulted in significant increase in the number of *C. perfringens* in the small intestine and/or feces. In broiler chickens high protein diets and especially diets consisting of high level of animal source proteins have been reported to predispose birds to NE (Drew *et al.,* 2004; Kaldhusdal, 2000; Kocher, 2003; Wilkie *et al.,* 2005). In a longitudinal study of NE and feeding practices over a 20 year period, Kaldhusdal and Skjerve (1996) found that the use of animal source protein in broiler diets in Norway was associated with an increased incidence of NE. This observation is based on feeding diets at or near NRC requirements. Although NE is associated with feeding high levels of dietary crude protein, the literature is limited regarding the direct effect of high levels of dietary crude protein on the composition of intestinal microflora in broiler chickens, particularly on the *C. perfringens* populations. There are some *in vitro* studies showing the association between certain amino acids and *C. perfringens* growth and/or α toxin production. A local increase in protein concentration or a particular amino acid in the small intestine may initiate the overgrowth of *C. perfringens* since the growth of this bacteria and production of α toxin are influenced by the presence of various amino acids (Titball *et al.,* 1999). Muhammed *et al.* (1975) documented that methionine, while not a required nutrient for *C. perfringens*, was highly stimulatory for growth and was required for sporulation. Another study reported

that glycine also accelerated the growth of *C. perfringens* and production of α toxin *in vitro* (Ispolatovskaya, 1971). In defined media, α toxin requires the presence of glycine-containing peptides (Nakamura *et al.,* 1968; Stevens and Rood, 2000). Hence, one factor in animal protein based diets that could predispose birds to NE is glycine content. Animal-source proteins contain from 2 to 4 times as much glycine as a percentage of crude protein compared to plant based proteins (AminoDat™, 2001). Animal-source protein diets may therefore oversupply glycine and contribute to the overgrowth of *C. perfringens* in the intestinal tract. The source of a particular amino acid is also important in influencing the microbial community in the gastrointestinal tract. A study was conducted to determine the apparent absorption of hydroxy analogue of methionine and DL-Methionine in germ free and conventionally reared broiler chickens and concluded that 2-hydroxy-4-methylthiobutoanic acid (MHA-FA) might be comparatively more available to gastrointestinal microbes than DL-Methionine (Drew *et al.,* 2003).

Preliminary work in our laboratory examined the effects of protein source (fish meal vs. soy protein concentrate, SPC) and protein concentration (230, 315 or 400 g/kg crude protein) on intestinal populations of *C. perfringens* in broiler chickens (Drew *et al.,* 2004). We observed a significant increase in *C. perfringens* counts in birds fed 400 g/kg crude protein fish meal diet, however, *C. perfringens* counts were low in ileum of birds fed SPC-based diets at all levels of crude protein. *C. perfringens* counts in ileum and cecum increased as crude protein level increased from 23 to 40% in birds fed fish meal-based diets but not in birds fed SPC diets. The feeds were analyzed for their amino acid content and a ratio was calculated by dividing amino acid present in 40% fish meal diet by amino acid present in 40% SPC diet. The ratio was higher for glycine (1.65) and

methionine (1.35) compared to other amino acids suggesting that these amino acids are in relative excess in the 40% fish meal diet compared to the 40% SPC diet. Wilkie *et al.* (2005) performed an experiment to assess *C. perfringens* colonization for a wide range of protein sources and to determine whether increased *C. perfringens* colonization could be correlated with the supply of specific amino acids. Seven different diets were formulated to contain 400 g/kg crude protein using fish meal, meat and bone meal, feather meal, potato protein concentrate, pea protein concentrate, soy protein concentrate or corn gluten meal as the primary source of protein. The number of *C. perfringens* in ileal and cecal digesta of chickens fed animal protein-based diets were significantly higher compared to those fed the control diet or the plant protein-based diets with the exception of potato protein concentrate diet. Correlation analysis of the amino acids content of eight experimental diets and intestinal *C. perfringens* populations revealed a significant positive correlation between the level of glycine present in the diet and the number of *C. perfringens* in both ileum and cecum. None of the other dietary amino acids were significantly correlated with the *C. perfringens* number neither in ileum nor in cecum. Further studies are required to confirm a cause and effect relationship between dietary glycine concentration and intestinal *C. perfringens* populations in broilers.

2.5. Conclusions

Whether or not the livestock industry is to blame for the emergence of antibiotic-resistant bacteria, the reality is that the consuming public does not like the idea of routinely feeding antibiotics to animals, for whatever reason, and so North America may follow the European lead, either by government regulation or voluntarily, to reduce or discontinue the use of AGPs in animal feed. Experiences of many European countries

yield important information about how we might best cope with a restricted use of anibiotics and what changes we might expect in North America. Experiments from Sweden and Denmark have confirmed that one of the key problems for countries reliant on wheat-based formulations is the control of NE once AGPs have been removed. Currently, many parts of the world are experimenting alternative feed additives that may be used to alleviate the problems associated with the withdrawal of antibiotics from feed. To date no single preventive therapy that can substitute for in-feed antibiotics in poultry feeds has been found. Effective non-antibiotic prevention of *C. perfringens*-associated health and performance problems will only be attained by means of extensive multidisciplinary research efforts. There are number of alternatives that may be used strategically to decrease the incidences of NE in poultry. However, limited data has been presented to support the efficacy of AGPs alternatives.

Increased feed particle size, the feeding of whole wheat, feeding corn-based diets, reducing non-starch polysaccharides and reducing the level of animal-based proteins in the diet all seem to help reduce the incidence of NE. Furthermore, the effect of feed composition, particularly the effect of grain source and amino acid profile on NE requires significantly more study. The bacteria in the gastrointestinal tract of birds derive most of their energy for reproduction and growth from various dietary compounds, and several bacterial species have different substrate preferences and growth requirements. The chemical composition of the digesta largely determines the microbial composition in gastrointestinal tract. Today there is a pressing need to better understand factors that predispose birds to NE and to develop alternative dietary strategies to control the incidence and severity of NE in broiler chickens in the “post-antibiotic” era.

Based on these observations, the overall objective of this study was to develop a model of *C. perfringens* colonization and/or NE in broiler chickens without using any coccidial challenge, and then use this model to investigate the effect of various dietary components especially amino acids on various intestinal microbes, especially *C. perfringens* and lactobacilli populations in the gastrointestinal tract of broiler chickens. An improved understanding of the relationship between feed chemistry and gastrointestinal microflora will help us to formulate poultry diets through appropriate ingredient selection to control or reduce various enteric pathogens including *C. perfringens* in absence of in-feed sub-therapeutic antibiotics.

3.0. INTESTINAL BACTERIAL POPULATIONS IN BROILER CHICKENS FED HIGH PROTEIN DIETS WITH AND WITHOUT ADDED GUAR GUM

3.1. Abstract

An experiment was conducted to study the effects of two different dietary protein sources and a soluble non-starch polysaccharide (guar gum) on the intestinal bacterial populations of broiler chickens. Six groups of 12 birds (in duplicate) were provided with a medicated ideal protein-balanced corn-based starter crumble feed from days 0 to 21 of age. From day 21 onwards, six experimental diets with 2 levels of guar gum (0 or 1%) and 3 levels of protein (40% using fish meal as the primary protein source, 40% using soy protein concentrate (SPC) as the main protein source, and 23% using both fish meal and SPC) in a 2 x 3 factorial arrangement of treatments. All birds were orally challenged with *C. perfringens* type A broth culture from days 21 to 27 and *C. perfringens*, lactobacilli, total anaerobes and total aerobes were enumerated in ileal and cecal contents on days 35, 39 and 42. Nine (6.25%) birds died during the first 4 days of diet shift, but lesions typical of NE were seen in only three birds. The birds that were given guar gum supplemented diets performed poorly. The viscosity and the pH of ileal contents were significantly ($P < 0.05$) higher and lower, respectively, in birds fed diets with added guar gum. There was a significant ($P < 0.05$) increase in the *C. perfringens* and total anaerobes count in cecum at all time points due to guar gum addition. However, there were no significant effects of protein on the *C. perfringens* numbers except on day 35 in ileum and day 42 in the cecum where counts were significantly ($P < 0.05$) higher in birds fed SPC-based diet. Significantly higher ($P < 0.05$) populations of lactobacilli were observed in the ileum and cecum of birds fed diets with added guar gum. Ileal viscosity, clostridial

counts and lactobacilli counts were significantly ($P < 0.05$) correlated in the ileum on day 42. The pH of ileal contents was significantly ($P < 0.05$) higher in SPC-fed birds compared to control birds on day 35 and 39. Also there was a significant increase in number of total goblet cells in ileum with the addition of guar gum. Hence, it was concluded that the increased *C. perfringens* and total anaerobes growth in guar gum-fed birds was associated with higher intestinal viscosity and lower intestinal pH. Protein source had no significant effect on various bacterial populations in this experiment with few exceptions.

3.2. Introduction

Necrotic enteritis (NE) is a common disease found in all poultry-growing areas of the world and was first described by Parish in 1961 (Ficken and Wages, 1997). The causative agent of NE is *Clostridium perfringens*, types A (Long and Truscott, 1976) or C (Shane *et al.,* 1985). *C. perfringens* is a ubiquitous gram positive, spore forming anaerobic bacteria found in soil, dust, feces, feed, poultry litter, and intestinal contents. However, high populations of *C. perfringens* may also be detected in the gut of birds with no visible signs of NE, thus breaking Koch's postulate that a disease-causing organism should not be present in healthy individuals. This has resulted in continuing uncertainty as to the etiology of NE and resulted in great deal of research to determine the factors that precipitate an outbreak of clinical disease.

Disease outbreaks have been minimized to a significant extent through the use of in-feed antibiotics. However, the incidence of NE in broiler flocks has increased in Western Europe since the implementation of a ban on sub therapeutic antibiotic usage by the European Union. It has been estimated that the total estimated loss to the poultry

industry globally due to NE is nearly US$ 2 billion based on US$ 0.05 per bird production performance losses (Van der Sluis, 2000a, 2000b). The sub-clinical form of NE may be most economically important because it has been shown to impair feed conversion in broilers (Stutz and Lawton, 1984). Impaired feed conversion, reduced live weight at slaughter and increased condemnation percentage are major causes of production losses associated with *C. perfringens* infection (Lovland and Kaldhusdal, 2001). Although an outright ban of AGPs has not occurred in North America, a growing consumer demand for safer foods is creating pressure on producers to limit their use. As a result, there is a pressing need to better understand factors that predispose birds to NE and to develop alternative management and dietary strategies to control the incidence and severity of infection.

Although *C. perfringens* is recognized as the etiological agent of NE, other contributing factors are usually required which predispose the flocks to disease. These precipitating factors include management stress, sub-clinical intestinal coccidiosis, and diet composition (Kaldhusdal, 2000; Shane *et al.,* 1985). Elevated concentrations of dietary wheat and barley (Riddell and Kong, 1992), animal proteins (Kaldhusdal and Skjerve, 1996) and zinc (Baba *et al.,* 1992) may also exacerbate clostridial-induced NE, but the reason(s) for the increased susceptibility has not been determined. Kaldhusdal and Skjerve (1996) and Kaldhusdal (2000) reported that the use of animal source protein in broiler diets in Norway was associated with an increased incidence of NE. Previous work in our laboratory examined the effects of protein source (animal vs. plant) and protein concentration on intestinal populations of *C. perfringens* in broiler chickens (Drew *et al.,* 2004). *C. perfringens* counts in ileum and cecum increased significantly ($P < 0.05$) as

crude protein level increased from 23 to 40% in birds fed fish meal-based diets but not in birds fed SPC diets. The crude protein alone does not adequately explain the changes in *C. perfringens* populations. Other possible factors linked to this effect include lipids, carbohydrates, anti-nutritional factors and amino acid balance. Diets containing small grains, including wheat, rye, barley and oats, are associated with higher levels of NE in broilers than corn-based rations (Riddell and Kong, 1992). These small grains all contain relatively high levels of complex carbohydrates, including arabinoxylans and β-glucans, which may elevate the digesta viscosity, increase intestinal transit time and thus interfere with digestion. It has been suggested that the viscous non-starch polysaccharides may influence intestinal bacterial populations and broiler growth performance (Choct *et al.,* 1996; Langhout *et al.,* 1999).

Although NE is an economically important disease of broiler chickens, the development of a reproducible disease model has not been successful. A number of epidemiological as well as experimental studies have shown that there is a strong relationship between certain feed ingredients and the incidence of NE in poultry. Dietary cereal grains rich in non-starch polysaccharides (NSP) and dietary proteins especially of animal origin encourage the development of NE (Annett *et al.,* 2002; Kaldhusdal and Skjerve, 1996; Riddell and Kong, 1992; Wilkie *et al.,* 2005). The combination of NSP and protein in the diet can greatly exacerbate the problem.

Previously in our lab, we were able to produce high *C. perfringens* colonization in the intestinal tract of broiler chickens using high protein diets but have not produced clinical disease using high protein diets alone. We hypothesized that a dietary combination of high protein and high non-starch polysaccharides may promote the *C.*

perfringens overgrowth in the intestinal tract of broiler chickens, thus increase the risk of clinical NE. The objectives of this study were two pronged: to evaluate the effect of two sources of dietary protein (fish meal and soy protein concentrate) and a soluble dietary fiber, guar gum, on intestinal colonization of *C. perfringens,* and the development of a working model of clinical NE in broiler chickens.

3.3. Materials and Methods

Experimental protocols were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with recommendations of the Canadian Council on Animal Care as specified in the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

3.3.1. Experimental Animals, Diets, and Design

One hundred and forty four, one-day-old mixed sex conventional broiler chicks (*Gallus gallus*, Hubbard x Hubbard) were obtained from a local broiler hatchery (Lilydale Hatchery, Wynyard, SK, Canada) and placed randomly into 12 electrically heated battery cages (12 birds per cage) at the Animal Care Unit, Western College of Veterinary Medicine of the University of Saskatchewan. From day 1 through 21 of the experiment, birds were provided with a medicated, ideal protein balanced (lysine 12%) corn-based starter crumble (Co-op Feeds, Saskatoon, SK) **(Table 3.1)**. On day 21, the birds were weighed and randomly assigned to one of the 12 battery cages. Two cages were assigned to one of the six experimental diets **(Table 3.2)** up to the end of the experiment (day 42). The dietary treatments were arranged in a 2 x 3 factorial design with 2 levels of guar gum (0 or 1%) and 3 levels of crude protein (40% using fish meal as the primary protein source, 40% using soy protein concentrate (SPC) as the main protein

source, and 23% using both fish meal and SPC). The diets were isocaloric and met or exceeded the NRC requirements for broiler chickens (NRC, 1994). All diets contained 250 ppm of zinc since zinc is known to increase α toxin production from *C. perfringens in vitro* and also it might protect the α toxin from inactivation by intestinal proteases.

The research facility was thoroughly cleaned and disinfected prior to bird placement. The battery cages were arranged in four tires with a wire floor and were equipped with external feed and water troughs. Lighting schedule was 16 L: 8 D throughout the experiment. Room temperature was maintained according to industry standards. None of the experimental diets contained antibiotics or coccidiostats and were not pelleted. Throughout the experimental period in each experiment, birds were fed and watered *ad libitum.* Feed consumption and body weight for each cage was recorded for the period days 21-28, 28-35 and 35-42 for calculation of feed conversion. When calculating feed conversion the body weight of dead birds was taken into account.

Table 3.1. Ingredient composition of starter diet used for all birds up to day 21 of age[1]

Ingredients	Inclusion (%)
Soybean meal	40.90
Corn	39.50
Wheat	10.00
Tallow	4.52
Dicalcium phosphate	1.53
Calcium carbonate	1.29
Canola oil	1.00
DL-Methionine	0.26
Choline chloride	0.10
Vitamin/Mineral premix[2]	0.50
Salinomycin sodium[3]	0.06
Bacitracin[4]	0.05
Bio-cox 120[5]	0.05

[1] Diet was formulated to contain 31.5% crude protein and meet NRC requirements for broiler chickens.

[2] Supplied per kilogram of diet: vitamin A, 3.3 mg; cholecalciferol, 55 µg**;** vitamin E, 30 mg; vitamin K, 0.5 mg; vitamin B12, 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; sodium, 511 mg; iron, 80 mg; maganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

[3] Phibro Animal Health Ltd.

[4] Alpharma Canada Corp.

[5] Anticoccodial Premix, OzBioPharma Ltd.

Table 3.2. Experimental diet formulations fed to birds on days 21-42 of the experiment[1]

	Without Guar Gum			With Guar Gum		
Ingredients	Control	FM	SPC	Control	FM	SPC
Corn	51.23	12.40	22.36	50.23	11.40	21.36
Wheat	20.00	20.00	20.00	20.00	20.00	20.00
Fish meal	16.99	56.96	0.00	16.99	56.96	0.00
Soy protein conc.	7.96	0.00	53.86	79.6	0.0	53.86
Guar gum	0.00	0.00	0.00	1.00	1.00	1.00
Canola oil	2.00	7.42	2.00	2.00	7.42	2.00
Calcium carbonate	1.21	2.62	1.18	1.21	2.62	1.18
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10
Vit./Min. premix[2]	0.50	0.50	0.50	0.50	0.50	0.50

[1]Diets were formulated to contain either 23% (control) or 40% crude protein (Fish meal- or Soy protein concentrate-based) and meet NRC requirements for broiler chickens.

[2] Supplied per kilogram of diet: vitamin A, 3.3 mg; cholecalciferol, 55 µg**;** vitamin E, 30 mg; vitamin K, 0.5 mg; vitamin B12, 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; sodium, 511 mg; iron, 80 mg; maganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

Note: 100 mg/kg zinc was supplied by mineral premix. In addition to this, 150 mg/kg zinc was also supplemented in all diet i.e. each diet contained 250 ppm of zinc.

FM = Fish meal, SPC = Soy protein concentrate

3.3.2. *Clostridium perfringens* Challenge Model

An avian *C. perfringens* field strain isolated from a clinical case of NE was obtained from Dr. Manuel Chirino, College of Veterinary Medicine, University of Saskatchewan and characterized by PCR technique as type A toxin producer. The organism was cultured anaerobically on BBL™ Blood Agar Base (Becton, Dickinson and Co., Sparks, MD, USA) containing 5.0% sheep blood and 100 mg/L Neomycin Sulfate (The Upjohn Company, Orangeville, ON, Canada) for 18 hours at 37°C, then aseptically inoculated into brain heart infusion (Difco Labs, Detroit, MI), and incubated anaerobically overnight at 37°C. All birds were orally challenged in the crop with 1.0 mL on days 21 to 27 inclusive with this overnight culture of *C. perfringens* using a 12.0 mL syringe equipped with vinyl tubing (I.D. 0.97mm, O.D. 1.27mm). Bacterial counts were performed on the culture daily prior to inoculation and the numbers ranged from 6.00 x 10^4 to 9.59 x 10^6 CFU/mL.

3.3.3. Pathological Examination

Birds were observed on a pen basis at least once daily for any sign of NE. On days 35, 39 and 42 of the experiment, four birds from each pen were killed by cervical dislocation, weighed and necropsied immediately. Intestinal tracts were removed and examined for any gross lesions of NE. On day 42, following postmortem examination, a 1.5-2.0 cm long piece of intestinal tissue from ileum (just below the Mackel's diverticulum) was collected in phosphate-buffered formaldehyde solution and processed routinely for paraffin embedding, sectioned at approximately 5 μm, and stained with hematoxylin and eosin. In addition to this, four randomly selected ileal tissues from each treatment were also processed for PAS-Alcian blue staining to differentially stain the

neutral and acid mucin producing goblet cells. Neutral mucin and acid mucin producing cells were counted differentially by digital image capture analysis per 10 random high power fields (40x) using Northern Eclipse Software version 6.0 (2002).

3.3.4. Bacteriological Examination

On day 35, 39 and 42, four birds were selected at random from each pen, weighed, killed and their intestinal tracts were removed. Samples of fresh digesta (0.1 to 0.2 g) from ileum (Meckel's diverticulum to 1 cm proximal to ileocecal junction) and paired ceca were collected aseptically in pre-weighed 15 mL sterilized plastic tubes containing 1 mL 0.1% sterile peptone buffer with 5 g/L cysteine hydrochloride (Sigma Chemical Co., St. Louis MO). The samples were immediately placed and kept on ice until plated within 4 h of collection. The samples were weighed and diluted in peptone water to an initial 10^{-1} dilution. Ten-fold dilutions were spread in duplicate using an automated spiral plater (Autoplate, Spiral Biotech Inc., Bethesda MD, USA) on BBL™ blood agar base (Becton, Dickinson and Co., Sparks, MD, USA) containing 5.0% sheep blood and 100 mg/L neomycin sulfate (The Upjohn Company, Orangeville, ON, Canada) for *C. perfringens* enumerations. In addition, the digesta samples were also cultured on MRS agar (Becton, Dickinson and Co., Sparks, MD, USA) and blood agar (Becton, Dickinson and Co., Sparks, MD, USA) for the enumeration of lactobacilli, total aerobes and total anaerobes. The plates were incubated at 37°C for 16-24 h anaerobically for *C. perfringens,* lactobacilli and total anaerobes while aerobically for total aerobes. The *α*- and *β*- hemolytic colonies on blood agar/neomycin plates were counted as *C. perfringens* **(Appendix 9.1A)** with presumptive colonies being randomly picked, gram stained, plated on Mannitol Yolk Polymixin agar (Oxoid Inc., Napean, ON, Canada) and examined

microscopically to confirm them as *C. perfringens*. Counts were expressed as the Log_{10} CFU/g of intestinal contents.

3.3.5. Measurement of Viscosity and pH of Ileal Digesta

On day 35, 39 and 42, the viscosity and pH of ileal digesta was measured within 5-10 min of digesta collection. Immediately after digesta collection, the ileal digesta (1-2 g) was centrifuged at 14,000 rpm for 3 min and supernatant was diluted in water (1:1000 w/v). Samples were then stored on ice for maximum 10 min until viscosity (centipoise) was determined using a digital viscometer (Brookfield digital viscometer, Model LVTDVCP-II, Brookfield Engineering Laboratories, Stoughton, MA., USA). For pH estimation, the ileal digesta (1 g) was immediately mixed with 4.0 mL deionised water, vortexed well to make slurry and pH was determined using digital pH/mV/°C meter (Cole Palmer Instrument Company, Chicago, IL, USA). The probe of pH meter was washed well with deionised water between samples.

3.3.6. Statistical Analysis

The data were analyzed as a 2 x 3 factorial design using the General Linear Model of SPSS (v.12.0, SPSS Inc, Chicago IL, USA). Individual treatment means were compared using the Ryan-Einot-Gabriel-Welsch multiple F test and differences between the means were considered significant when $P < 0.05$. Bivariate correlation analysis was performed between *C. perfringens* and lactobacilli populations in ileum, and ileal pH and viscosity. Pearson's correlation coefficient was considered significant when $P < 0.05$.

3.4. Results

The birds did not show any signs of disease subsequent to challenge with *C. perfringens*. Nine birds died (mortality 6.25%) during the course of experiment, but necropsy examination could be conducted only on 5 birds. The dead birds were in good condition and small necrotic patches in the distal ileum were seen in 3 birds, however, the lesions were very mild and possibly might not be the cause of death of these birds. Overall the intestinal lesions of NE were reported in 27 birds (18.75%) in all treatment groups; however, there were no treatment effects on the number of birds with NE lesions. The intestinal gross lesions in most of the birds were very mild and inconclusive of NE (e.g. intestinal wall was thin and hyperemic with engorged mesenteric blood vessels). NE lesion scoring was not done in this experiment. Microscopic examination of formalin fixed intestinal tissues from 42-day-old broiler chickens revealed no lesions specific of NE and there was no evidence of gram-positive rod-shaped organisms attached to the intestinal mucosa.

The average daily feed intake (ADFI) was measured during week 4, 5 and 6 while average daily gain (ADG) and feed:gain ratio (FC) were measured during week 4 and 5 only. At all time points, the ADFI and ADG were significantly lower ($P < 0.05$) in birds which were fed SPC-based diets compared to the other two diets **(Table 3.3)**. The birds that received guar gum supplemented diets had a significantly lower ($P < 0.05$) ADG and poor feed conversion efficiency during week 4 and 5. There was no significant interaction between protein source and guar gum for performance parameters except for feed efficiency during week 4.

The pH and viscosity of ileal digesta were measured on day 35, 39 and 42 of the experiment. The birds which were fed SPC-based diets had a significantly ($P < 0.05$) lower pH of ileal digesta on day 35 and 39, but there was no effect of protein source on ileal pH on day 42 **(Table 3.4)**. Also guar gum supplementation was associated with a significant decline ($P < 0.05$) in ileal pH at all three time points. Protein source had no significant effect on ileal digesta viscosity, whereas guar gum supplementation resulted in a significant elevation ($P < 0.05$) in ileal viscosity at all three time points. The goblet cells numbers (acid, neutral or total) in the ileal tissue were not affected by dietary protein source, but the birds fed guar gum supplemented diets has significantly ($P = 0.007$) higher number of total goblet cells **(Figure 3.1)** while the *P*-values for the effect of guar gum supplementation on acidic and neutral mucin producing goblet cells were 0.072 and 0.085, respectively.

C. perfringens were enumerated in the ileum and ceca of these birds on day 35, 39 and 42 of age whereas lactobacilli, total aerobes and total anaerobes were quantified on day 39 and 42 only. The populations of all these bacterial species were higher in ceca than the ileum at all time points. On day 35, the SPC-based diets resulted in a significant increase ($P = 0.048$) in *C. perfringens* populations in ileum compared to control diets, whereas on day 42 in cecum the birds fed SPC-based diets had a significantly higher ($P = 0.036$) *C. perfringens* populations than the birds given either control or fish meal-based diets **(Figure 3.2 A)**. Guar gum supplementation had no effect on *C. perfringens* growth in ileum except on day 42 where the numbers increased significantly ($P = 0.005$) with addition of guar gum. On the other hand, in cecum the *C. perfringens* populations

Table 3.3. Average daily feed intake (ADFI, g/day), average daily gain (ADG, g/day) and feed:gain ratio (FC, feed intake/ weight gain, g/g) of birds during weeks 4, 5 and 6 of the experiment.

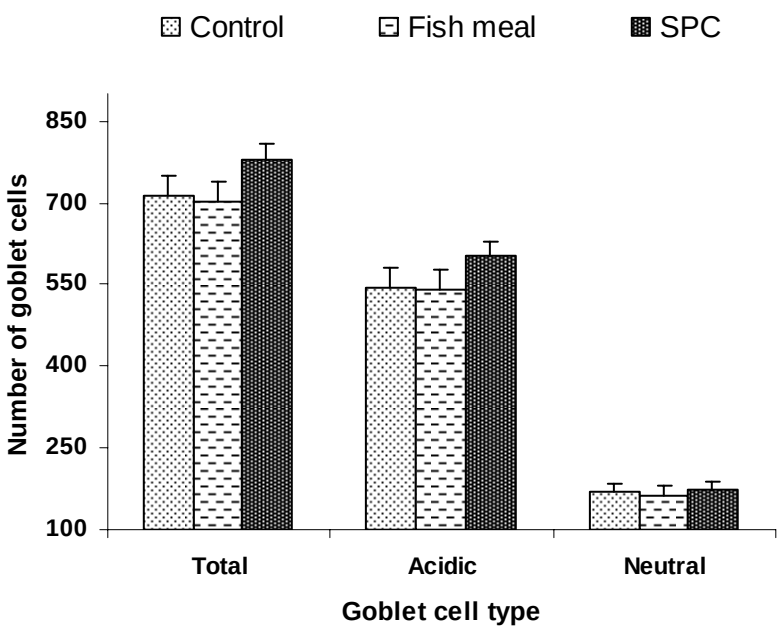
	ADFI			ADG		FC	
	Week 4	Week 5	Week 6	Week 4	Week 5	Week 4	Week 5
Protein							
Control	82.4[a]	117.4[b]	142.3[a]	41.3[a]	58.4[a]	2.1	2.2
Fishmeal	83.4[a]	127.6[a]	149.2[a]	41.1[a]	60.4[a]	2.1	2.2
SPC	60.1[b]	90.2[c]	100.4[b]	30.4[b]	39.8[b]	1.9	2.4
Pooled SEM	13.15	19.32	26.43	6.24	11.33	0.05	0.09
Guar gum							
Absent	72.8	113.7	136.6	41.1	63.9	1.8	1.8
Present	77.8	109.8	124.6	34.1	41.7	2.3	2.7
Pooled SEM	3.55	2.69	8.49	4.96	15.75	0.34	0.63
***P*-values**							
Protein	0.000	0.000	0.000	0.002	0.011	0.204	0.822
Guar gum	0.079	0.286	0.055	0.005	0.002	0.000	0.011
Protein x guar gum	0.231	0.282	0.597	0.058	0.247	0.000	0.930

[abc] Means with the different superscripts within a column differ significantly ($P < 0.05$)

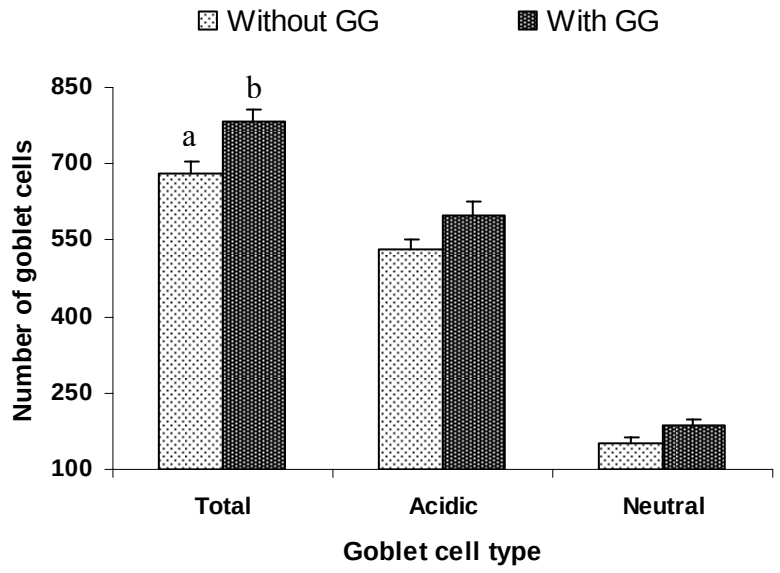
Table 3.4. Effect of protein source and guar gum on the pH and viscosity (centipoise) of ileal digesta on days 35, 39 and 42 of the experiment.

	Ileal pH			Ileal viscosity		
	Day 35	Day 39	Day 42	Day 35	Day 39	Day 42
Protein						
Control	7.05[a]	7.18[a]	7.79	1.76	1.59	1.77
Fishmeal	7.21[ab]	7.42[a]	7.51	1.20	1.24	1.41
SPC	7.65[b]	8.02[b]	7.96	1.43	1.45	1.43
Pooled SEM	0.30	0.40	0.26	0.28	0.18	0.20
Guar gum						
Absent	7.72	7.84	8.06	1.15	0.93	0.95
Present	6.89	7.24	7.41	1.78	1.92	2.14
Pooled SEM	0.52	0.41	0.43	0.41	0.69	0.78
***P* value**						
Protein	0.040	0.029	0.124	0.077	0.090	0.111
Guar gum	0.001	0.018	0.002	0.007	0.000	0.000
Protein x guar gum	0.438	0.242	0.803	0.179	0.432	0.158

[ab] Means with the different superscripts within a column differ significantly ($P < 0.05$)



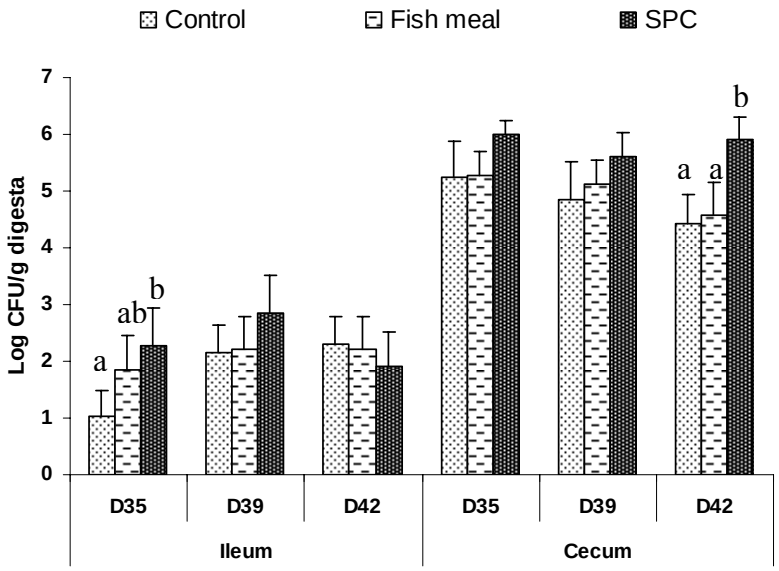
A



B

Figure 3.1. Effect of different protein sources (A) and guar gum (B) on acid, neutral and total goblet cell counts (per 10 random high power fields) in ileum of broiler chickens on day 42 of the experiment. Bars represent the mean ± SEM. SPC = Soy protein concentrate, GG = Guar gum

[ab]Bars with different labels are significantly different ($P < 0.05$)



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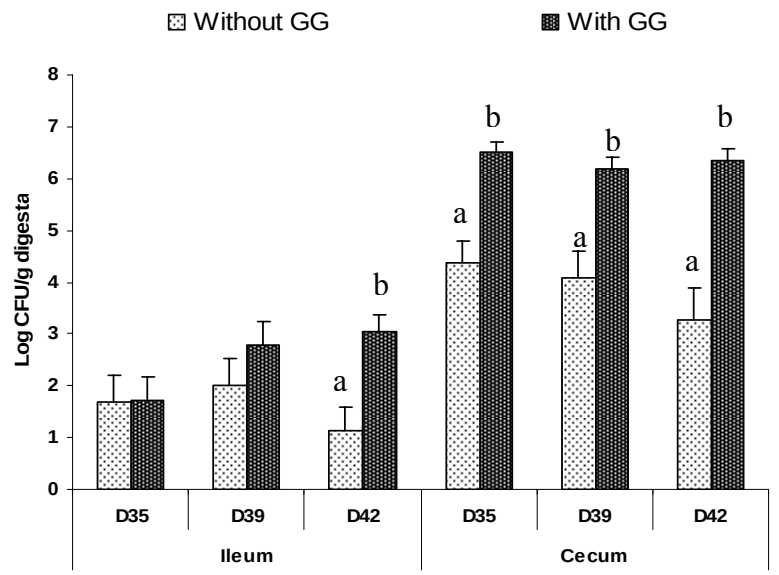


B

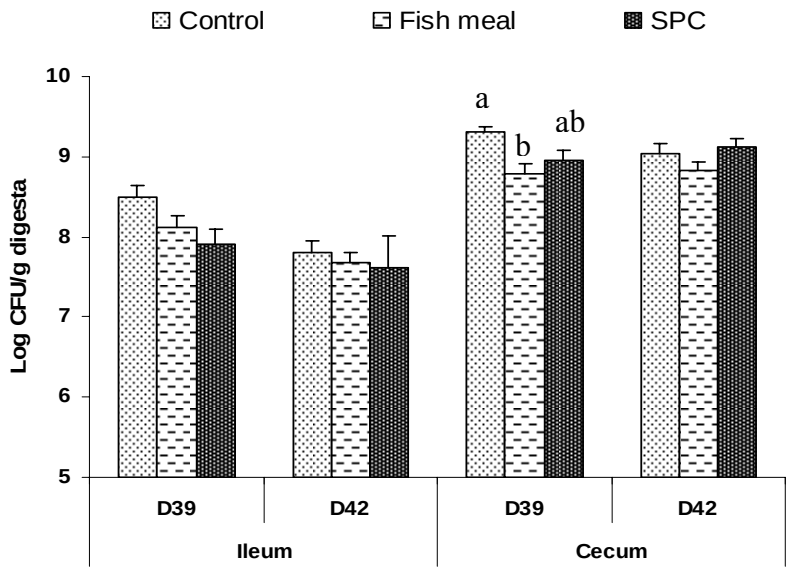
Figure 3.2. Effect of different protein source (A) and guar gum (B) on *C. perfringens* populations in ileum and cecum of broiler chickens on days 35, 39 and 42 of the experiment. Bars represent the mean ± SEM. SPC = Soy protein concentrate, GG = Guar gum

[ab]Bars with different labels are significantly different ($P < 0.05$)

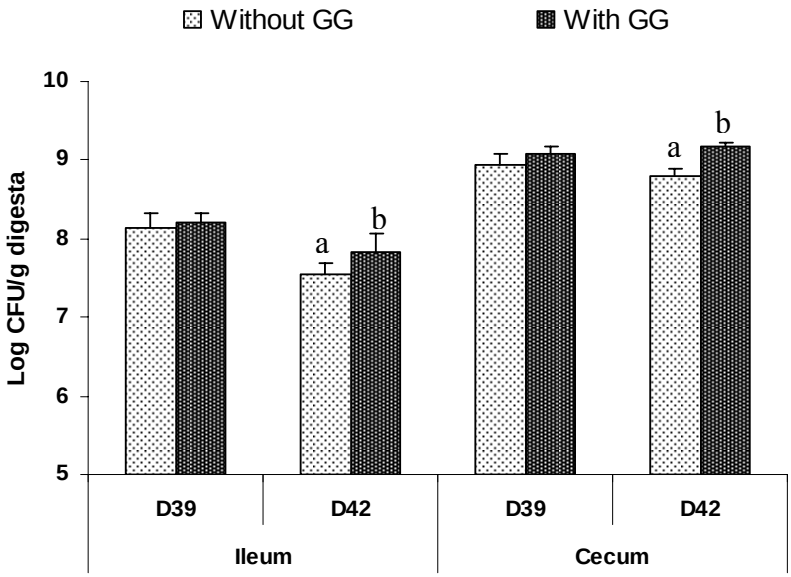
increased significantly ($P < 0.05$) in response to guar gum supplementation at all time points **(Figure 3.2 B)**. Also we observed a significant ($P = 0.035$) interaction between protein source and guar gum for *C. perfringens* growth in cecum on day 42.

On day 39, the fish meal-based diets supported a significantly lower ($P = 0.022$) population of lactobacilli in cecum than control diets, whereas guar gum supplementation was associated with a significant increase ($P < 0.05$) in lactobacilli in both ileum and cecum on day 42 **(Figure 3.3 A and B)**. There was no significant effect of protein source on total anaerobe populations except that SPC-based diets resulted in significantly higher ($P = 0.014$) total anaerobes in cecum on day 42 compared to fish meal diet. On day 42, we also observed a significant increase ($P = 0.001$) in total anaerobes in cecum of birds fed guar gum added diets **(Figure 3.4 A and B).** On day 39, the total aerobes decreased significantly ($P = 0.038$) in the ileum of fish meal fed birds than the SPC fed birds, whereas guar gum supplementation was associated with a significantly higher ($P = 0.004$) total aerobes in cecum on day 42 **(Figure 3.5 A and B)**.

Results of a bivariate correlation analysis between *C. perfringens* and lactobacilli populations in ileum, and ileal pH and viscosity are presented in **Table 3.5.** We observed a significant negative correlation ($P < 0.05$) between ileal pH and *C. perfringens* on day 42 and lactobacilli on day 39. However, *C. perfringens* growth on day 39 and 42 was positively correlated ($P < 0.01$) with ileal viscosity. Also there was a significant ($P < 0.05$) positive correlation between ileal viscosity and lactobacilli population on day 42.



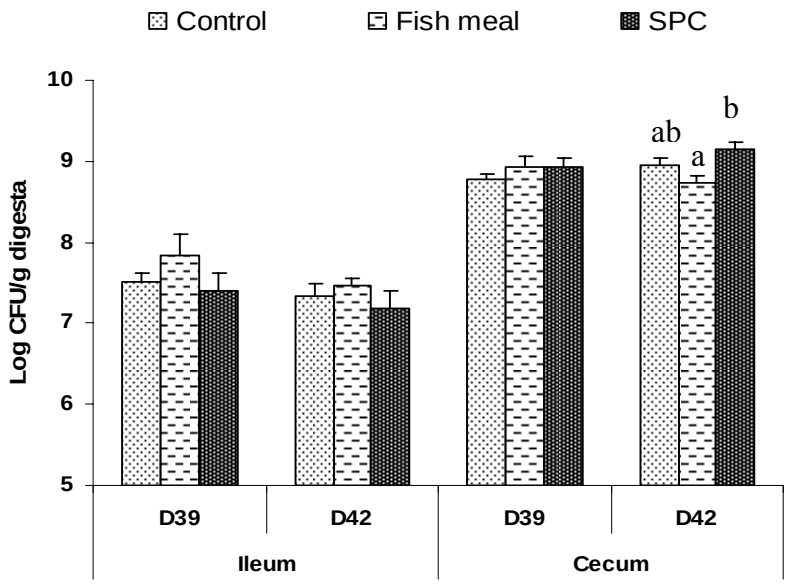
A



B

Figure 3.3. Effect of different protein source (A) and guar gum (B) on lactobacilli populations in ileum and cecum of broiler chickens on days 39 and 42 of the experiment. Bars represent the mean ± SEM. SPC = Soy protein concentrate, GG = Guar gum.

[ab]Bars with different labels are significantly different ($P < 0.05$)



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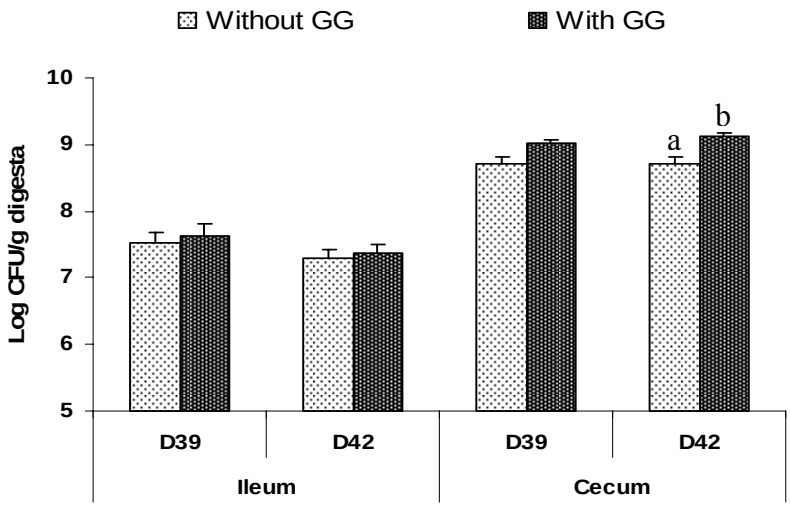
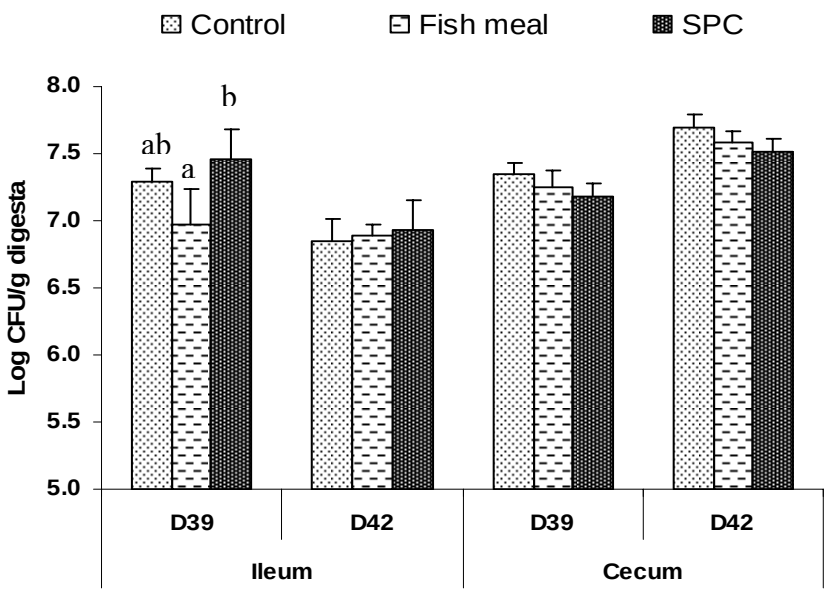


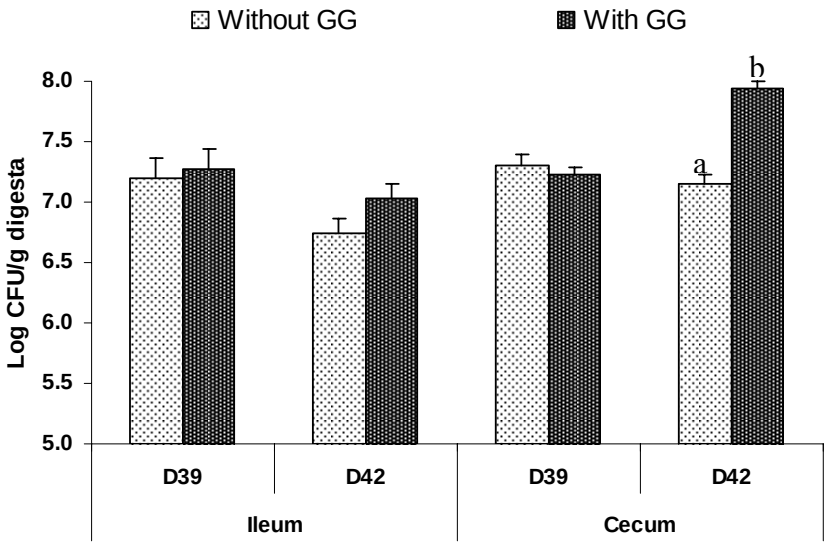
B

Figure 3.4. Effect of different protein source (A) and guar gum (B) on total anaerobes in ileum and cecum of broiler chickens on days 39 and 42 of the experiment. Bars represent the mean ± SEM. SPC = Soy protein concentrate, GG = Guar gum

[ab]Bars with different labels are significantly different ($P < 0.05$)



A



B

Figure 3.5. Effect of different protein source (A) and guar gum (B) on total aerobes in ileum and cecum of broiler chickens on days 39 and 42 of the experiment. Bars represent the mean ± SEM. SPC = Soy protein concentrate, GG = Guar gum

[ab]Bars with different labels are significantly different ($P < 0.05$)

Table 3.5. Pearson correlation (r) coefficients between the pH and viscosity of ileal digesta and ileal populations of *C. perfringens* and lactobacilli at different days of experiment.

		C. perfringens			**Lactobacilli**	
		Day 35	Day 39	Day 42	Day 39	Day 42
Ileal pH	Day 35	0.247	0.276	-0.226	-	-
	Day 39	-0.020	0.351	-0.365	-0.542**	-0.140
	Day 42	0.132	0.294	-0.504*	0.020	-0.232
Ileal viscosity	Day 35	-0.151	-0.190	0.316	-	-
	Day 39	-0.069	0.640**	0.138	0.050	0.391
	Day 42	-0.011	0.206	0.562**	0.247	0.419*

**Correlation is significant at 0.01 level.
* Correlation is significant at 0.05 level.

3.5. Discussion

The *C. perfringens* populations in a healthy chicken gut vary considerably from sporadic to low numbers to 10^7 CFU/g digesta or even more (Dumonceaux *et al.*, 2006; Engberg *et al.,* 2002; Ficken and Wages, 1997). Although the intestinal tracts of birds were highly colonized with *C. perfringens*, however, no overt clinical signs of NE were noted in the present study. Kaldhusdal *et al.* (1999) suggested that median counts of *C. perfringens* above one million per gram predict a high probability of concurrent NE-specific gut lesions. Other authors found *C. perfringens* concentrations ranging from 10^5 to 2 x 10^8/g sample in the small intestine of birds that died of NE (Kondo, 1988; Long *et al.,* 1974). We observed some mild lesions of NE in these birds, but mortality and NE lesions typical of field cases were not seen. Previous controlled studies of chickens challenged with *C. perfringens* have also failed to induce mortality or other symptoms of NE, even though high *C. perfringens* populations were reported in the intestinal tract (Cowen *et al.,* 1987). Takeda *et al.* (1995) reported that even 50% inclusion of rye in the diets of caged young leghorn chicks significantly increased *C. perfringens* populations, and although the chicks suffered from diarrhea and ateliosis, clinical NE did not develop and no NE lesions were observed. There is evidence that the normal gut microflora in healthy birds inhibits the pathogenicity of *C. perfringens* (Fukata *et al.,* 1991; Fukata *et al.,* 1988). Furthermore, digestive enzymes such as trypsin inactivate the α-toxin of *C. perfringens* type A and β-toxin of *C. perfringens* type C (Baba *et al.,* 1992; Niilo, 1965). Since *C. perfringens* is the etiological agent of NE it seems reasonable to assume that increased populations of this organism are associated with an increased predisposition to clinical NE.

The ADFI and ADG were significantly lower in birds which were fed SPC-based diets compared to control and fish meal-based diets. It seems that palatability was an issue with the SPC-based diets because SPC has a very fine particle size making it difficult to consume in a mash diet and it has a relatively poor amino acid balance. Although there was no effect of SPC on ileal viscosity, the pH of ileal contents was significantly higher in SPC-fed birds. It is possible that digestibility of SPC was reduced because of higher intestinal pH in these birds and that's why ADG was lower in SPC-fed birds. As expected, guar gum supplementation at 1.0% level was associated with a significantly lower ADG and poor feed efficiency. The ADFI was very close to significant especially during the week 4 and 6 (P = 0.079 and 0.055, respectively). Also the intestinal viscosity and pH were significantly higher and lower, respectively in birds that received guar gum supplemented diets. Guar gum is a high molecular weight galactomannan polysaccharide consisting of approximately 35% galactose, 63% mannose and 5-7% protein (Horton, 1997) which is known to have an impact on gastrointestinal tract viscosity, and digestion and absorption of nutrients. On a weight basis, galactomannan is five times as viscous as starch, and is one of the most viscous polysaccharides known (Whistler and Smart, 1953). The physiological action of guar gum has been reported to depend on its capacity to hydrate rapidly and thus increase the viscosity of digesta in gizzard and small intestine (Blackburn *et al.,* 1984). Guar gum decreases growth and performance of broiler chickens even when guar gum-containing meals are fed at low concentrations (Vohra and Kratzer, 1964). Maisonnier *et al.* (2001) reported that guar gum addition (0, 1, 3 g/kg) in broiler chicken diets increased digesta viscosity and decreased nutrient digestibilities, with most pronounced effects being

observed for lipids, then for proteins, and lowest for starch. In the current study, we measured the ileal viscosity because ileal viscosities are more sensitive and consistent to changes in diet composition than other segments of the small intestine (Lee *et al.,* 2003). Ingredients in broiler diets that cause increased intestinal viscosity, such as wheat, barley and rye, are cited as the cause of growth depression and poor feed efficiency (Bedford and Classen, 1992; Choct *et al.,* 1995). High intestinal viscosity decreases digestibility coefficients of all macronutrients and decreases digestive enzyme (amylase and lipase) activity throughout the small intestine (Smits *et al.,* 1997). It is apparent that some NSP interact with protein and glycoproteins of the epithelial tissue, causing damage or inducing the secretion of mucin from the tissues (Kleessen *et al.,* 2003). A second means by which water soluble NSP may reduce performance of chicks is through excessive stimulation of the intestinal microflora which in turn increases deconjugation of bile acids in the small intestine which in turn reduce the formation of micelles and thus limit absorption of fatty acids (Coates *et al.,* 1981). As expected, addition of guar gum resulted in reduced pH of ileal contents. Since guar gum is a soluble NSP, it is easily, rapidly and completely fermented mainly in terminal ileum releasing various short chain fatty acids, in particular acetate, propionate, butyrate, lactate and succinate thus aids in reducing the pH of ileal digesta (Nyman *et al.,* 1986, Langhout *et al.*, 2000).

In the current experiment, we observed a significantly higher numbers of total goblet cells in the ileum of guar gum fed birds, however, acid and neutral mucin cells did not differ significantly due to guar gum supplementation. It is well known that the intestinal epithelial surface is covered with a mucus gel secreted by epithelial goblet cells that acts as a protective barrier against harmful intraluminal components. Dietary factors

have a profound effect on the goblet cell numbers and mucin composition (Smirnov *et al.,* 2005). In agreement with our results, Schneeman *et al.* (1982) reported a significant increase in the number of mucin-producing goblet cells in the epithelium of small intestine of rats fed a diet containing 200 g wheat bran/kg compared to a fibre-free diet. Improved capacity of mucin synthesis stimulated by dietary fibre intake can be regarded as an adaptation to a chronic mechanical irritation caused by dietary fibre. This effect may also occur by way of SCFA production that favors secretion of mucins in the colon (Montagne *et al.,* 2003). Mucus offers a number of ecological advantages to intestinal bacteria e.g. it is a source of carbohydrates and peptides for microbes and it protects bacteria from rapid expulsion due to peristalsis. In this regard, it is possible that mucus secretion is typically enhanced in response to intestinal microbes. So both commensals and pathogenic bacteria regulate mucus synthesis, secretion and composition from host goblet cells (Deplancke and Gaskins, 2001).

In contrast to our previous findings (Drew *et al.,* 2004), there was no significant effect of fish meal on *C. perfringens* growth in ileum and cecum in the present study. Whereas birds fed SPC-based diets had significantly elevated numbers of *C. perfringens* in ileum on day 35 and in cecum on day 42 compared to birds given control diets. The *C. perfringens* lacks the ability to produce at least 13 amino acids out of 20 essential amino acids, and its growth is therefore enhanced in an environment rich with protein (Mcdevitt *et al.,* 2006). The clostridial overgrowth and thus an increased risk of NE is often associated with animal protein ingredients such as fish meal or meat and bone meal (Kocher, 2003). One possible explanation may be that the very high quantities of fish meal (around 57.0%) used in these diets might be responsible for some physio-chemical

changes in the GIT of these birds which do not support *C. perfringens* growth. It is also possible that it may not be fish meal *per se*, but rather the use of poor quality fish meal, which aids in the proliferation of *C. perfringens* and thus contributes to the development of NE, possibly via production of biogenic amines.

We observed that SPC fed birds had significantly higher numbers of total aerobes in ileum on day 39 and total anaerobes in cecum on day 42. Diets that contain relatively high concentration of proteins, as well as those with imbalanced profile of amino acids, have reduced digestibility in upper GIT and thus larger concentrations of these compounds, as well as their metabolites, are found in the lower GIT (Mcdevitt *et al.,* 2006). Similarly, poorly digested proteins concentrate in the lower GIT and thus act as substrates for microflora (Williams *et al.,* 2001). The proteins can be degraded to ammonia and amines, thus encouraging the proliferation of pathogenic bacteria such as *C. perfringens* (Juskiewicz *et al.,* 2004). The birds fed SPC-based diets had comparatively higher intestinal pH and *C. perfringens* counts than the other two groups. It is possible that the presence of various nitrogenous degradation products (e.g. ammonia and amines) may raise the pH of lower GIT because of their high pKa values and counteract pH changes that would normally occur due to bacterial production of lactic and acetic acids, thereby enhancing proliferation of *C. perfringens* (Juskiewicz *et al.,* 2004; Lan *et al.,* 2005). It is interesting to note that lactobacilli populations were higher in SPC fed birds on day 39 and 42 ($P = 0.022$ and 0.081, respectively). It is very important because lactobacilli are known to protect against colonization of various enteric pathogens. Fukata *et al.* (1991) reported that pathogenic effect of *C. perfringens* could be reduced by feeding chicks a monoflora of *Lactobacillus acidophilus* or

Streptococcus faecalis. The probiotic strains of lactobacilli which are able to colonize the human intestinal tract induce epithelial cells to secret mucins that diminish enteric pathogens binding to mucosal epithelial cells (Johansson *et al.,* 1993).

Feeding guar gum diets increased the intestinal microflora (*C. perfringens*, lactobacilli, total aerobes and anaerobes) of broiler chickens in the current study. The increase in intestinal microflora corresponds with the increase in intestinal viscosity due to added guar gum. A similar increase in microbial activity in the small intestine has previously been reported in birds fed with diets containing rye (Wagner and Thomas, 1978), wheat pentosans (Choct *et al.,* 1996), highly methylated citrus pectin (Wagner and Thomas, 1978) or caroxymethyl cellulose (Smits *et al.,* 1998). Different hypotheses have been put forward concerning effect of NSP on *C. perfringens* growth and NE. Barley, wheat, rye and oats contain high levels of soluble NSP, which are known to increase digesta viscosity, decrease digesta passage rate and nutrient digestibility, in contrast to corn (Choct *et al.,* 1996). This is proven to lead to increase in intestinal anaerobic populations (Hubener *et al.,* 2002; Kocher, 2003). The NSP compounds also act as substrates for microflora within the GIT and thus allow the proliferation of pathogenic bacteria (Choct *et al.,* 1996) including *C. perfringens*.

3.6. Conclusions

This experiment suggests that diet is only one of the numerous factors predisposing birds to NE. Not only protein source and concentration *per se*, but the quality of protein is important in terms of *C. perfringens* overgrowth, α toxin production and thus NE predisposition. Various dietary factors (e.g. protein and fibre) have profound effects on intestinal physiology and morphology which in turn influence the microbial

colonization in the GIT. With such as multi-factorial disease, a working and reproducible experimental model is an essential tool to determine various control or management strategies. The NE challenge model used in this study was not successful in producing clinical disease, so we decided to make some significant changes in our subsequent model. The first important change was that in our subsequent experiments, we also challenged the birds with *C. perfringens* when they were one day old because early stage of post-hatch is a critical period for the establishment of the gut microbial community in broiler chickens. Newly-hatched chicks are particularly vulnerable to invasion by pathogens before their intestinal microbial communities become established. Therefore, it seems reasonable to administer *C. perfringens* to chicks during the early period after hatching to achieve high intestinal colonization of *C. perfringens* and thus clinical disease. Second important change was that instead of using overnight culture of *C. perfringens* in brain heart infusion agar, we decided to use cooked meat media (CMM) to grow *C. perfringens* for challenging the birds. It was decided on the basis of the results of our *in vitro* experiments **(Appendices 9.2A and 9.2B).** Also all the subsequent experiments were conducted only for day 28 of age instead of day 42 because most of the field cases of NE have been reported during that period.

4.0. EFFECT OF DIFFERENT DIETARY METHIONINE SOURCES ON INTESTINAL MICROBIAL POPULATIONS IN BROILER CHICKENS

4.1. Abstract

Previous work in our laboratory showed a significant reduction in *C. perfringens* growth in media containing DL-Methionine (DL-Met). Two experiments were therefore conducted to study the effect of various levels of DL-Met or 2-hydroxy-4-methylthiobutoanic acid (MHA-FA) on *C. perfringens* and other intestinal bacteria in broiler chickens. In each experiment, two cages of 6 birds (14 day post-hatch) were assigned to one of 7 different diets in a 2 x 4 factorial arrangement. The main effects were methionine source (either DL-Met or MHA-FA) and methionine level (0, 0.2, 0.4 or 0.8% DL-Met or 0, 0.227, 0.454 and 0.908% MHA-FA, thus providing 4 corresponding equimolar levels of each methionine source). All birds were orally gavaged with a *C. perfringens* type A broth culture on day 1 as well as from days 14 to 20 and euthanized on day 28. Intestinal populations of *C. perfringens*, lactobacilli, *Streptococcus* group D and coliforms were enumerated in ileum and cecum, and NE intestinal lesions were scored. There were no significant differences in growth of various bacterial species in the intestinal tract of broiler chickens fed two different methionine sources. However, we observed a significant reduction ($P < 0.05$) in *C. perfringens* growth with methionine supplementation in cecum (Experiment 1), or both in ileum and cecum (Experiment 2). Also there was a significant interaction ($P < 0.05$) between methionine source and level in experiment 2. *C. perfringens* populations were generally higher both in ileum and cecum in experiment 2 compared to experiment 1. In experiment 2, the lactobacilli populations were significantly higher ($P < 0.05$) in ceca of birds receiving 0.80%

methionine than the birds given diets with other levels of methionine tested. Significantly lower ($P < 0.05$) coliforms and *Streptococcus* group D were enumerated in ileum of birds fed 0.80% methioinine supplemented diet than the other dietary treatments. There were no significant differences ($P > 0.05$) in NE intestinal lesion scores and performance of birds fed different methionine sources and concentrations. The results suggest that both DL-Met and MHA-FA may reduce intestinal populations of *C. perfringens* in broiler chickens when used in relatively high concentrations and thus may reduce the risk of NE.

4.2. Introduction

Clostridial or necrotic enteritis (NE), caused by *Clostridium perfringens* type A and C (Ficken and Wages, 1997), is a potentially fatal poultry disease of global significance, and is both an animal welfare and economic problem. Outbreaks of NE are sporadic and may result in high mortality, reduced growth rate, impaired feed conversion and increased condemnation rates (Lovland and Kaldhusdal, 2001). NE is estimated to cost the poultry industry as much as US$ 0.05 per bird with total global loss of almost US$ 2 billion (Van der Sluis, 2000a, 2000b). However, this might be an underestimate, given the difficulty in diagnosing mild or sub clinical forms of NE. In the recent years, the banning of prophylactic use of antibiotics in Europe, as well as consumer concerns about their use in other jurisdictions, has stimulated interest in finding alternative management or dietary strategies to control the incidence and severity of this disease in post-antibiotic era.

C. perfringens is frequently found in the intestinal tract of healthy poultry, usually at low levels ($< 10^4$ CFU/g digesta) and is spread in poultry production units and processing through feces and intestinal rupture. Although *C. perfringens* is recognized as

main etiological agent of NE, but some other co-factors are usually required to precipitate an outbreak of NE. The physical and chemical composition of broiler diets have been reported to have a marked effect on intestinal microflora of chickens and it has been shown to have important impact on the incidence of NE in broiler chickens (Branton *et al.,* 1987; Dahiya *et al.,* 2006b; Drew *et al.,* 2004; Riddell and Kong, 1992). Branton *et al.* (1987) found that the mortality due to NE was increased in birds fed ground feed (hammer-mill) compared with coarsely ground feed (roller-mill). Dietary cereal grains rich in non-starch polysaccharides (NSP) and dietary proteins especially proteins of animal origin encourage the development of NE (Annett *et al.,* 2002; Kaldhusdal and Skjerve, 1996; Riddell and Kong, 1992; Wilkie *et al.,* 2005). Kaldhusdal and Skjerve (1996) reported that two major outbreaks of NE in broiler chickens in Norway from 1969-1989 were associated with increased use of barley and wheat in broiler diets. Drew et al. (2004) observed a significant increase in *C. perfringens* counts in birds fed 400 g/kg crude protein fish meal diet, however, *C. perfringens* counts were low in ileum of birds fed SPC-based diets at all levels of crude protein. Dahiya *et al.* (2005) and Wilkie *et al.* (2005) reported a significant positive correlation between glycine content of the diets and digesta, and *C. perfringens* populations in ileum and cecum of broiler chickens. There are some *in vitro* studies showing the association between certain amino acids and *C. perfringens* growth and/or α toxin production (Ispolatovskaya, 1971; Muhammed *et al.,* 1975; Titball *et al.,* 1999). Although Muhammed *et al.* (1975) documented that methionine was stimulatory for growth of *C. perfringnes in vitro*; however, previous experiments in our lab demonstrated an antibacterial effect of DL-Met against *C. perfringens* in an *in vitro* experiment (Wilkie *et al.,* 2005). These researchers reported a

significantly ($P < 0.05$) reduced growth of *C. perfringens* after 24 h of incubation of mixed bacterial culture in minimal salt media supplemented with 10 mg/mL solution of DL-Met compared to unsupplemented media.

Methionine is the first limiting amino acid in poultry diets and protein synthesis will come to a halt in the absence of methionine. It is commonly supplemented as dry DL-Met (99% pure) or as liquid MHA-FA at a concentration of around 0.10 to 0.25% in poultry diets. We hypothesized that high dietary concentrations of methionine are responsible for reduced *C. perfringens* growth in the gastrointestinal tract of boiler chickens and thus may decrease the risk of NE. Hence, the purpose of this experiment was to determine the effect of nutritional and supranutritional levels of two methionine sources (DL-Met and MHA-FA) on intestinal *C. perfringens* and other microbial populations, and NE lesion scores in broiler chickens using previously established *C. perfringens* challenge model with slight modifications.

4.3. Materials and Methods

Experimental protocols were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with recommendations of the Canadian Council on Animal Care as specified in the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

4.3.1. Experimental Animals, Diets, and Design

In each experiment, a total of 84 one-day old conventional male broiler chicks (Ross 308) were obtained from a local broiler hatchery (Lilydale Hatchery, Wynyard, SK, Canada) and housed randomly in seven electrically heated battery cages for the first 2 weeks of age (12 birds per cage) at the Animal Care Unit, Western College of

Veterinary Medicine of the University of Saskatchewan. The birds received a medicated, ideal protein-balanced (1.20% lysine) corn-based starter crumble diet (Co-op feeds, Saskatoon, Canada) for the first 14 days of the experiment **(Table 4.1)**. On day 14, the birds were weighed and 2 cages of 6 birds each were assigned in a completely randomized design to one of the seven different ideal protein-balanced experimental diets in a 2 x 4 factorial arrangment. The main effects were methionine source (DL-Met or MHA-FA) and methionine level (0, 0.2, 0.4 and 0.8% DL-Met or 0.227, 0.454 and 0.908% MHA-FA, thus providing 4 corresponding equimolar levels of each methionine source) **(Table 4.3 and 4.4)**. The control diet was formulated to contain 23% crude protein, 1.2% lysine, 0.38% methionine and 3200 Kcal/kg ME. The control diet was then supplemented with either dry DL-Met or liquid MHA-FA to achieve four equimolar levels of each methionine source (0, 0.2, 0.4 and 0.8%). The calculations were based on the fact that the commercial product contained 88% MHA-FA **(Table 4.2)**. The diets met or exceeded the NRC nutrient requirements for broiler chickens for all other nutrients (NRC, 1994).

The research facility was thoroughly cleaned and disinfected prior to bird placement. The battery cages were arranged in four tires with a wire floor and were equipped with external feed and water troughs. Lighting schedule was 16L:8D throughout the experiment with controlled temperature and humidity. Room temperature was maintained according to industry standards. None of the experimental diets contained antibiotics or coccidiostats and were not pelleted. Throughout the experimental period in each experiment, birds were fed and watered *ad libitum.* Feed consumption and body weight for each cage was recorded for the period day 14-21 and day 21-28 in each

experiment for calculation of feed conversion. When calculating feed conversion the body weight of dead birds was taken into account. Amino acid analysis of the different diets was performed by Degussa Corporation (Degussa Canada Inc., Burlington, ON, Canada).

4.3.2. *Clostridium perfringens* Challenge Model

An avian *C. perfringens* field strain isolated from a clinical case of NE was obtained from Dr. Manuel Chirino, College of Veterinary Medicine, University of Saskatchewan and characterized by PCR technique as a type A toxin producer. The organism was cultured anaerobically on BBL^{TM} Blood Agar Base (Becton, Dickinson and Co., Sparks, MD, USA) containing 5.0% sheep blood and 100 mg/L neomycin sulfate (The Upjohn Company, Orangeville, ON, Canada) for 18 h at 37°C, then aseptically inoculated into either brain heart infusion (Difco Labs, Detroit, MI, USA) (experiment 1) or cooked meat medium (Difco Labs, Detroit, MI, USA) (experiment 2) and incubated anaerobically overnight (experiment 1) or for 8 h (experiment 2) at 37°C. All birds were orally challenged in the crop with 0.5 mL on day 1 and 1.0 mL on days 14 to 20 inclusive with this actively growing culture of *C. perfringens* using a 12.0 mL syringe equipped with vinyl tubing (I.D. 0.97mm, O.D. 1.27mm). Bacterial counts were performed on the culture daily prior to inoculation and the numbers ranged from 4.51×10^4 to 3.73×10^6 CFU/mL.

Table 4.1. Composition of starter diet used in both experiments up to day 14 of age[1].

Ingredients	Inclusion (%)
Soybean meal	40.90
Corn	39.50
Wheat	10.00
Tallow	4.52
Dicalcium phosphate	1.53
Calcium carbonate	1.29
Canola oil	1.00
DL-Methionine	0.26
Choline chloride	0.10
Vitamin/Mineral premix[2]	0.50
Salinomycin sodium[3]	0.06
Bacitracin[4]	0.05
Bio-cox 120[5]	0.05

[1] Diet was formulated to contain 31.5% crude protein and meet NRC requirements for broiler chickens.

[2] Supplied per kilogram of diet: vitamin A, 3.3 mg; cholecalciferol, 55 µg**;** vitamin E, 30 mg; vitamin K, 0.5 mg; vitamin B12, 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; sodium, 511 mg; iron, 80 mg; maganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

[3] Phibro Animal Health Ltd.

[4] Alpharma Canada Corp.

[5] Anticoccodial Premix, OzBioPharma Ltd.

Table 4.2. Experimental design.

Treatment	Methionine source[1]	Addition of Met Source (% of product)	Addition of Met equivalents (%)
1	Control diet	-	-
2	DL-Met	0.200	0.200
3	DL-Met	0.400	0.400
4	DL-Met	0.800	0.800
5	Liquid MHA-FA	0.227[2]	0.200
6	Liquid MHA-FA	0.454[2]	0.400
7	Liquid MHA-FA	0.908[2]	0.800

[1] DLM = DL-Methionine; Liquid MHA-FA = Liquid Methionine hydroxyl analog-free acid

[2] Based on a liquid MHA-FA content of 88% in the commercial product

Table 4.3. Ingredient composition of experimental diets used in experiments 1 and 2 (% as is basis).

Ingredients	Experimental diets						
	Control	0.2 DL-Met	0.4 DL-Met	0.8 DL-Met	0.2 MHA-FA	0.4 MHA-FA	0.8 MHA-FA
Corn	63.75	63.55	63.35	62.95	63.52	63.30	62.84
Meat/Bone meal	14.16	14.16	14.16	14.16	14.16	14.16	14.16
PPC[x]	6.88	6.88	6.88	6.88	6.88	6.88	6.88
Soybean meal	6.65	6.65	6.65	6.65	6.65	6.65	6.65
Wheat	5.90	5.90	5.90	5.90	5.90	5.90	5.90
Canola oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cal. carbonate	0.88	0.88	0.88	0.88	0.88	0.88	0.88
Dical. phosphate	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-threonine	0.03	0.03	0.03	0.03	0.03	0.03	0.03
L-lysine HCl	0.10	0.10	0.10	0.10	0.10	0.10	0.10
DL-methionine	0.00	0.20	0.40	0.80	0.00	0.00	0.00
MHA-FA[z]	0.00	0.00	0.00	0.00	0.23	0.45	0.91
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vit/Min premix[1]	0.50	0.50	0.50	0.50	0.50	0.50	0.50

[1]Supplied per kilogram of diet: vitamin A, 3.3 mg; cholecalciferol, 55 µg**;** vitamin E, 30 mg; vitamin K, 0.5 mg; vitamin B12, 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; sodium, 511 mg; iron, 80 mg; maganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

[x]PPC = Potato protein concentrate

[z]MHA-FA = Methionine hydroxy analog

Table 4.4. Crude protein and amino acid composition (analyzed) of experimental diets used in experiments 1 and 2 (% as is basis).

	Experimental diets						
	Control	0.2 DL-Met	0.4 DL-Met	0.8 DL-Met	0.2 MHA-FA	0.4 MHA-FA	0.8 MHA-FA
Crude protein	23.23	23.37	23.21	23.03	22.83	23.42	23.15
Essential amino acids							
Arginine	1.31	1.36	1.28	1.30	1.28	1.32	1.36
Histidine	0.52	0.53	0.52	0.52	0.51	0.52	0.53
Isoleucine	0.90	0.92	0.89	0.90	0.88	0.91	0.91
Leucine	2.05	2.06	2.04	2.04	2.02	2.05	2.05
Lysine	1.24	1.28	1.24	1.28	1.24	1.28	1.34
MHA-FA[x]	-	-	-	-	0.22	0.50	0.86
Methionine	0.43	0.63	0.78	1.19	0.43	0.43	0.43
Meth. + cystine	0.77	0.95	1.12	1.51	0.75	0.75	0.76
Phenylalanine	1.08	1.10	1.07	1.07	1.04	1.08	1.09
Threonine	0.98	1.03	0.96	1.01	0.95	0.97	1.00
Valine	1.11	1.15	1.10	1.12	1.09	1.13	1.13
Nonessential amino acids							
Alanine	1.47	1.51	1.47	1.46	1.46	1.48	1.49
Aspartate	2.11	2.14	2.08	2.11	2.04	2.12	2.12
Cysteine	0.33	0.33	0.33	0.32	0.32	0.32	0.33
Glycine	1.63	1.72	1.60	1.62	1.60	1.63	1.69
Glutamate	3.56	3.60	3.56	3.56	3.53	3.58	3.57
Proline	1.51	1.56	1.50	1.50	1.50	1.54	1.55
Serine	1.15	1.16	1.14	1.16	1.12	1.17	1.15

[x]MHA-FA = Methionine hydroxy analog

4.3.3. Pathological Examination

Birds were observed on a pen basis at least once daily for any signs of NE and all birds that died during the course of experiments were necropsied to determine the cause of death. On day 28, the surviving chickens were euthanized by cervical dislocation, weighed and necropsied immediately. Intestinal tracts were removed and intestinal lesions were scored blindly according to the method of Truscott and Al-Sheikhly (1977) with slight modifications on a scale 0 to 4 where 0 was apparently normal, no lesion; 0.5, severely congested serosa and mesentery engorged with blood; 1, thin walled and friable intestines with small red petechiae (> 5); 2, focal necrotic lesions; 3, patches of necrosis (1-2 cm long); and 4, diffused necrosis typical of field cases. Following postmortem examination, if the score was ≥1 then a 1.5-2.0 cm long piece of intestinal tissue with the gross NE lesion was collected in phosphate-buffered formaldehyde solution and processed routinely for paraffin embedding, sectioned at approximately 5 μm, and stained with hematoxylin and eosin.

4.3.4. Bacterial Enumeration

On day 28, birds were selected at random from each pen, weighed and killed by cervical dislocation and their intestinal tracts were removed. Samples of fresh digesta (0.1 to 0.2 g) from ileum (Meckel's diverticulum to 1 cm proximal to ileocecal junction) and ceca were collected aseptically in pre-weighed 15 mL sterilized plastic tubes containing 1 mL 0.1% sterile peptone buffer with 5 g/L cysteine hydrochloride (Sigma Chemical Co., St. Louis MO). The digesta samples were pooled from two birds from each cage. The samples were immediately placed and kept on ice until plated within 3 h of collection. The samples were weighed and diluted in peptone water to an initial 10^{-1} dilution. Ten-

fold dilutions were spread in duplicate using an automated spiral plater (Autoplate, Spiral Biotech Inc., Bethesda MD, USA) on BBL™ blood agar base (Becton, Dickinson and Co., Sparks, MD, USA) containing 5% sheep blood and 100 mg/L neomycin sulfate (The Upjohn Company, Orangeville, ON, Canada) for *C. perfringens* enumerations. In addition, all the digesta samples were also cultured on MRS agar (Becton, Dickinson and Co., Sparks, MD, USA), MacConkey's agar (Becton, Dickinson and Co., Sparks, MD, USA) and Bile esculin agar (Becton, Dickinson and Co., Sparks, MD, USA) for the enumeration of lactobacilli, coliforms and *Streptococci* group D, respectively. The plates were incubated at 37°C for 16-24 h anaerobically for *C. perfringens* and lactobacilli while aerobically for coliforms and *Streptococcus* group D bacteria. The α- and β-hemolytic colonies on blood agar/neomycin plates were counted as *C. perfringens* with presumptive colonies being randomly picked, gram stained, plated on Mannitol Yolk Polymixin agar (Oxoid Inc., Napean, ON, Canada) and examined microscopically to confirm them as *C. perfringens.* Counts were expressed as the Log_{10} CFU/g of intestinal contents.

4.3.5. Statistical Analysis

The data were analyzed using the General Linear Model procedure of SPSS (v.12.0, SPSS Inc, Chicago IL, USA) as a 2 x 4 factorial (two methionine sources each with 4 levels). All the effects were considered as fixed and the interaction between methionine source and levels were used in the model with pen was the experimental unit. The treatment means were compared using Ryan-Einot-Gabriel-Welch multiple *F*-test and comparisons were deemed significant at $P < 0.05$.

4.4. Results

The crude protein and amino acid composition of the experimental diets used in this study from day 14-28 of age is shown in **Table 4.4.** The dietary CP, DL-Met and MHA-FA concentrations were very close to that of our planned levels. The Rest of the amino acids and MHA-FA were in similar concentrations in all experimental diets in both experiments.

Subsequent to challenge with *C. perfringens* some of the birds were dull, depressed and had abnormally wet droppings for the first 3-5 days of each experiment. During the course of this study, 2 birds died in experiment 1 (one each from control and 0.4% DL-Met supplemented groups) and 3 birds died in experiment 2 (one each from control, 0.2 and 0.8% MHA-FA supplemented groups) of unknown causes. Majority of the dead birds were in good condition and did not have any full blown gross lesions of NE either in intestine or any other organ which might cause the death. Only one bird had distended jejunum and ileum with thin and friable intestinal wall, and the lumen was filled with gas and dark brown fluid content. Rest of the dead birds had few petechial hemorrhages in distal jejunum and proximal ileum. Surviving birds had no apparent signs of morbidity 7-10 day post-challenge.

In both experiments, the average daily feed intake (ADFI), average daily gain (ADG) and feed conversion efficiency (FC) at the end of 14-21 and 21-28 day period were not significantly different among various dietary treatments **(Table 4.5)**. The only exception is that the ADG increased significantly ($P < 0.05$) with methionine supplementation during 21-28 day period in experiment 2 and there was significant interaction between methionine source and level for ADG. However, there was no

difference in ADG of the birds fed either DL-Met or MHA-FA at various dietary concentrations. Also, there was no difference in performance of these birds fed either DL-Met or MHA-FA in any of the experiments at any time point.

Various bacterial species (*C. perfringens*, lactobacilli, *Streptococcus* group D and coliforms) were enumerated in ileum and cecum of chickens on day 28 of the two experiments. The populations of all four bacterial species enumerated in this study were higher in cecum than ileum. There were no significant differences in growth of various bacterial species in the intestinal tract of broiler chickens fed two different methionine sources, whereas methionine concentration had significant effect on various bacterial species either in ileum or cecum or both. There was no significant interaction between methionine source and methionine concentration for various bacterial species populations either in ileum or cecum in both experiments. The only exception is *C. perfringens* growth in ileum and cecum in experiment 2 where there was a significant ($P < 0.05$) interaction between methionine source and level. There was decrease in *C. perfringens* numbers with increase in dietary DL-Met both in ileum and cecum whereas *C. perfringens* counts were higher in ileum (3.38 versus 3.90 $\log_{10}$ CFU/g wet digesta) and cecum (3.98 versus 4.98 $\log_{10}$ CFU/g wet digesta) when dietary MHA-FA increased from 0.2 to 0.4% **(Figure 4.1)**. However, there were no significant differences in *C. perfringens* counts between DL-Met and MHA-FA at 0.2 or 0.4% inclusion rates.

In experiment 2, significantly higher ($P < 0.05$) *C. perfringens* numbers were observed in ileum of birds fed control diet compared to birds given methionine supplemented diets. Also the *C. perfringens* populations in ileum decreased significantly ($P < 0.05$) when methioine supplementation increased from 0.2 to 0.8%. Whereas, in

experiment 1, the *C. perfringens* growth in ileum was not significantly different among various dietary treatments. In experiment 1, the ceca of birds fed either 0.2 or 0.4% methionine contained significantly lower ($P < 0.05$) *C. perfringens* counts than those received control diet with no supplemental methionine. The control diet supported a significantly higher growth of *C. perfringens* than methionine supplemented diets in experiment 2. An important point to be noted is that the *C. perfringens* populations were generally higher both in ileum and cecum in experiment 2 compared to experiment 1.

The lactobacilli populations were not significantly altered in ileum of broiler chickens fed different levels or sources of methionine in both experiments **(Figure 4.2)**. Similarly, there were no significant differences in lactobacilli growth in cecum in experiment 1. However, in experiment 2, the lactobacilli populations were significantly higher ($P < 0.05$) in ceca of birds receiving 0.8% methionine than the birds given diets with other levels of methionine tested.

In experiment 2, *Streptococcus* group D populations were significantly lower ($P < 0.05$) in ileum of birds receiving 0.8% methionine supplemented diets compared to those given either control or 0.4% methionine supplemented diets **(Figure 4.3)**. We observed a significant difference in *Streptococcus* group D growth in ceca of control and 0.2% added methionine fed birds in experiment 1, whereas there were no significant differences in experiment 2.

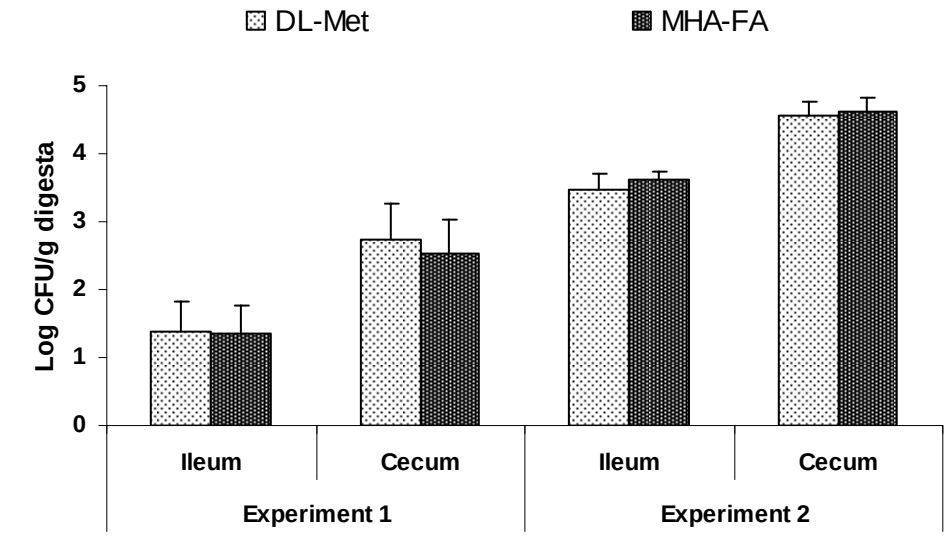
Table 4.5. Average daily feed intake (ADFI, g/day), average daily gain (ADG, g/day) and feed:gain ratio (FC, feed intake/weight gain, g/g) of birds during days 14-21 and 21-28 in both experiments.

	Days 14-21			Days 21-28		
Experiment 1	ADFI	ADG	FC	ADFI	ADG	FC
Level (%)						
0.0	66.4	40.5	1.6	140.6	66.3[a]	2.1
0.2	75.9	42.3	1.8	135.7	76.7[b]	1.8
0.4	77.0	42.4	1.8	147.6	76.3[b]	1.9
0.8	76.6	43.8	1.7	126.4	77.9[b]	1.6
Pooled SEM	5.43	1.10	0.06	12.44	4.09	0.18
Source						
DLM	72.9	43.2	1.7	140.8	75.3	1.9
MHA-FA	77.2	41.8	1.8	133.6	75.4	1.8
Pooled SEM	5.28	2.74	0.19	5.86	1.72	0.12
***P* value**						
Level	0.403	0.879	0.532	0.279	0.007	0.131
Source	0.245	0.441	0.086	0.329	0.352	0.463
Level*Source	0.093	0.607	0.459	0.624	0.011	0.429
Experiment 2						
Level (%)						
0.0	71.7	39.9	1.8	113.9	57.2	2.0
0.2	73.7	40.7	1.8	121.3	57.2	2.2
0.4	81.5	45.3	1.8	119.8	51.6	2.4
0.8	72.0	41.1	1.7	119.9	57.6	2.1
Pooled SEM	4.41	2.27	0.05	2.53	2.84	0.13
Source						
DLM	76.9	42.7	1.8	121.1	54.3	2.3
MHA-FA	73.3	41.4	1.7	117.8	57.1	2.1
Pooled SEM	4.30	1.84	0.08	4.73	2.97	0.19
***P* value**						
Level	0.560	0.778	0.738	0.775	0.701	0.555
Source	0.571	0.740	0.506	0.449	0.622	0.418
Level*Source	0.935	0.760	0.171	0.575	0.133	0.099

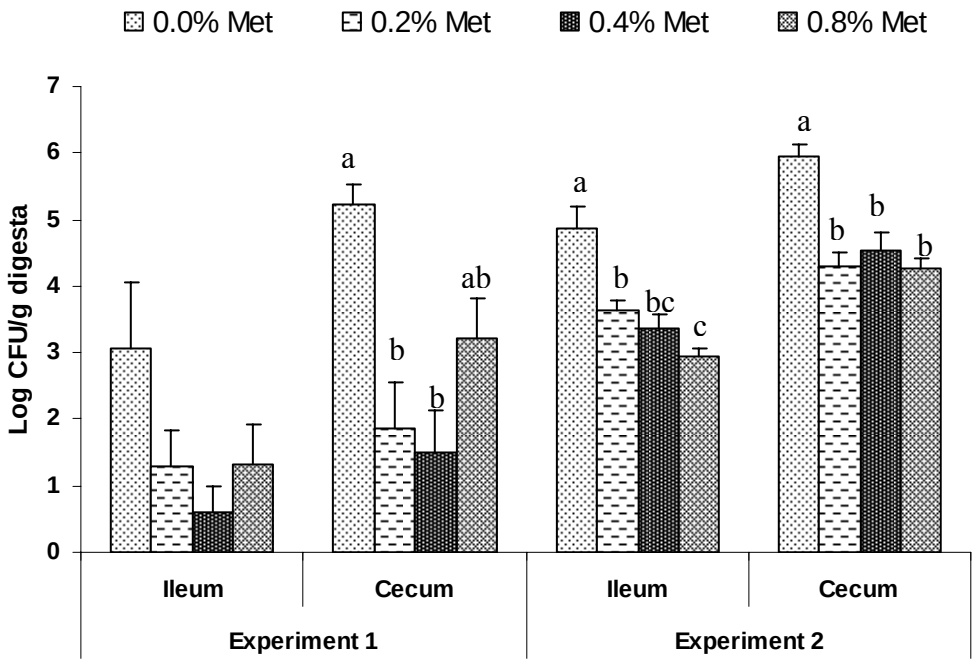
[abc] Means with the different superscripts within a column (within an experiment) differ significantly ($P < 0.05$)

In experiment 2, the coliforms counts were significantly higher ($P < 0.05$) in ileum of broiler chickens which were given control diets with no added methionine compared to birds receiving rest of the experimental diets with different concentrations of methionine. Methioine source or level had no significant impact on coliforms counts in cecum either in experiment 1 or 2 **(Figure 4.4)**. The coli form counts were substantially lower than those of lactobacilli and streptococci in the intestines of chickens on day 28 of age.

The mean NE lesion scores of chickens fed different experimental diets and killed on day 28 are presented in **Figure 4.5.** Dietary methionine source or concentration had no significant effect ($P > 0.05$) on NE specific intestinal lesion scores in any of the experiments. Irrespective of the dietary treatment, a large number of birds had thin and friable intestinal wall with congested serosa with blood engorged mesenteric vessels. Some birds had focal patches of hemorrhagic lesions in various intestinal regions, more frequently in distal jejunum, proximal ileum and cecal tonsils. But typical field type lesions specific to NE were not observed in any of the birds in either experiments. Although methionine source or concentration had no significant effect on intestinal lesion scores, however, there was a trend of decreasing the lesions with increase in methionine concentration which corresponds well with *C. perfringens* colonization in these birds.



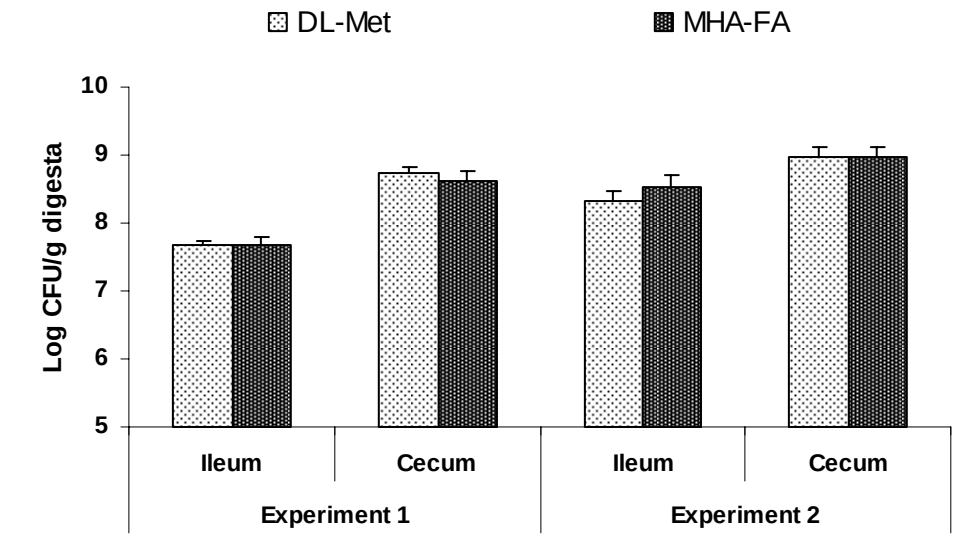
A



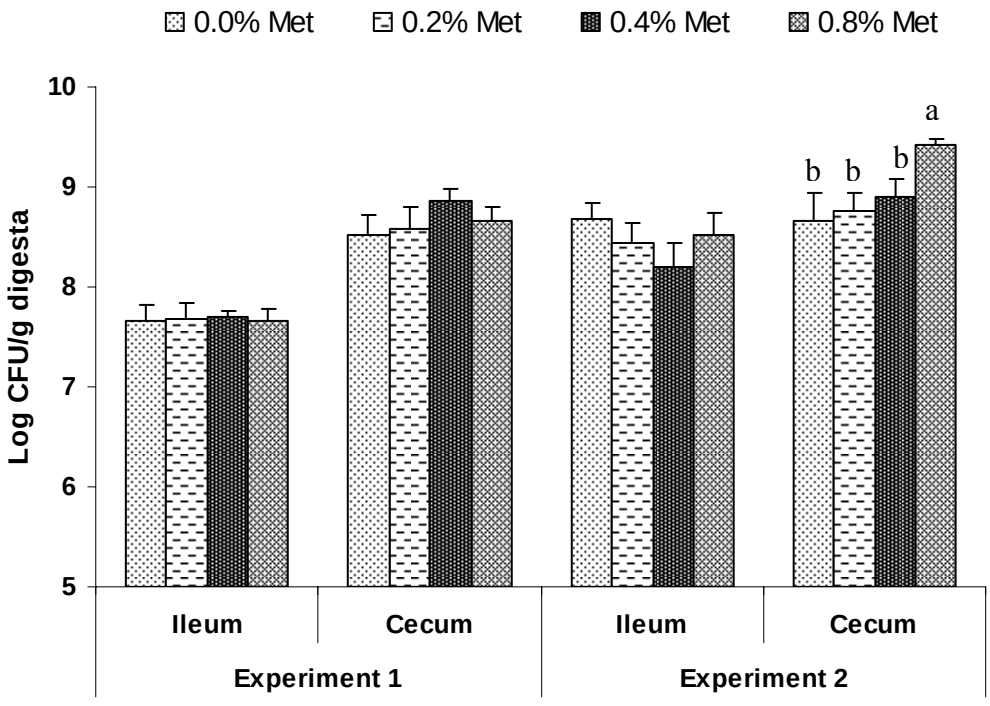
B

Figure 4.1. Effect of different methionine sources (A) and levels (B) on *C. perfringens* populations in ileum and cecum of broiler chickens on day 28 of both experiments. Bars represents the mean ± SEM.

[abc]Bars with different labels are significantly different ($P < 0.05$)



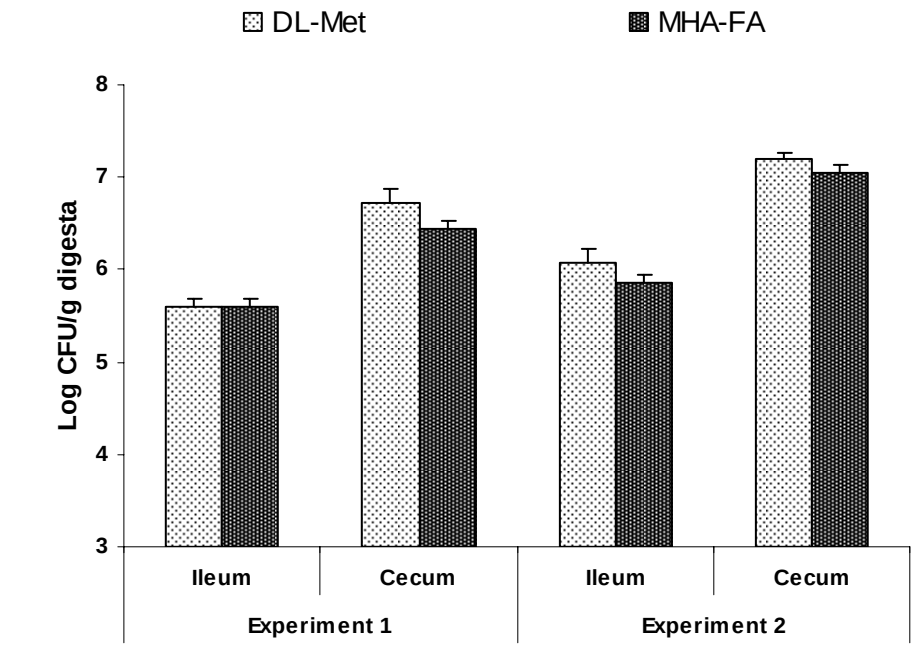
A



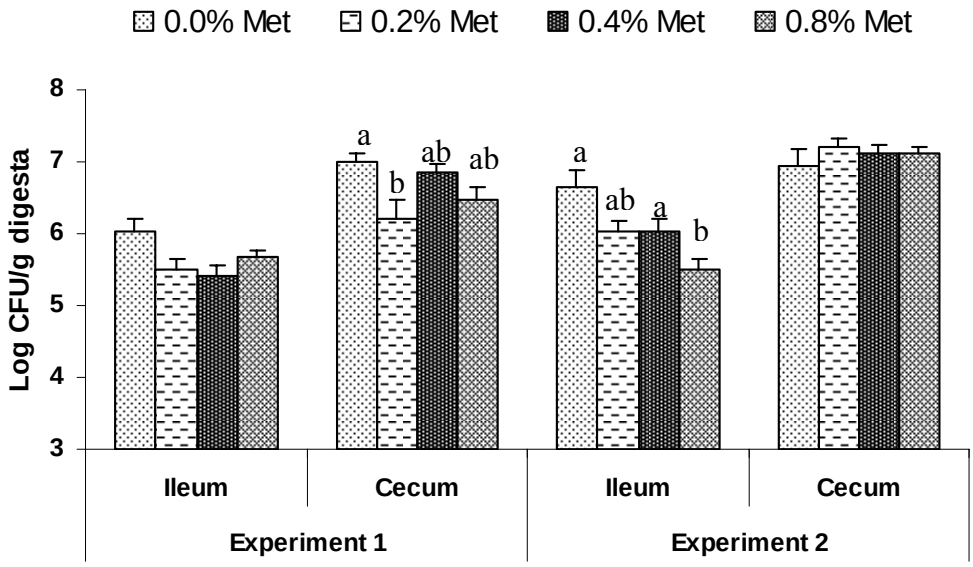
B

Figure 4.2. Effect of different methionine sources (A) and levels (B) on lactobacilli populations in ileum and cecum of broiler chickens on day 28 of both experiments. Bars represents the mean ± SEM.

[ab]Bars with different labels are significantly different ($P < 0.05$)



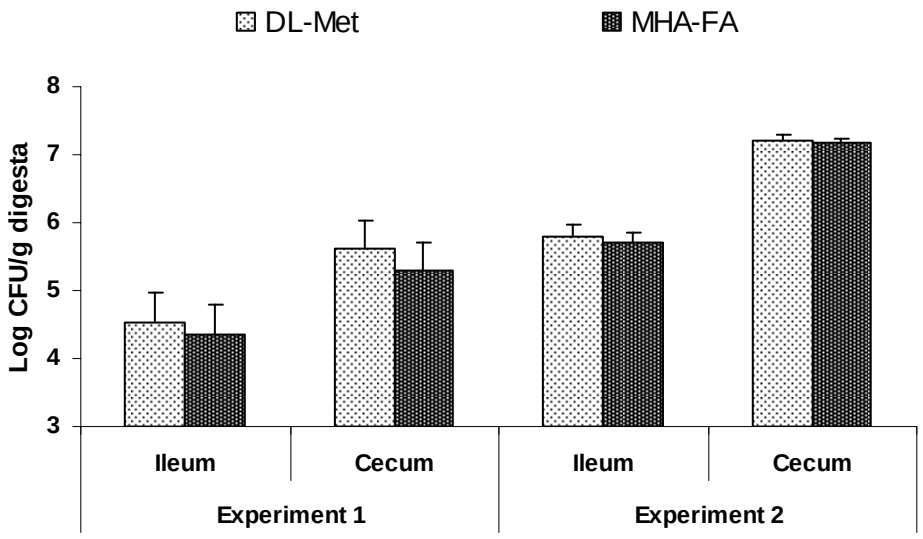
A



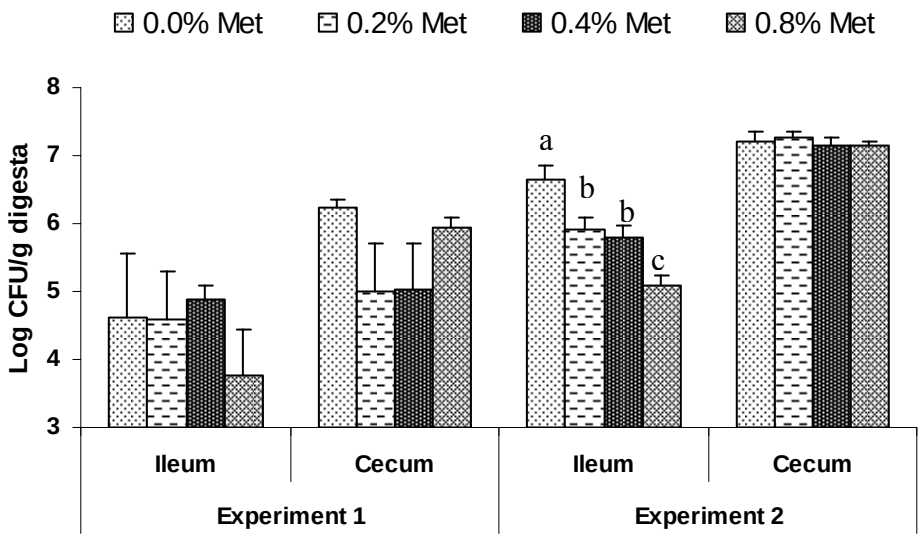
B

Figure 4.3. Effect of different methionine sources (A) and levels (B) on *Streptococcus* group D populations in ileum and cecum of broiler chickens on day 28 of both experiments. Bars represents the mean ± SEM.

[ab]Bars with different labels are significantly different ($P < 0.05$)



A



B

Figure 4.4. Effect of different methionine sources (A) and levels (B) on coliforms populations in ileum and cecum of broiler chickens on day 28 of both experiments. Bars represents the mean ± SEM.

[abc]Bars with different labels are significantly different ($P < 0.05$)

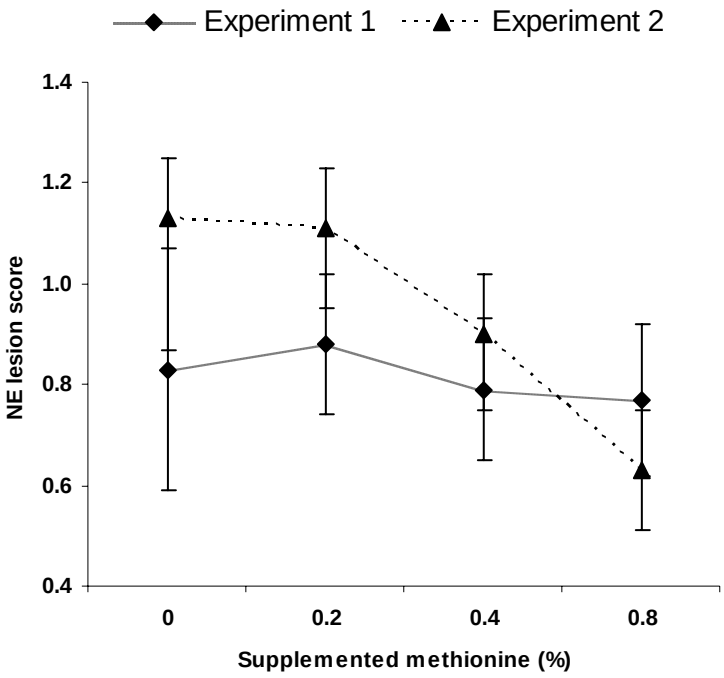


Figure 4.5. Mean necrotic enteritis lesion scores in experiments 1 and 2 in 28-day-old broiler chickens given experimental diets (days 14 to 28) containing different levels/sources of methionine.

Histological examination of formalin fixed intestinal tissues from 28-day-old broiler chickens euthanized in both experiments revealed no frank lesions of NE. The lesions include only slight edema and diffuse hemorrhages in lamina propria in some sections. There was no necrosis or desquamation of epithelial cells in the villi. There was no evidence of gram-positive rod-shaped organisms attached to intestinal mucosa. PMN cells infiltration in the lamina propria was not seen in any of the sections. We did not find any evidence of coccidial oocysts in any sections examined.

4.5. Discussion

Amino acid composition of various protein sources might be an important determinant of intestinal microbial growth in broiler chickens (Dahiya *et al.* 2006a). Excessive concentration of some amino acids might have a toxic effect on some bacterial species. Wlikie (2005) demonstrated a reduced growth of *C. perfringens* in the presence of methionine *in vitro*. In one of his studies, he demonstrated that *C. perfringens* counts were virtually reduced to zero in minimal salt media supplemented with 10 mg/mL of DL-Met after 24 h incubation. So the current study was designed to determine whether two commonly used methioinine sources have the similar antibacterial effects against *C. perfringens in vivo.* The two experiments reported in this study support our hypothesis that high levels of methionine supplementation may reduce *C. perfringens* populations in the intestinal tract and, thus decrease the risk of NE in broiler chickens.

No significant differences in ADFI, ADG and FC were observed during the period day 14-21 or 21-28 in either of the experiments. One exception was that ADG increased significantly with methionine supplementation during day 21-28 in experiment 1. The overall performance of the birds was relatively poor in both experiments and that

might be due to the fact that there was more feed wastage. Another explanation for poor performance is that the birds were under stress of *C. perfringens* challenge and they had high *C. perfringens* populations in their intestinal tract which is evident from **Figure 4.1.** The ADFI and ADG were lower and FC was comparatively poor in experiment 2 during the period day 21-28. As evidenced by intestinal lesion scores and high intestinal colonization of *C. perfringens*, most of the birds in present study had a subclinical NE as documented by Lovland and Kaldhusdal (2001). A highly significant inverse correlation was obtained between the number of *C. perfringens* in the ileum and weight gain of chicks fed the glucose, sucrose, fructose and practical diets (Stutz and Lawton, 1984). Decreased growth rate and poor feed conversion efficiency have already been reported in broilers having high numbers of *C. perfringens* in the intestinal tract (Dahiya *et al.,* 2005; Kaldhusdal and Hofshagen, 1992; Stutz *et al.,* 1983).

Numerous studies have been conducted to compare the efficacy of DL-Met and liquid MHA-FA in broiler chickens (Baker and Boebel, 1980; Waldroup *et al*., 1981; Rostagno and Barbosa, 1995; Lemme *et al*., 2002). These studies have been inconsistent in the value assigned to the efficacy of MHA-FA due to a number of reasons. The differences in the efficacy between DL-Met and MHA-FA might be due to both differences in absorption in intestinal tract, in microbial conversion in the intestinal tract and to differences in post-absorptive conversion of two molecules for metabolic purposes (Maenz and Engele-Schaan, 1996; Drew *et al.,* 2003). It is well known that DL-Met is transported by the System B amino acid transporter, whereas it's hydroxy analog (MHA-FA) is absorbed by a $^{+}$H-dependent nonstereospecific lactate transport system. A number of studies with broilers using radiolabeled methionine sources indicated a significantly

lower absorption of MHA-FA compared to DL-Met, which means that the DL-Met is removed more quickly from the intestinal lumen than MHA-FA and therefore has less exposure to intestinal bacteria resulting in decreased methionine uptake (Maenz and Engele-Schaan, 1996; Drew *et al.,* 2003). In agreement with Esteve-Garcia and Austic (1993), Maenz and Engele-Schaan (1996) concluded that there is a substantial conversion of dietary MHA-FA, during passage through the small intestine, to compounds that cannot be used as a source of methionine by the bird. This might be due to degradation of a substantial fraction of the hydroxy analog by microbial fermentation during passage through the small intestine (Lemme *et al.,* 2001). Drew *et al.* (2003) compared the apparent absorption of ^{3}H-labelled L-methionine with MHA-FA in germ-free and conventionally reared broiler chickens and observed a significantly lower residual MHA-FA in distal ileum of germ-free birds than in conventional birds whereas there was no difference in residual methionine level. Questions remain regarding the effect of these two methionine sources on intestinal microbial ecology of the birds. As far as we know this is the first study where we directly examine the impact of these two methionine sources on intestinal microflora in broiler chickens.

As evident in **Figure 4.1**, *C. perfringens* populations were higher in ileum and cecum of birds in experiment 2 than in experiment 1. It might be due to the fact that in experiment 2, the cooked meat media was used for culturing the *C. perfringens*, which supports the *C. perfringens* growth and α toxin production much better than brain heart infusion which was used in experiment 1 **(See Appendices 9.2A and 9.2B)**. In the present study, we observed a significant reduction in *C. perfringens* growth with methionine supplementation in cecum (Experiment 1), or both in ileum and cecum

(Experiment 2). However, there were no significant differences between the two methionine sources. We could not find any literature regarding the toxic effects of methionine on *C. perfringens in vivo*. Earlier, in *in vitro* experiments, Wilkie *et al.* (2005) reported a significantly reduced growth of *C. perfringens* in minimal salt media supplemented with 10 mg/mL DL-Met compared to control. In contrast to this, Muhammed *et al.* (1975) reported that alanine, aspartic acid and methionine are stimulatory for the growth and sporulation of *C. perfringens in vitro.* But we cannot extrapolate the results of *in vitro* to *in vivo* because the conditions are entirely different in the gastrointestinal tract. It should be remembered that there are differences in amino acid and vitamin requirements among various strains of *C. perfringens* (Fuchs and Bonde, 1957). They also reported a significant increase in *C. perfringens* growth in the presence of glycine, lysine and serine, and proved that not only individual amino acid but the balance of amino acids is important for maximum growth of *C. perfringens.* There might be antagonism between amino acids i.e. the action of certain amino acids being prevented by several other amino acids. Previously, we have found that amino acid glycine supports the *C. perfringens* growth and α toxin production both *in vitro* as well as *in vivo* (Dahiya *et al.,* 2005; Wilkie *et al.,* 2005). Glycine accelerated the *C. perfringens* growth (Ispolatovskaya, 1971) while α toxin production required the presence of glycine-containing peptides in defined media (Nakamura *et al.,* 1968; Stevens and Rood, 2000).

We are aware of no study that directly examines the effect of methionine sources/concentration on *C. perfringens* growth in broiler chickens. The methionine level used in this study was much higher (2 to 4 times) than commonly used in commercial poultry diets. The supranutritional concentrations of methionine were associated with

reduced *C. perfringens* growth in broiler chickens, however, it might not be commercially viable method to control NE due to high cost of synthetic methionine. The mode of action by which methionine influences the intestinal populations of these important groups of bacteria in broiler chickens is unclear. Not all bacteria have the ability to utilize DL-Met or MHA-FA. Intestinal microflora of the bird might be involved in nitrogen recycling mechanism (Mattocks, 1971). Schmidt *et al.* (1952) reported that *C. perfringens* are involved in degradation of arginine into ammonia and carbon dioxide through citrulline and ornithine. As *C. perfringens* is strictly dependent on carbohydrates, the effect of higher levels of dietary methionine on intestinal *C. perfringens* populations might be indirect. It is possible that high dietary methionine concentration might cause an excessive growth of lactic acid producing bacteria (mainly *Lactobacillus, Streptococci*, and *Staphylococci*) which are predominant in the intestines of normal healthy chickens and generally considered to be protective against colonization of pathogenic micro-organisms.

In the present study, we observed a significant elevation in lactobacilli population in cecum of birds fed 0.8% methionine supplemented diets compared to rest of the diets in experiment 2. In contrast to this, *Streptococcus* group D populations were decreased with methionine supplementation in ileum (experiment 2) and cecum (experiment 1). Hofshagen and Kaldhusdal (1992) reported a higher population of lactobacilli in intestinal tract of broiler chickens fed corn-based diets and hypothesized that higher lactobacilli colonization impeded the development of NE. This effect might be indirect through inhibition of *C. perfringens* colonization. We observed substantially lower populations of coliforms than of lactobacilli and streptococci in the intestines of chickens

which is in corresponding with the findings of Barnes *et al.* (1972) and Stutz *et al.* (1983) and opposed to the similar levels of the three bacterial species reported by Hofshagen and Kaldhusdal (1992). This discrepancy may be attributed to variations in bacteriological isolation procedures, feed composition, feed antibiotics, or environmental conditions between the studies. Hegedus *et al.* (1993) documented that some lactic acid producing bacteria (*Lactobacillus plantarum, Leuconostoc mesenteriodes, Lactobacillus casei*) have the ability to utilize DL-Met for their growth. An antagonistic effect of lactobacilli against *Helicobacter pylori* (Aiba *et al.,* 1998), *Salmonella* spp. (Gill *et al.,* 2001) and *E.coli* (Mangell *et al.,* 2002) has been demonstrated in murine model. Lactic acid bacteria can significantly affect both the systemic and mucosal-associated immune responses and lactobacilli have been characterized for their potential as probiotic agents against *Salmonella* and *Campylobacter* in poultry (Chang and Chen, 2000; Gusils *et al.,* 2003; Lan *et al.,* 2003). Fukata *et al.* (1991) reported that pathogenic effect of *C. perfringens* could be reduced by feeding chicks a monoflora of *Lactobacillus acidophilus* or *Streptococcus faecalis*. The antagonistic activity may be mediated either directly by the production of inhibitory substances such as lactic acid, hydrogen peroxide and bacteriocins or by competing with the pathogen for binding sites on the epithelial cell surface. It is possible that the protein/amino acids are serving as energy and/or nitrogen source for other community members that in turn modify the intestinal environment in a favorable way for lactobacilli proliferation. This may occur by providing positive selection pressure on those gastrointestinal microbes which can directly ferment amino acids for carbon and nitrogen. Alternatively, the high methionine diets might affect the

physiochemical characteristics of gastro-intestinal tract of host in such a manner that the gastrointestinal milieu is made less favorable for *C. perfringens* proliferation.

Irrespective of the dietary treatments, some birds were dull, depressed and diarrheic for first 4-5 days after we start challenging them on 14 day of age. But the signs of illness disappeared after some days and all birds looked apparently healthy during the rest of period. There were some mortalities but necropsy examination showed that none of them was NE-specific. So despite inoculation of very high doses of *C. perfringens,* we did not observe any NE-specific mortality in any of the experiments. This is in agreement with some previous studies where chickens challenged with *C. perfringens* have also failed to induce mortality or other signs of NE, even though high *C. perfringens* colonization was reported in the intestinal tract of the birds (Craven, 2000; Pedersen *et al.,* 2003). In contrast to this, Al-Sheikhly and Truscott (1977a) and Vissiennon *et al.* (2000) were able to induce various pathological changes and mortality in chickens when inoculated orally with *C. perfringens* directly into the duodenum. It is therefore possible that some of the vegetative cells are inactivated by the acidic pH in the gizzard when given orally. Kaldhusdal *et al.* (1999) documented that lesions are more sensitive disease indicator than mortality, and subclinical NE is more frequent than clinical disease in broiler chickens. Since *C. perfringens* is the etiological agent of NE it seems reasonable to assume that increased numbers of this organism in the gut are associated with an increased predisposition to clinical NE.

In the present study, gross intestinal lesions of varying degrees among various treatment groups were observed although there were no significant differences among various dietary treatments i.e. dietary methionine source or concentration had no

significant effect ($P > 0.05$) on NE specific intestinal lesion scores in any of the experiments. However, there was a trend of decreasing the lesions with increase in methionine concentration which corresponds well with *C. perfringens* colonization in these birds. The lesion scores were higher and there were more differences among various groups in experiment 2 compared to experiment 1. The differences might be attributed to the fact that cooked meat media (used in experiment 2) supports the *C. perfringens* growth and α toxin production much better than the brain heart infusion used in experiment 1 (Appendices 9.2A and 9.2B)**.** The demonstration of a relationship between NE lesion scores, performance data and *C. perfringens* numbers is an important feature of both experiments. Al-Sheikhly and Truscott (1977a) and Vissiennon *et al.* (2000) suggested that high *C. perfringens* populations and slight intestinal damage were apparently necessary for disease production when a broth culture was used. We believe that whereas the aforementioned may be major factors, other factors, including the chemical/physical nature of the diet and the interaction of other intestinal microflora, may contribute to pathogenesis of the disease. In the present study, even clinically healthy birds were highly colonized with *C. perfringens* which confirms the sub clinical nature of the disease because high numbers of *C. perfringens* with macroscopically visible, focal necrotic lesions in the small intestine is a strong indicator of an occurrence of NE (Kaldhusdal and Hofshagen, 1992).

In contrast to some earlier findings, the microscopic lesions in intestine were not conclusive of NE in the present study (Fukata *et al.,* 1988; Kaldhusdal *et al.,* 1995; Long *et al.,* 1974; Shane *et al.,* 1985). Presence of slight edema and hemorrhages in lamina propria were observed in quite a few numbers of birds. However, desquamated epithelial

cells and polymorphonuclear cells (PMN) were not detected in any of the sections. Bryant *et al.* (1993) and Stevens *et al.* (1997) also demonstrated an absence of PMN cells at the site of *C. perfringens* infection. In the absence of host response (suppression of PMN influx), clostridia proliferates rapidly, leading to local accumulation of toxins. Higher *in situ* concentrations of *C. perfringens* toxins, especially α toxin, further inhibit PMN influx and reach concentration sufficient to cause membrane destruction (Stevens and Rood, 2000). So in spite of high numbers of *C. perfringens* in the intestinal tract of these birds, the clinical disease could not be produced. It is possible that it is not high *C. perfringens* counts *per se*, but some other factors possibly associated with high *C. perfringens* colonization that are required to produce a full blown disease. Intestinal damage caused by toxins of *C. perfringens* could represent such a factor. Lesions of NE were produced by the administration of either purified α toxin or the supernatant of broth cultures of *C. perfringens* to conventional (Al-Sheikhly and Truscott, 1977c) and germ free chicks (Fukata *et al.,* 1988), whereas no lesions were detected in birds receiving supernatant of broth cultures neutralized by anti-α toxin serum.

4.6. Conclusions

Results of the present study demonstrated an antibacterial effect of both methionine sources against *C. perfringens* when used in high concentrations. Thus, the understanding of the effects of different dietary methionine sources and levels on intestinal microbial ecology including *C. perfingens* growth is important for developing dietary formulations for broiler chickens that may reduce the risk of an outbreak of clinical NE. There was no difference in bird's performance under stress of *C. perfringens* challenge when these two methionine sources are fed on equimolar basis. Because there

are some limitations with the culture based methods used in the present study for bacterial enumeration, it would be interesting to use culture-independent approaches such as PCR with denaturing gradient gel electrophoresis in studying wide group of microbiota with respect to different methionine sources. A further assessment of the putative associations between these methionine sources and NE is of considerable interest. Further studies to determine which bacterial species are involved in competition for these two methionine sources with the host would be an interesting addition to the present study.

5.0. DIETARY GLYCINE CONCENTRATION AFFECTS INTESTINAL *CLOSTRIDIUM PERFRINGENS* AND LACTOBACILLI POPULATIONS IN BROILER CHICKENS

5.1. Abstract

Previous studies have reported that intestinal populations of *C. perfringens*, the causative agent of necrotic enteritis (NE), are correlated with diets high in glycine. To establish a direct causative link, three experiments were conducted to examine the effect of dietary glycine levels on gut *C. perfringens* populations, α-toxin production and NE lesion scores in broiler chickens. In experiments 1 and 2, 12 groups of 4 birds were fed 4 different ideal protein-balanced diets formulated to contain 0.75, 1.58, 3.04 or 4.21% glycine on day 14-28 of age. In experiment 3, 24 groups of 4 birds were given 6 different ideal protein-balanced diets formulated to contain 0.50, 0.75, 1.00, 1.50, 2.00 or 4.00% glycine. All birds were orally challenged with broth culture of *C. perfringens* type A on day 1 and between days 14 - 21 of age, and killed on day 28. The majority of birds showed clinical signs of NE with 4.16-8.33% mortality in the 3 experiments. The highest mortality and intestinal lesion scores were observed in chickens receiving 3.04% glycine in experiments 1 and 2; and 4.00% glycine in experiment 3. *C. perfringens* populations in the cecum varied quadratically with increasing dietary glycine with the maximal response seen at 3.30, 3.89 and 3.51% dietary glycine in experiments 1, 2 and 3, respectively. Number of lactobacilli in cecum declined significantly ($P < 0.05$) with increasing levels of glycine. The results suggest that dietary glycine level has a significant effect on *C. perfringens* and lactobacilli populations and may be a predisposing factor for NE in broiler chickens.

5.2. Introduction

Necrotic enteritis is a worldwide disease of poultry caused by *Clostridium perfringens*, usually belonging to toxin type A (Ficken and Wages, 1997). *C. perfringens* is a ubiquitous, spore-forming anaerobic bacteria found in soil, dust, feces, feed, poultry litter, and in the intestinal tract of poultry and other animals. Although *C. perfringens* is a normal inhabitant of the intestine, under certain circumstances it proliferates rapidly increasing to 10^7 to 10^9 CFU/g of digesta, and may cause sub clinical or clinical NE. Impaired feed conversion, reduced growth rate, increased condemnation rates and mortality are major causes of production losses associated with NE (Lovland and Kaldhusdal, 2001). The α toxin of *C. perfringens* has been detected in feces and intestinal contents of chickens and other birds with NE and is believed to be responsible for the intestinal mucosal necrosis that is characteristics of this disease. More α toxin is produced by isolates from birds with NE than by isolates from birds not displaying symptoms (Hofshagen and Stenwig, 1992).

NE may be controlled prophylactically with certain in-feed antibiotic growth promoters and ionophore coccidiostats. However, in the recent years, growing political and consumer demands for safer foods have put pressure on the prophylactic use of AGPs and ionophore coccidiostats as feed additives. Hence, there is a pressing need to better understand the factors that cause the overgrowth of *C. perfringens* in the intestine and predispose birds to NE in order to develop alternative management or dietary strategies to control the incidence and severity of this disease.

Diet composition has a great influence on the gastrointestinal microbial ecology of birds and several dietary factors have been shown to contribute to the incidence of NE.

There appear to be two major dietary factors which predispose birds to NE. The first factor is dietary cereal grains rich in water soluble non-starch polysaccharides such as wheat (Branton *et al.,* 1997; Riddell and Kong, 1992), barley and rye (Annett *et al.,* 2002; Kaldhusdal and Hofshagen, 1992; Kaldhusdal and Skjerve, 1996; Riddell and Kong, 1992). The second dietary factor predisposing broilers to NE is high protein diets, particularly those consisting of high percentage of protein from animal sources (Drew *et al.,* 2004; Kaldhusdal and Skjerve, 1996; Truscott and Al-Sheikhly, 1977). A number of studies have demonstrated that source and level of protein in diets had a profound effect on the number of *C. perfringens* in intestinal contents in chickens (Drew *et al.,* 2004; Kaldhusdal, 2000), pigs (Mansson and Olhagen, 1967; Mansson and Smith, 1962) and dogs (Zentek *et al.,* 1998).

There are various studies in literature that show the mechanism(s) behind the effect of cereal grains on *C. perfringens* numbers and NE but little is known about the factors or mechanisms responsible for the increased incidence of NE in broilers fed high protein diets. Previous work has shown that there might be a correlation between certain amino acids, especially glycine, and *C. perfringens* populations and/or α toxin production (Drew *et al.,* 2004; Muhammed *et al.,* 1975; Titball *et al.,* 1999). Ispolatovskaya (1971) reported that glycine accelerated the growth of *C. perfringens* and production of α toxin *in vitro.* In defined media, there was increased production of α toxin in the presence of glycine-containing peptides (Nakamura *et al.,* 1968; Stevens and Rood, 2000). Drew *et al.* (2004) reported a significantly greater population of *C. perfringens* in the intestinal tract of chickens fed fish meal-based diets versus soy protein concentrate diets, and significantly higher levels of glycine were reported in fish meal diets compared to soy

protein diets. More recently, Wilkie *et al.* (2005) documented a significant correlation between dietary glycine and *C. perfringens* populations in ileum and cecum of broiler chickens. Corzo *et al.* (2004) documented that dietary glycine might become a limiting factor in all-vegetable diets fed to broiler chicks when crude protein is low and suggested that the current NRC recommendations (NRC, 1994) for glycine + serine of 1.25% may be insufficient.

Taken together, the results of previous studies and preliminary results of our laboratory suggest that an increase in the concentration of glycine in the lower small intestine may be a triggering event for the proliferation of *C. perfringens* and/or up regulation of virulence factors chiefly α toxin which may ultimately lead to clinical disease. The purpose of the current study was to examine the effect of various levels of dietary glycine on intestinal *C. perfringens* populations, α toxin production and NE lesion scores in broiler chickens.

5.3. Materials and Methods

Experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) and were performed in accordance with recommendations of the Canadian Council on Animal Care as specified in the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

5.3.1. Animal Management and Experimental Diets

5.3.1.1. Experiments 1 and 2

In each of these two experiments, a total of 48, one-day old conventional male broiler chicks (Ross 308) were obtained from a local broiler hatchery (Lilydale Hatchery, Wynyard, SK, Canada) and housed randomly in two electrically heated battery cages for

the first 2 weeks of age (24 birds per cage). The birds received a medicated, ideal protein-balanced (1.20% lysine) corn-based starter crumble (Co-op feeds, Saskatoon, Canada) for the first 14 days of experiment **(Table 5.1)**. On day 14 the birds were randomized and 3 cages of 4 birds each were assigned to one of four different ideal protein-balanced experimental diets formulated to contain 0.75, 1.58, 3.04 or 4.21% glycine **(Tables 5.2 and 5.3)**. The diets were isocaloric (3,200 Kcal of ME/kg), contained 1.20% lysine with other essential amino acid levels formulated to be within 10 per cent of ideal protein ratio. The diets met or exceeded requirements for broiler chickens for all other nutrients (NRC, 1994).

5.3.1.2. Experiment 3

Ninety six one-day old conventional male broiler chicks (Ross 308) were placed randomly into four electrically heated battery cages (24 birds per cage) for first 2 weeks of age. On day 1 through 14 of the experiment, birds were provided with the same starter diet described above. On day 14, birds were randomly assigned to one of the 24 battery cages (4 birds per cage). Four cages were assigned in a randomized complete block design to one of the six ideal protein-balanced experimental diets up to the end of experiment (day 28). Diets were formulated to contain six different glycine concentrations viz. 0.50, 0.75, 1.00, 1.50, 2.00 and 4.00% glycine **(Tables 5.4 and 5.5)**. The diets were isocaloric (3,200 Kcal ME/kg), contained 1.20% lysine with other essential amino acid levels formulated to be within 10 per cent of ideal protein ratio. The diets met or exceeded the NRC nutrient requirements for broiler chickens for all other nutrients (NRC, 1994).

In all the three experiments, the experimental diets did not contain antibiotics or coccidiostats and were not pelleted. An indigestible marker, Celite (Celite Corporation, Lompoc, California, USA) was mixed in each diet in all the three experiments at a concentration of 2%. Throughout the experimental period in each experiment, birds were provided continous lighting, and were fed and watered *ad libitum*. Feed intakes and individual bird weights were recorded on day 14, 21 and 28 of the experiment. Amino acid analysis of the different diets was performed by Degussa Corporation (Degussa Canada Inc., Burlington, ON).

5.3.2. *Clostridium perfringens* Challenge

An avian *C. perfringens* field strain isolated from a clinical case of NE was obtained from Dr. Manuel Chirino, College of Veterinary Medicine, University of Saskatchewan and characterized by PCR technique as a type A toxin producer. The organism was cultured anaerobically on BBL^{TM} Blood Agar Base (Becton, Dickinson and Co., Sparks, MD, USA) containing 5.0% sheep blood and 100 mg/L neomycin sulfate (The Upjohn Company, Orangeville, ON, Canada) for 18 h at 37°C, then aseptically inoculated into cooked meat medium (Difco Labs, Detroit, MI, USA) and incubated anaerobically for 8 h at 37°C. All birds were orally challenged in the crop with 0.5 mL on day 1 and 1.0 mL on days 14 to 21 inclusive with this actively growing culture of *C. perfringens* using a 12.0 mL syringe equipped with vinyl tubing (I.D. 0.97mm, O.D. 1.27mm). Bacterial counts were performed on the culture daily prior to inoculation and the numbers ranged from 3.95×10^6 to 4.64×10^6 CFU/mL.

Table 5.1. Composition of starter diet used in each of the three experiments from 0-14 days of age[1].

Ingredients	Inclusion (%)
Soybean meal	40.90
Corn	39.50
Wheat	10.00
Tallow	4.52
Dicalcium phosphate	1.53
Calcium carbonate	1.29
Canola oil	1.00
DL-Methionine	0.26
Choline chloride	0.10
Vitamin/Mineral premix[2]	0.50
Salinomycin sodium[3]	0.06
Bacitracin[4]	0.05
Bio-cox 120[5]	0.05

[1] Diet was formulated to contain 31.5% crude protein and meet NRC requirements for broiler chickens.

[2] Supplied per kilogram of diet: vitamin A, 3.3 mg; cholecalciferol, 55 μg**;** vitamin E, 30 mg; vitamin K, 0.5 mg; vitamin B12, 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; sodium, 511 mg; iron, 80 mg; maganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

[3] Phibro Animal Health Ltd.

[4] Alpharma Canada Corp.

[5] Anticoccodial Premix, OzBioPharma Ltd.

Table 5.2. Ingredient composition of experimental diets used in experiments 1 and 2 (% as is basis).

	Experimental diets			
Ingredients	0.75% Gly	1.58% Gly	3.04% Gly	4.21% Gly
Gelatin	0.00	5.54	14.09	20.10
Corn gluten meal	0.17	0.00	0.00	2.34
Soy protein concentrate	13.59	6.24	0.00	0.00
Barley	80.37	77.08	73.20	63.82
Canola oil	1.00	4.03	4.50	4.22
Limestone	1.67	2.32	2.27	2.26
Dicalcium phosphate	1.63	2.82	2.93	2.95
L-threonine	0.21	0.29	0.38	0.56
L-tryptophan	0.01	0.03	0.07	0.19
DL-methionine	0.30	0.33	0.49	0.70
L-lysine	0.26	0.46	0.48	0.67
L-valine	0.07	0.05	0.11	0.45
L-isoleucine	0.12	0.16	0.44	0.62
L-leucine	0.00	0.05	0.44	0.52
Choline choride	0.10	0.10	0.10	0.10
Vit./Min. premix[1]	0.50	0.50	0.50	0.50

[1] Supplied per kilogram of diet: vitamin A, 11,000 IU; cholecalciferol, 2,200 IU**;** vitamin E, 30 IU; vitamin K, 0.5 mg; vitamin B_{12}, 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; pantothenic acid 0.02 mg; chloride, 788 mg; sodium, 511 mg; iron, 80 mg; manganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

Table 5.3. Crude protein and amino acid composition (analyzed) of experimental diets for experiments 1 and 2 (% as is basis).

	Experiment 1				Experiment 2			
	0.75% Gly	1.58% Gly	3.04% Gly	4.21% Gly	0.75% Gly	1.58% Gly	3.04% Gly	4.21% Gly
Crude protein (%)	19.49	19.88	24.42	32.54	19.45	20.23	24.31	32.91
Essential amino acids								
Arginine	1.12	1.20	1.52	2.00	1.13	1.24	1.45	2.06
Histidine	0.45	0.36	0.32	0.37	0.44	0.36	0.31	0.37
Isoleucine	0.85	0.71	0.90	1.13	0.85	0.73	0.83	1.14
Leucine	1.36	1.15	1.40	1.79	1.34	1.16	1.37	1.82
Lysine	1.18	1.16	1.22	1.52	1.15	1.26	1.21	1.55
Methionine	0.54	0.57	0.74	0.98	0.56	0.61	0.70	0.98
Meth. + cystine	0.86	0.83	0.94	1.18	0.89	0.86	0.90	1.18
Phenylalanine	0.92	0.78	0.72	0.86	0.89	0.76	0.69	0.85
Threonine	0.87	0.87	0.92	1.15	0.89	0.89	0.88	1.12
Valine	0.97	0.82	0.86	1.29	0.95	0.82	0.85	1.31
Non essential amino acids								
Alanine	0.79	1.07	1.58	2.21	0.80	1.11	1.62	2.31
Aspartate	1.55	1.35	1.33	1.70	1.64	1.42	1.38	1.77
Cysteine	0.32	0.25	0.20	0.20	0.33	0.26	0.21	0.21
Glycine	0.78	1.87	3.48	4.87	0.76	1.95	3.45	5.07
Glutamate	3.80	3.44	3.45	4.07	3.61	3.31	3.30	4.02
Serine	0.83	0.79	0.83	1.07	0.86	0.81	0.85	1.10

Table 5.4. Ingredient composition of experimental diets used in experiment 3 (% as is basis).

Ingredients	Experimental diets					
	0.50% Gly	0.75% Gly	1.00% Gly	1.50% Gly	2.00% Gly	4.00% Gly
Gelatin	0.00	0.00	1.67	3.34	5.01	14.09
Casein	9.43	0.00	0.00	0.00	0.00	0.00
Corn gluten meal	0.00	0.17	0.12	0.07	0.02	0.00
SPC	0.00	13.59	11.38	9.16	6.95	0.00
Barley	77.92	80.37	79.37	78.38	77.37	73.20
Canola oil	3.91	1.00	1.91	2.83	3.74	4.50
Limestone	2.33	1.67	1.87	2.06	2.26	2.27
Dical. phosphate	2.55	1.63	1.99	2.35	2.71	2.93
L-threonine	0.46	0.21	0.23	0.26	0.28	0.38
L-tryptophan	0.06	0.01	0.02	0.02	0.03	0.07
DL-methionine	0.50	0.30	0.31	0.32	0.33	0.49
L-lysine	0.54	0.26	0.32	0.38	0.44	0.48
L-valine	0.23	0.07	0.06	0.06	0.05	0.11
L-arginine	0.93	0.00	0.00	0.00	0.00	0.00
L-isoleucine	0.31	0.12	0.13	0.14	0.16	0.44
L-leucine	0.23	0.00	0.02	0.03	0.05	0.44
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10
Vit./Min. premix[1]	0.50	0.50	0.50	0.50	0.50	0.50

[1] Supplied per kilogram of diet: vitamin A, 11,000 IU; cholecalciferol, 2,200 IU**;** vitamin E, 30 IU; vitamin K, 0.5 mg; vitamin B_{12}, 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; pantothenic acid 0.02 mg; chloride, 788 mg; sodium, 511 mg; iron, 80 mg; manganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

SPC = Soy protein concentrate

Table 5.5. Crude protein and amino acid composition (analyzed) of experimental diets for experiment 3 (% as is basis)

	Experimental diets					
	0.50% Gly	0.75% Gly	1.00% Gly	1.50% Gly	2.00% Gly	4.00% Gly
Crude protein (%)	23.70	22.07	22.41	22.78	23.94	29.43
Essential amino Acids						
Arginine	1.74	1.30	1.32	1.35	1.45	1.54
Histidine	0.51	0.49	0.46	0.43	0.41	0.35
Isoleucine	1.25	0.98	0.93	0.89	0.84	1.02
Leucine	1.89	1.53	1.47	1.40	1.35	1.64
Lysine	1.60	1.22	1.19	1.27	1.28	1.29
Methionine	1.02	0.68	0.64	0.65	0.67	0.91
Methionine+cystine	1.26	1.03	0.96	0.95	0.95	1.12
Phenylalanine	1.05	1.08	1.05	0.99	0.94	0.88
Threonine	1.26	0.97	0.97	0.95	0.97	0.99
Valine	1.44	1.10	1.06	1.00	0.97	1.01
Non essential amino acids						
Alanine	0.71	0.87	0.96	1.05	1.11	1.44
Aspartate	1.32	1.65	1.67	1.59	1.57	1.51
Cysteine	0.24	0.35	0.32	0.31	0.28	0.21
Glycine	0.58	0.84	1.20	1.59	2.14	4.35
Glutamate	4.52	4.39	4.34	4.19	4.06	4.07
Serine	0.98	0.93	0.93	0.91	0.92	0.96

5.3.3. Pathological Examination

Birds were kept under constant observation for any signs of NE and all birds that died during the course of experiments were necropsied. On day 28, the surviving chickens were euthanized by cervical dislocation, weighed and necropsied immediately. Intestinal tracts were removed and intestinal lesions were scored blindly according to the method of Truscott and Al-Sheikhly (1977) with slight modifications on a scale 0 to 4 where 0 was apparently normal, no lesion; 0.5, severely congested serosa and mesentery engorged with blood; 1, thin walled and friable intestines with small red petechiae (> 5); 2, focal necrotic lesions; 3, patches of necrosis (1-2 cm long); and 4, diffused necrosis typical of field cases. Following postmortem examination, if the score was ≥1 then a 1.5-2.0 cm long piece of intestinal tissue with the gross NE lesion was collected in phosphate-buffered formaldehyde solution and processed routinely for paraffin embedding, sectioned at approximately 5 µm, and stained with hematoxylin and eosin.

5.3.4. Bacteriological Examination

In experiment 1 and 2, the fresh intestinal contents were collected aseptically from jejunum (distal duodenal loop to 1 cm proximal to Meckel's diverticulum), ileum (Meckel's diverticulum to 1 cm proximal to ileocecal junction) and paired ceca into sterilized plastic dram vials and mixed well. In experiment 3 samples were not collected from the jejunum. Using a sterile spatula, pea-sized samples were transferred into pre-weighed 15 mL sterile plastic tubes containing 1 mL 0.1% sterile peptone buffer with 5 g/L cysteine hydrochloride (Sigma Chemical Co., St. Louis MO.). The ceca samples were directly collected into the pre-weighed 15 mL sterile tubes. The samples were immediately placed and kept on ice until plated within 3 h of collection. The samples

were weighed and diluted in peptone water to an initial 10^{-1} dilution. Ten-fold dilutions were spread in duplicate using an automated spiral plater (Autoplate, Spiral Biotech Inc., Bethesda MD, USA) on BBL™ Blood Agar Base (Becton, Dickinson and Co., Sparks, MD, USA) containing 50 mL/L sheep blood and 0.01% neomycin sulfate (The Upjohn Company, Orangeville, ON, Canada) for the enumeration of *C. perfringens* and MRS agar (Becton, Dickinson and Co., Sparks, MD, USA) for the enumeration of lactobacilli. The blood agar/neomycin plates were incubated anaerobically for 24 h at 37°C while MRS agar plates were incubated anaerobically for 48 h at 37°C. The *α*- and *β*- hemolytic colonies on blood agar/neomycin plates were counted as *C. perfringens* with presumptive colonies being randomly picked, gram stained, plated on Mannitol Yolk Polymixin agar (Oxoid Inc., Napean, ON, Canada) and examined microscopically to confirm them as *C. perfringens.* Bacterial populations were expressed as Log_{10} CFU/g of intestinal contents.

5.3.5. Samples Collection and Processing for Alpha Toxin Estimation

The concentrations of α toxin were estimated in ileal contents in experiments 2 and 3. About 1.0 g of fresh ileal content from each bird was collected into 2.0 mL sterile Eppendorf tubes (Eppendorf AG, Hamburg, Germany), and immediately stored at -80° C. The α toxin activity was estimated in ileal digesta using Amplex Red Phosphatidylcholine-Specific Phospholipase C Assay Kit (Molecular Probes; Cedarlane Lab. Ltd., ON, Canada) according to the manufacture's instructions. Briefly, 100µl of 1X reaction buffer diluted sample was used in duplicate for each reaction into separate wells of a fluorescence multiwell plate. 100µl of 10µM H_2O_2 solution was used as positive control while 1X reaction buffer without PC-PLC served as negative control. Standard was prepared by diluting the 10 U/mL PC-PLC stock solutions into 1X reaction buffer to

produce a 0.1 U/mL PC-PLC solution and then two fold serial dilutions were prepared for the calibration curve. 100µl of working buffer containing Amplex Red reagent/HRP/alkaline phosphatase/choline oxidase/lecithin was added to each well containing the samples, controls and standard. Plate was incubated for 1 h at 37°C protected from light and then fluorescence was measured with a fluorescence mircoplate reader (Fluoroskan Ascent FL 2.5) using excitation at 544 nm and emission detection at 612 nm.

5.3.6. Samples for Amino Acids Digestibility Determination

In each of the three experiments, the remainder of the ileal contents of 4 birds from each pen were pooled, lyophilized and analyzed for amino acid content (Degussa Canada Inc., Burlington, ON). The acid insoluble ash in experimental diets and freeze dried ileal digesta samples was determined using method of MaCarthy *et al.* (1974). Digestibilities of various amino acids were calculated using the formula:

$$\frac{\text{(Amino acid/acid insoluble ash) diet - (Amino acid/acid insoluble ash) digesta}}{\text{(Amino acid/acid insoluble ash) diet}}$$

5.3.7. Statistical Analysis

Data were analyzed using General Linear Model procedure of SPSS (Version 12.0, SPSS Inc, Chicago IL, USA). Treatment means were compared using the Ryan-Einot-Gabriel-Welsch multiple F test. The linear and quadratic effects of dietary glycine on growth parameters and bacterial populations were determined using regression analysis. Comparisons were considered significant when $P < 0.05$.

5.4. Results

The amino acid composition of the experimental diets is shown in **Tables 5.3** (experiments 1 and 2) and **5.5** (experiment 3) while protein and amino acid analyses of ileal digesta in each of the three experiments are depicted in **Tables 5.6** (experiments 1 and 2) and **5.7** (experiment 3). The ileal digestibilities of various amino acids have been presented in **Tables 5.8 and 5.9.** Approximately 70-80 per cent of the birds were dull, depressed and diarrheic for first 5-10 days post-challenge (i.e. after day 14) in each of the three experiments. Huddling, ruffled feathers and apathy were obvious. NE-specific deaths occurred in all the three experiments with 8.33, 6.25 and 4.16% mortality in experiments 1, 2 and 3, respectively. Together in experiments 1 and 2, three birds died in the groups fed the 3.04% glycine while two birds fed 1.58% glycine and two birds fed 4.21% glycine diets also died. There was no mortality in the 0.75% glycine group in either of the experiments. In experiment 3, two birds died in the group that received 4.00% glycine diet, while one bird died each from group fed 0.75 and 2.00% glycine diets. Most of these birds that died during the course of experiment had marked distension of jejunum and ileum, and the lumen was filled with gas and foul-smelling dark brown fluid content. Some birds had small necrotic ulcers (0.5-1.5 cm long), mainly in the proximal ileum, though not as pronounced as in typical field cases. At places, petechial hemorrhages were also noticed chiefly in the duodenum and proximal jejunum. No detectable gross lesions were observed in any other organ. Surviving birds had no apparent signs of morbidity 7 to 10 days post-challenge.

The experimental diets were readily accepted in each of the three experiments and there were no significant differences in feed intake except on day 21 to 28 in experiment

3 where feed intake was significantly higher in chickens receiving diets with 1.00, 1.50 and 4.00% glycine compared to rest of the diets **(Table 5.10)**. The linear and quadratic effects of glycine on feed intake were significant during days 14-21 in experiments 1 and 2 and during days 21-28 in experiment 3. In experiments 1 and 2, a significant decline in the average daily gain (ADG) was observed in birds fed 3.04 and 4.21% glycine diets compared to those with 0.75% glycine diets both on day 14-21 and day 21-28. The linear and quadratic effects of glycine on average daily gain were significant in both time periods in experiments 1 and 2 and the quadratic effect was significant during days 14-21 in experiment 3. There were no significant differences in the feed:gain ratio in experiments 1 and 2, however there was a significant linear effect of glycine on feed:gain ratios in both periods for experiment 1. In experiment 3 during the day 14-21 and 21-28 periods, a significantly higher feed:gain ratio was observed in birds on the 4.00% glycine diets compared to those receiving the other 5 diets. The linear and quadratic effects of glycine on feed:gain ratio were significant for both periods during experiment 3.

Bacterial populations in the digestive tracts of chickens on day 28 of the three experiments are presented in **Table 5.11.** The number of lactobacilli and *C. perfringens* increased from proximal part to the distal part of the intestine with highest counts measured in the ceca. No *C. perfringens* were enumerated in the jejunum and ileum from birds in experiment 1 because the numbers were below the detection limit of the assay. In the jejunum, no significant difference was noticed among various treatment groups in lactobacilli counts in experiment 1, and both lactobacilli and *C. perfringens* populations in each of the first two experiments so lactobacilli and *C. perfringens* were not enumerated in the jejunum in experiment 3.

In each of the three experiments, there was a decrease in population of lactobacilli with increase in the dietary glycine concentration, and this effect was more pronounced in the cecum than in the ileum. A significant decline in the lactobacilli populations was observed in ileum of chickens fed 3.04% dietary glycine compared to the diet with 1.58% glycine in experiment 1 while in experiment 3, the numbers were significantly less in chickens which were provided with 4.00% dietary glycine than in those with other levels of dietary glycine tested **(Table 5.11)**. In experiment 2, no statistically significant difference was observed in the lactobacilli populations in the ileum although there was decrease in the numbers ranging from 7.22 to 6.84 $\log_{10}$ CFU/g digesta with a corresponding increase in dietary glycine level from 0.75 to 4.21%.

The effect of dietary glycine concentration on lactobacilli populations in cecum of 28-day-old broiler chickens was statistically significant in experiment 1 and 2. There was a significant decrease in the lactobacilli counts in cecum between chickens that received 4.21% glycine and those that received 0.75 or 1.58% glycine diets. No difference was detected among various treatments groups with respect to lactobacilli populations in cecum in experiment 3 except in birds which received diets with 4.00% glycine where the numbers declined significantly ($P < 0.05$) compared to other experimental groups.

Mean *C. perfringens* counts in the jejunum were not influenced by the level of dietary glycine in experiment 2 **(Table 5.11)**. Significantly higher *C. perfringens* numbers were observed in the ileum of day-28-old birds fed 1.58, 3.04 or 4.21% dietary glycine compared with those given 0.75% glycine in experiment 2. In experiment 3, *C. perfringens* counts in ileum increased from 3.14 to 4.57 $\log_{10}$ CFU/g of intestinal

contents as dietary glycine concentration increased from 0.50 to 2.00% but the counts decreased again with 4.00% glycine.

Glycine supplementation resulted in a significant quadratic response for *C. perfringens* growth in cecum of 28-day-old broiler chickens with maximum response at 3.30, 3.89 and 3.51% dietary glycine concentration with r^2 values of 0.95, 0.99 and 0.95 in experiments 1, 2 and 3, respectively **(Figure 5.1)**. Compared with 0.75 and 1.58% glycine-fed chickens the population of *C. perfringens* was significantly greater in the ceca of 3.04% glycine-fed chickens both in experiment 1 and 2, however, no difference was noticed in birds that received either 1.58 or 4.21% glycine diets. In experiment 3, cecal *C. perfringens* counts were significantly greater in the birds fed 1.50, 2.00 and 4.00% glycine diets compared to those fed 0.50, 0.75 or 1.00% glycine diets and there was an almost 2 log difference in cecal *C. perfringens* populations when dietary glycine concentrations were increased from 0.50 to 2.00%.

Table 5.6. Protein and amino acid content (%) of ileal digesta in experiments 1 and 2.

	Experiment 1				Experiment 2			
	0.75% Gly	1.58% Gly	3.04% Gly	4.21% Gly	0.75% Gly	1.58% Gly	3.04% Gly	4.21% Gly
CP (%)	9.86	10.68	13.60	18.01	9.21	9.98	11.94	14.91
Essential amino acids								
Arginine	0.43	0.43	0.45	0.53	0.37	0.36	0.38	0.42
Histidine	0.19	0.18	0.17	0.22	0.18	0.16	0.16	0.18
Isoleucine	0.33	0.29	0.25	0.29	0.28	0.24	0.20	0.21
Leucine	0.57	0.49	0.45	0.54	0.47	0.40	0.35	0.39
Lysine	0.37	0.36	0.37	0.35	0.33	0.31	0.31	0.34
Methionine	0.13	0.12	0.12	0.16	0.10	0.10	0.10	0.11
Meth. + Cyst.	0.35	0.31	0.29	0.35	0.32	0.29	0.26	0.29
Phenylalanine	0.60	0.52	0.51	0.56	0.36	0.33	0.28	0.29
Threonine	0.47	0.42	0.42	0.51	0.43	0.39	0.36	0.40
Valine	0.44	0.39	0.37	0.43	0.38	0.34	0.31	0.33
Non essential amino acids								
Alanine	0.45	0.48	0.62	0.82	0.39	0.40	0.50	0.59
Aspartate	0.87	0.88	1.09	1.49	0.85	0.89	0.97	1.26
Cysteine	0.23	0.19	0.17	0.19	0.21	0.19	0.16	0.17
Glycine	0.51	0.91	2.00	2.95	0.47	0.87	1.64	2.36
Glutamate	1.44	1.39	1.56	1.96	1.18	1.15	1.28	1.46
Proline	0.61	0.83	1.54	2.36	0.56	0.88	1.41	2.13
Serine	0.46	0.44	0.54	0.70	0.42	0.43	0.46	0.57

Gly = Dietary glycine; CP = Crude protein

Table 5.7. Protein and amino acid content (%) of ileal digesta in experiment 3.

	Experimental diets					
	0.50% Gly	0.75% Gly	1.00% Gly	1.50% Gly	2.00% Gly	4.00% Gly
CP (%)	10.02	10.75	11.00	10.99	11.32	14.40
Essential amino acids						
Arginine	0.43	0.50	0.51	0.50	0.48	0.61
Histidine	0.19	0.22	0.23	0.21	0.20	0.24
Isoleucine	0.37	0.34	0.33	0.31	0.30	0.34
Leucine	0.51	0.58	0.57	0.55	0.53	0.62
Lysine	0.38	0.42	0.42	0.41	0.40	0.42
Methionine	0.14	0.12	0.12	0.12	0.11	0.15
Meth. + Cys.	0.33	0.37	0.35	0.34	0.31	0.34
Phenylalanine	0.60	0.68	0.64	0.64	0.61	0.68
Threonine	0.49	0.52	0.50	0.48	0.46	0.51
Valine	0.47	0.46	0.46	0.44	0.42	0.50
Non essential amino acids						
Alanine	0.42	0.45	0.48	0.48	0.47	0.65
Aspartate	0.74	1.19	1.14	1.12	1.09	1.22
Cysteine	0.19	0.26	0.23	0.22	0.20	0.19
Glycine	0.49	0.56	0.67	0.76	0.87	1.69
Glutamate	1.63	1.56	1.66	1.57	1.46	1.82
Proline	0.73	0.70	0.78	0.82	0.91	1.13
Serine	0.57	0.51	0.49	0.49	0.48	0.60

Gly = Dietary glycine; CP = Crude protein

Table 5.8. Ileal digestibilities (%) of various amino acids in experiments 1 and 2[1].

	Experiment 1				Experiment 2			
	0.75% Gly	1.58% Gly	3.04% Gly	4.21% Gly	0.75% Gly	1.58% Gly	3.04% Gly	4.21% Gly
Essential amino acids								
Arginine	78.47	81.90	87.07	89.25	85.41	86.96	87.10	91.25
Histidine	76.10	74.75	77.42	76.04	82.10	79.62	75.12	78.95
Isoleucine	78.18	79.61	87.80	89.78	85.49	85.43	88.34	92.03
Leucine	76.55	78.63	86.07	87.77	84.26	84.51	87.42	90.65
Lysine	82.71	84.47	86.73	88.16	87.34	89.06	87.25	90.60
Methionine	86.95	89.37	92.77	93.56	91.78	92.88	92.97	94.99
Meth. + Cyst.	77.36	81.14	86.63	88.16	83.97	84.85	85.60	89.49
Phenylalanine	63.51	66.12	69.49	73.91	82.14	80.69	80.26	85.23
Threonine	79.73	75.62	80.21	82.13	78.47	80.14	80.05	84.67
Valine	74.57	75.98	81.35	86.46	82.17	81.19	82.05	88.99
Non essential amino acids								
Alanine	68.54	77.50	82.90	85.05	78.46	83.67	84.81	88.88
Aspartate	68.77	67.21	64.36	64.60	76.99	71.73	65.28	69.19
Cysteine	60.59	61.62	63.87	61.71	71.19	67.17	62.49	64.28
Glycine	63.39	75.43	75.09	75.59	72.63	79.88	76.65	79.83
Glutamate	78.92	79.55	80.44	80.56	85.47	84.44	80.96	84.32
Serine	69.17	71.66	71.97	73.63	78.23	75.96	73.55	77.70

[1] Ileal digestibilities were calculated using the formula:

$$\frac{\text{(Amino acid/acid insoluble ash) diet - (Amino acid/acid insoluble ash) digesta}}{\text{(Amino acid/acid insoluble ash) diet}}$$

Table 5.9. Ileal digestibilities (%) of various amino acids in experiment 3[1].

	Experimental diets					
	0.50% Gly	0.75% Gly	1.00% Gly	1.50% Gly	2.00% Gly	4.00% Gly
Essential amino acids						
Arginine	89.00	82.88	81.03	83.15	86.42	86.02
Histidine	83.30	80.84	75.74	77.40	79.74	71.48
Isoleucine	86.99	85.69	82.27	83.94	85.22	86.08
Leucine	88.00	83.86	80.69	82.04	83.82	84.05
Lysine	89.63	84.89	82.71	85.24	88.18	85.33
Methionine	93.93	92.33	91.09	91.56	93.11	93.20
Meth + Cys.	88.50	85.93	81.92	83.64	86.50	87.20
Phenylalanine	74.71	73.81	69.77	70.32	73.38	67.57
Threonine	82.88	78.25	74.69	76.90	80.75	78.41
Valine	85.63	82.30	78.48	80.00	82.24	79.26
Non essential amino acids						
Alanine	73.82	74.95	75.46	79.21	84.06	85.18
Aspartate	85.92	71.25	66.07	67.86	71.65	66.02
Cysteine	64.51	73.84	63.97	67.55	71.06	61.17
Glycine	62.42	62.12	72.41	78.29	83.37	83.65
Glutamate	92.76	84.55	81.06	82.89	85.22	81.14
Serine	74.15	76.20	74.14	75.63	78.71	73.79

[1] Ileal digestibilities were calculated using the formula:

$$\frac{\text{(Amino acid/acid insoluble ash) diet - (Amino acid/acid insoluble ash) digesta}}{\text{(Amino acid/acid insoluble ash) diet}}$$

Table 5.10. Average daily feed intake (ADFI, g/day), average daily gain (ADG, g/day) and feed:gain ratio (FC, feed intake/ weight gain, g/g) of birds during days 14-21 and 21-28 of the three experiments.

	Days 14-21			Days 21-28		
Glycine (%)	ADFI	ADG	FC	ADFI	ADG	FC
Experiment 1						
0.75	193.9	54.6[a]	3.6	357.9	81.6[a]	4.4
1.58	187.2	53.9[a]	3.5	333.2	79.4[a]	4.2
3.04	166.6	33.8[b]	5.0	352.7	64.5[b]	5.5
4.21	164.1	30.9[b]	5.7	345.8	62.5[b]	5.5
Pooled SEM	5.04	3.97	0.38	9.07	2.69	0.25
Linear	0.006	0.003	0.015	0.845	0.000	0.030
Quadratic	0.029	0.017	0.050	0.888	0.001	0.105
Experiment 2						
0.75	220.5	81.8[a]	2.7	264.5	87.9[a]	3.0
1.58	235.5	74.4[ab]	3.2	248.6	71.8[ab]	3.5
3.04	193.4	49.9[bc]	4.7	223.6	59.3[bc]	3.8
4.21	185.7	42.7[c]	4.4	224.6	53.2[c]	4.4
Pooled SEM	7.84	5.92	0.40	10.88	4.39	0.29
Linear	0.028	0.002	0.062	0.144	0.000	0.084
Quadratic	0.071	0.009	0.170	0.338	0.000	0.242
Experiment 3						
0.50	182.2	74.3[a]	2.5[a]	241.3[a]	66.7[ab]	3.6[a]
0.75	177.4	68.3[a]	2.6[a]	247.3[a]	63.9[a]	3.9[a]
1.00	174.7	74.4[a]	2.4[a]	303.6[b]	81.3[c]	3.7[a]
1.50	182.5	73.3[a]	2.5[a]	313.0[b]	80.0[bc]	3.9[a]
2.00	188.4	74.4[a]	2.5[a]	243.6[a]	61.3[a]	3.9[a]
4.00	177.9	51.4[b]	3.6[b]	321.9[b]	60.6[a]	5.4[b]
Pooled SEM	2.18	2.36	0.11	8.73	2.21	0.16
Linear	0.665	0.077	0.036	0.000	0.326	0.010
Quadratic	0.906	0.026	0.006	0.000	0.156	0.006

[abc] Means with the different superscripts within a column (within a experiment) differ significantly ($P < 0.05$)

Table 5.11. Mean *Lactobacilli* and *C. perfringens* populations[1] in jejunum, ileum and cecum of broiler chickens on day 28 of the experiments.

	Lactobacilli[2]			*C. perfringens*[2]		
Glycine (%)	Jejunum	Ileum	Cecum	Jejunum	Ileum	Cecum
Experiment 1						
0.75	6.57	7.13^{ab}	9.36^{a}	BD	BD	4.88^{c}
1.58	6.75	7.30^{a}	9.33^{ab}	BD	BD	5.87^{bc}
3.04	6.43	6.90^{b}	8.99^{bc}	BD	BD	6.91^{a}
4.21	6.46	6.92^{ab}	8.77^{c}	BD	BD	5.91^{ab}
Pooled SEM	0.05	0.05	0.06			0.18
Experiment 2						
0.75	6.35	7.22	9.21^{a}	2.54	3.39^{b}	5.83^{c}
1.58	6.74	7.07	8.93^{ab}	3.73	4.35^{a}	7.35^{b}
3.04	6.24	6.95	8.71^{b}	3.37	3.99^{a}	8.11^{a}
4.21	6.36	6.84	8.31^{c}	3.57	4.28^{a}	7.89^{ab}
Pooled SEM	0.07	0.07	0.06	0.18	0.11	0.16
Experiment 3						
0.50	ND	7.64^{a}	9.09^{a}	ND	3.14^{e}	5.27^{c}
0.75	ND	7.45^{a}	9.01^{a}	ND	3.30^{de}	5.82^{bc}
1.00	ND	7.67^{a}	9.01^{a}	ND	3.55^{cd}	6.10^{b}
1.50	ND	7.56^{a}	8.81^{a}	ND	3.79^{bc}	7.23^{a}
2.00	ND	7.49^{a}	8.75^{a}	ND	4.57^{a}	7.23^{a}
4.00	ND	7.28^{b}	8.33^{b}	ND	4.12^{b}	7.01^{a}
Pooled SEM		0.07	0.05		0.06	0.12

[1]Means are Log_{10} CFU/g of intestinal contents.

[2]Means with the different superscripts within a column (within an experiment) differ significantly ($P < 0.05$)

BD: Below detection; ND: Not determined

The mean NE lesion scores of chickens fed different experimental diets and killed on day 28 are presented in **Figure 5.2.** In a majority of birds, the intestinal serosa was congested and mesentery engorged with blood. Focal necrotic lesions were observed in various intestinal regions, including the cecal tonsils **(Appendix 9.3A)**. In experiments 1 and 2, there was a significant elevation ($P < 0.05$) in the intestinal lesion score in birds that received 3.04% glycine diets compared to those receiving the 0.75% glycine diet. Intestinal lesion scores were less in chickens receiving 4.21% glycine than those receiving 3.21% glycine diets. In experiment 3, significantly higher ($P < 0.05$) intestinal lesion scores were observed in birds receiving 4.00% glycine diet compared to rest of the diets. Chickens that consumed 0.50% glycine diet exhibited the lowest lesion score and the mean intestinal lesion score of this treatment group was significantly different ($P < 0.05$) from scores of chickens that consumed any of the five diets. No significant difference in the lesion score was observed with increase in dietary glycine concentration from 0.75 to 2.00% in experiment 3.

Histological examination of fixed intestinal tissues from 28-day-old broiler chickens euthanized in each of the three experiments revealed lesions of NE at various stages. The intestinal lesions varied from slight edema and congestion in lamina propria **(Appendix 9.3B);** mild necrosis to desquamation of epithelial cells at the tips of villi. In some cases, especially in terminal jejunum and proximal ileum, the mucosa was devoid of villi completely and was covered with a homogenous diphtheritic membrane comprising of debris of necrotic epithelial cells, mucous membrane and fibrin. Interestingly, there were very few areas of polymorphonuclear cells (PMN) infiltration in the lamina propria. There was no evidence of coccidial oocysts in any sections examined.

The concentration of α toxin in the ileal contents of the birds from experiments 2 and 3 is presented in **Table 5.12.** The α toxin concentration was significantly higher in chickens given 1.58% glycine diets compared to rest of the diets in experiment 2 while no significant difference was observed among various treatment groups in experiment 3.

5.5. Discussion

Glycine is an essential amino acid in growing broiler chickens and Corzo *et al.* (2004) determined that chicks optimized body weight gain and feed conversion at 0.98% and 1.02% dietary glycine, respectively. In the current study, there was a significant decline in growth performance of birds with an increase in the dietary glycine content above the requirement level. Although the essential amino acid content of the diets was balanced for ideal protein, the increasing levels of glycine would have resulted in an imbalance of some nonessential amino acids and this may have been responsible for the decrease in growth performance. However, it is also possible that the effect of dietary glycine was indirect and mediated through the increase in the number of intestinal *C. perfringens.* As evidenced from the intestinal lesion scores and high numbers of *C. perfringens* in ileum and cecum, most of these birds had a sub-clinical form of NE

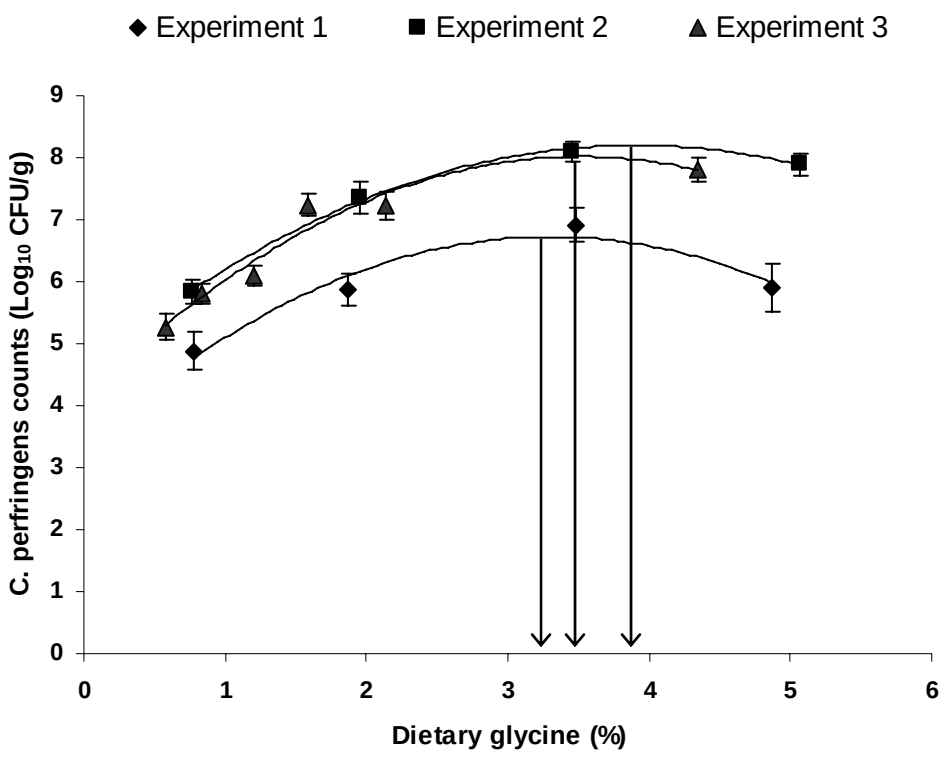


Figure 5.1. Relationship between dietary glycine concentration and *C. perfringens* populations in the ceca of 28-day-old broiler chickens in all the three experiments. Maximum numbers of *C. perfringens* were observed at 3.30, 3.89 and 3.51% dietary glycine concentration with r^2 values of 0.95, 0.99 and 0.95 in experiments 1, 2 and 3, respectively.

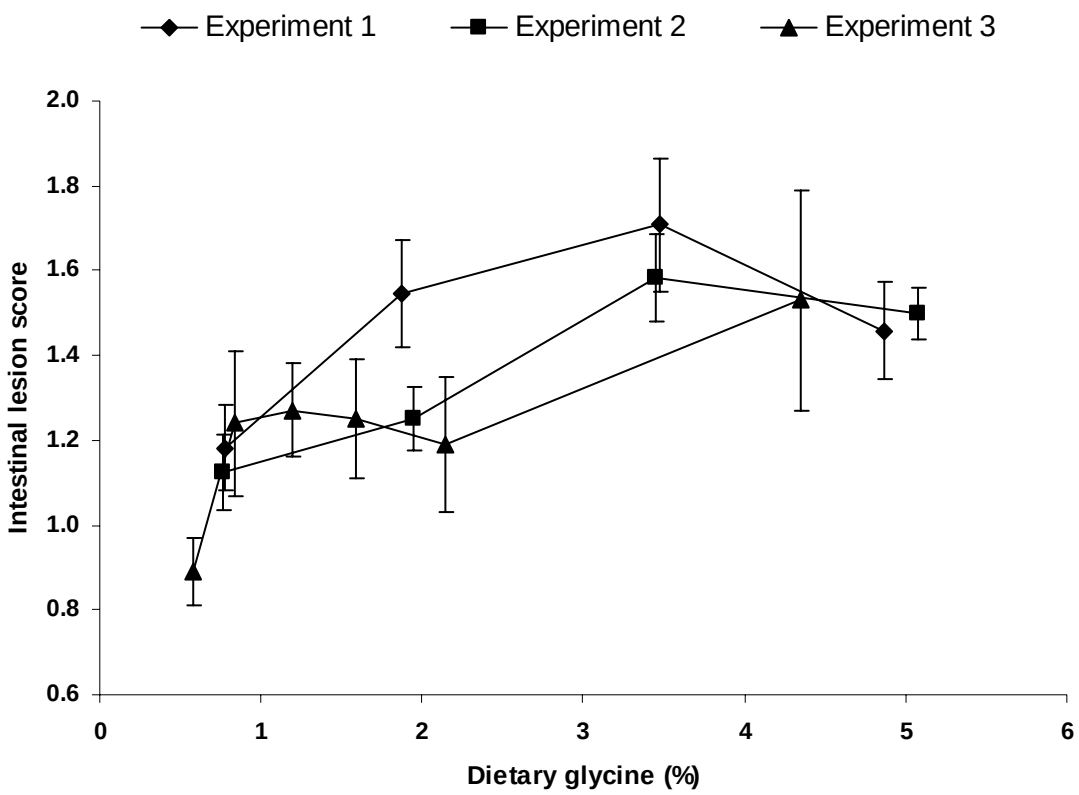


Figure 5.2. Mean necrotic enteritis lesion score in 28-day-old broiler chickens given experimental diets (day 14-28) containing different levels of glycine.

Table 5.12. Mean phospholipase C (PLC) concentration in the ileal contents of 28-day-old broiler chickens fed different experimental diets.

Glycine (%)	PLC concentration (mU/g of ileal digesta)
Experiment 2	
0.75	13971[b]
1.58	16321[a]
3.04	14076[b]
4.21	12738[b]
Pooled SEM	333.25
Experiment 3	
0.50	10643
0.75	10631
1.00	10656
1.50	10766
2.00	10692
4.00	10652
Pooled SEM	14.91

[ab] Means with the different superscripts within a column (within a experiment) differ significantly ($P < 0.05$)

as described by Lovland and Kaldhusdal (2001). Kaldhusdal and Hofshagen (1992) and Stutz *et al.* (1983) also reported a decreased growth rate and increased feed conversion rate in broilers having high number of *C. perfringens* in the gut. Increased populations of *C. perfringens* in the gut (as much as log 4.57 in ileum and log 8.11 in cecum) were observed even in the clinically healthy birds, which confirms the sub clinical nature of this disease because higher *C. perfringens* populations with grossly visible, focal necrotic lesions in the small intestine is strong indicator of an occurrence of NE (Kaldhusdal and Hofshagen, 1992; Kaldhusdal *et al.,* 1999).

A previous study in broiler chickens examining the effect of different protein sources (animal vs. plants) and dietary amino acid profile on intestinal populations of *C. perfringens* documented a significant correlation ($P < 0.05$) between the level of dietary glycine and the number of *C. perfringens* in both ileum and cecum (Wilkie *et al.,* 2005). They also observed a significant correlation between lysine and glycine content of ileal digesta and *C. perfringens* numbers in both ileum and cecum. However, no other ileal amino acids were significantly correlated with *C. perfringens* populations in ileum or cecum. In the present study, the lysine concentration of ileal digesta was constant (ranging from 0.31 to 0.37% in experiment 1 and 2, and from 0.38 to 0.42% in experiment 3) regardless of the diet and the lysine content of the diets with 0.75 to 3.04% glycine was also almost constant in each of the three experiments. Although gelatin is a rich source of glycine, it also contains high levels of alanine and proline (9.65 and 13.09% in CP). There was an increase in dietary alanine and ileal proline content with the addition of gelatin in the experimental diets, however, our results indicate that unlike glycine, alanine or proline do not enhance *C. perfringens* growth *in vivo* (Drew *et al.,*

2004) or *in vitro* (Wilkie *et al.,* 2005). Drew *et al.* (2004) reported that there was no difference in *C. perfringens* populations in ileum and cecum of broiler chickens that received soy protein concentrate-based diets containing 23.0, 31.5 or 40.0% CP. However, *C. perfringens* numbers increased significantly with fishmeal-based diets. It was concluded that it was not the protein, but the glycine concentration of fishmeal diets that was responsible for *C. perfringens* overgrowth in ileum and cecum of these birds.

In the current study, the number of lactobacilli decreased with increasing dietary glycine concentration, and this effect was clearer in the cecum than ileum. Interestingly, the effect of various dietary treatments on intestinal *C. perfringens* populations was opposite to that on lactobacilli populations. The mode of action by which glycine affects the intestinal populations of these important groups of bacteria in broiler chickens is unclear. Some strict anaerobic bacteria have a unique energy conserving mechanism and they catalyze glycine as substrate using an internal Stickland reaction by which glycine serves as electron donor during oxidation by a glycine cleavage system or as electron acceptor being reduced by glycine reductase. The glycine reductase system is present in several clostridia (*C. sticklandii, C. difficile, C. litorale* and *Eubacterium acidaminophilum*) and catalyzes the reductive deamination of glycine to acetylphosphate and ammonia (Andreesen, 1994; Costilow, 1977). As *C. perfringens* is not a protein fermentor, the effect of higher levels of dietary glycine on intestinal *C. perfringens* populations might be indirect. It is possible that high dietary glycine concentration might cause a reduction in lactic acid producing bacteria (mainly *Lactobacillus, Streptococci*, and *Staphylococci*) which are predominant in the intestines of normal healthy chickens and generally considered to be protective against colonization of pathogenic micro-

organisms. An antagonistic effect of lactobacilli against *Helicobacter pylori* (Aiba *et al.,* 1998), *Salmonella* spp. (Gill *et al.,* 2001) and *E.coli* (Mangell *et al.,* 2002) has been demonstrated in murine model.

It is conceivable that the protein/amino acids are serving as energy and/or nitrogen source for other community members that in turn modify the intestinal environment in a favorable way for *C. perfringens* proliferation. This may occur by providing positive selection pressure on those gastrointestinal microbes which can directly ferment amino acids for carbon and nitrogen. Alternatively, the high glycine diets might affect the host in such a manner that the gastrointestinal milieu is made more favorable for *C. perfringens* proliferation *i.e.* by the induction of an inflammatory response, which in turn leads to leakage of serum proteins into the gut lumen. This leakage of serum protein may ultimately provide the environmental stimulus required for an expansion of *C. perfringens* population, and perhaps even the up regulation of virulence factors (Shane *et al.,* 1985). Titball *et al.* (1999) documented that growth of *C. perfringens* and production of α toxin are influenced by various amino acids. Methinone is highly stimulatory for the growth and sporulation of *C. perfringens* (Muhammed *et al.,* 1975). Glycine accelerated the *C. perfringens* growth (Ispolatovskaya, 1971) while α toxin production required the presence of glycine-containing peptides in defined media (Nakamura *et al.,* 1968; Stevens and Rood, 2000). Drew *et al.* (2004) observed a positive correlation between glycine and methionine content of the diet, and intestinal *C. perfringens* counts in broiler chickens fed high protein diets. While several studies have been conducted to determine the effects of different dietary compositions on intestinal *C. perfringens* populations but we are aware of no study that directly examines the effect of

various concentrations of dietary glycine on *C. perfringens* proliferation and NE production in broiler chickens.

In the present experiments, gross intestinal lesions of varying degrees among various treatment groups were observed although we did not observe the typical field-type lesions of NE. Reason for absence of field-type lesions is unknown; one possible explanation may be that some of the vegetative cells are inactivated by the low pH in the gizzard when given orally. Al-Sheikhly and Truscott (1977a) and Vissiennon *et al.* (2000) were able to detect severe necrotic lesions and induce high mortality by inoculated fresh cultures directly into the duodenum. They suggested that high *C. perfringens* populations and slight intestinal damage were apparently necessary for disease production when a broth culture was used. However, the results of current experiments indicate that even though the aforementioned may be major factors, other factors, including the chemical/physical nature of the diet and the interaction of other intestinal microflora, may contribute to pathogenesis of the disease. On day 28, the mean gross intestinal NE lesion scores were highest in the chickens given the diets with 3.04% glycine in experiment 1 and 2; and 4.00% glycine in experiment 3. Interestingly, *C. perfringens* counts and lesion scores were comparatively less in groups that received 4.21% dietary glycine than those in 3.04% glycine level both in experiment 1 and 2. It might be due to the fact that very high level of dietary glycine (above 3.04%) may modify the intestinal environment in a way which is not favorable for *C. perfringens* proliferation and/or α toxin production.

The histological examination of the affected intestinal tissues indicated that pathological lesions were more or less similar to those of previous investigations of

spontaneous (Helmboldt and Bryant, 1971; Long *et al.,* 1974) as well as experimental (Al-Sheikhly and Truscott, 1977a; Kaldhusdal *et al.,* 1995; Shane *et al.,* 1985) NE, indicating that necrosis starts in apical villous epithelium. Presence of slight edema in lamina propria and detachment of the epithelial layer from the underlying tissues especially from tips of the villi indicate the initial changes in NE. It is of interest that the cellular response was not evident in this study in contrast to some other studies (Kaldhusdal *et al.,* 1995; Shane *et al.,* 1985). However, Bryant *et al.* (1993) and Stevens *et al.* (1997) also demonstrated an absence of PMN cells at the site of *C. perfringens* infection. In the absence of host response (suppression of PMN influx), clostridia proliferates rapidly, leading to local accumulation of toxins. Higher *in situ* concentrations of *C. perfringens* toxins, especially α toxin, further inhibit PMN influx and reach concentration sufficient to cause membrane destruction (Stevens and Rood, 2000). From the current experiments, it is not clear whether the intestinal lesions were produced by vegetative cells or *C. perfringens* toxins.

Lesions can be reproduced by the administration of a crude preparation of α toxin to conventional (Al-Sheikhly and Truscott, 1977c) and germ free chicks (Fukata *et al.,* 1988). In the current study, in spite of a constant increase in the number of *C. perfringens* with increase in dietary glycine content, there was no significant difference in α toxin concentration in the ileal contents among various treatment groups, except in chickens fed 1.58% glycine diets in experiment 2. However, this does not rule out the possibility that a higher concentration of α toxin is present in the lower intestinal tract especially in cecum. There is evidence that various digestive enzymes such as trypsin inactivates the α toxin of *C. perfringens* type A and the β toxin of *C. perfringens* type C (Baba *et al.,*

1992; Niilo, 1965). The production of α toxin by various non *C. perfringens* members of the gastrointestinal microflora such as *C. sordellii, C. bifermentans, Pseudomonas aeruginosa* and *Bacillus spp*. makes this analysis even more difficult. To overcome this limitation, we are investigating measuring *C. perfringens* α toxin gene transcript abundance using quantitative real-time PCR.

5.6. Conclusions

The results of the present study support our hypothesis that dietary glycine level plays a significant role in increasing *C. perfringens* populations in the intestinal tract of broiler chickens. Because animal-source proteins contain from 2 to 4 times as much glycine as a percentage of crude protein compared to plant based proteins, glycine content may be one factor in animal protein-based diets that could predispose chickens to clinical NE. However, there are two confounding factors in this study. The crude protein and proline levels were also increasing with increase in quantity of geltin in the experimental diets. So we could not conclusively establish a direct causative link between dietary glycine and *C. perfringens* growth in this study. The NE challenge model used in this study was effective in inducing death, *C. perfringens* colonization, intestinal lesions, poor feed utilization, and reduced body weight gain in broiler chickens. This model can be used further for assessing various other factors in animal proteins that increase susceptibility of broiler chickens to NE and will allow the development of dietary formulations for broiler chickens that reduce clinical outbreaks of this disease.

6.0. INTESTINAL *CLOSTRIDIUM PERFRINGENS* AND LACTOBACILLI POPULATIONS IN BROILER CHICKENS FED PROTECTED GLYCINE AND PROLINE SUPPLEMENTED DIETS

6.1. Abstract

This study was conducted to determine if there is a causative relationship between dietary glycine concentration and *C. perfringens* growth in broiler chickens. Since crystalline glycine is rapidly absorbed in the duodenum, fat encapsulated glycine was therefore used to slowly release the amino acid along the entire length of the gut. An initial study showed that glycine concentrations were significantly higher ($P < 0.05$) in jejunum and ileum of birds fed encapsulated glycine compared to crystalline glycine. In the main study, two experiments were conducted to examine the effect of dietary encapsulated glycine or proline on gut *C. perfringens* growth and necrotic enteritis (NE) lesion scores in broiler chickens. In experiment 1, two cages of 6 birds (14 day post-hatch) were assigned to one of 6 experimental diets formulated to contain 7.6 and 10.6 (control), 17.8 and 40.6 (G1P3), 27.8 and 30.6 (G2P2), 37.8 and 20.6 (G3P1), 47.7 and 10.6 (G4P0) and 7.8 and 50.6 (G0P4) g/kg total glycine and proline, respectively, provided primarily by supplementing with encapsulated glycine or proline as required. In experiment 2, 12 groups of 6 birds were fed 4 different diets formulated to contain 7.6 (LG), 21.0 (MG), 34.3 (HG) or 47.7 (UHG) g/kg glycine with same level of proline. The birds were orally challenged with *C. perfringens* type A on day 1 and day 14-21 and killed on day 28. In experiment 1, significantly higher ($P < 0.05$) *C. perfringens* populations were observed in ileum and cecum of birds which received either 37.8 or 47.7 g/kg dietary glycine compared to those given 7.6 g/kg glycine. Lactobacilli counts in

ileum declined significantly ($P < 0.05$) in the ceca of birds fed 47.7 g/kg glycine compared to rest of the diets. In experiment 2, *C. perfringens* numbers were significantly higher ($P < 0.05$) in ileum of birds which were fed either 7.6 or 21.0 g/kg dietary glycine than those given either 34.3 or 47.7 g/kg glycine. Lactobacilli populations were significantly higher in both ileum and cecum of chickens which were provided with 7.6 g/kg glycine than those with other levels of dietary glycine tested. The mean NE lesion score increased significantly ($P < 0.05$) in birds receiving 47.7 g/kg glycine than those fed either 7.6 or 21.0 g/kg glycine in experiment 2. Also birds having higher *C. perfringens* colonization and lesion scores had poor performance in both experiments. There was no significant effect of dietary proline concentration on *C. perfringens* as well as lactobacilli populations in either of the experiments. We conclude that glycine is an important determinant of *C. perfringens* growth in the intestinal tract of broiler chickens.

6.2. Introduction

Enteric pathogens result in huge losses to poultry industry annually, and their control or reduction could potentially save the producers millions of dollars. Recognized as the causative agent of necrotic enteritis (NE), *Clostridium perfringens* is one such pathogen. *C. perfringens* is a nearly ubiquitous gram positive, spore forming, extremely prolific, toxigenic anaerobic bacteria affecting many warm-blooded animals including humans (Ficken and Wages, 1997). High populations of *C. perfringens* may be present in the intestinal tract of animals with no visible signs of disease, thus breaking Koch's postulate that a disease-causing organism should not be present in healthy individuals. Most workers, therefore, consider various predisposing factors to be of major importance in spontaneous outbreaks of NE in poultry (Dahiya *et al*., 2006b). Despite considerable

investigation of this disease, the predisposing factors that promote overgrowth of *C. perfringens*, excessive release of α toxin, and thus subsequent progression to disease are numerous and ill-defined. The long list of contributing factors includes management and environmental conditions (Cowen *et al.,* 1987; Kaldhusdal *et al.*, 1999; Kaldhusdal and Skjerve, 1996), stress and immunosuppression (Fukata *et al.,* 1991; McReynolds *et al.,* 2004), co-infection with *Eimeria* spp. (Al-Sheikhly and Al-Saieg, 1980; Shane *et al.,* 1985), and diet composition. The estimated cost of clostridial enteritis to the poultry industry is as much as US$ 0.05 per bird with total global loss pegged at almost US$ 2 billion (Van der Sluis, 2000a; Van der Sluis, 2000b). Others have suggested this is an underestimate, given the difficulty in diagnosing mild forms of clostridial enteritis (Lovland and Kaldhusdal, 2001). Furthermore, *C. perfringens*-associated NE and subclinical infection is expected to emerge in broiler chickens after European ban on antimicrobial growth promoters increasing its economic impact. Concerns also arise on the high contamination rates of poultry by *C. perfringens* and risk of transmission to the food chain, posing public health problems (Immerseel *et al.*, 2004). These factors have stimulated interest in finding alternative management or dietary strategies to control the incidence and severity of clostridial enteritis in post-antibiotic era.

The physical and chemical attributes of diet can modify the gastrointestinal microbial ecology of birds and are important determinants of NE (Dahiya *et al.,* 2006a; Kaldhusdal and Skjerve, 1996; Riddell and Kong, 1992). Birds fed diets based on wheat, rye, oats or barley have increased risk of NE compared with birds fed corn-based diets (Branton *et al.,* 1997; Kaldhusdal and Hofshagen, 1992; Riddell and Kong, 1992). Proteins of animal origin are favorable substrates for clostridial growth, and high

concentrations in broiler feeds are often associated with NE (Drew *et al.,* 2004; Kaldhusdal and Skjerve, 1996; Truscott and Al-Sheikhly, 1977; Wilkie *et al.,* 2005). Although there are sufficient evidences regarding the mechanism(s) behind the effect of cereal grains on *C. perfringens* growth and NE, relatively little information are available in peer-reviewed literature regarding the factors or mechanisms responsible for the increased incidence of NE in broilers fed high protein diets.

Previous work has shown that there is a significant correlation between certain amino acids, specially glycine and *C. perfringens* numbers and/or phospolipase C production (Dahiya *et al.,* 2005; Drew *et al.,* 2004; Muhammed *et al.,* 1975, Titball *et al.,* 1999; Wilkie *et al.,* 2005). Drew *et al.* (2004) reported a positive association between crude protein derived from fish meal and numbers of ileal and cecal *C. perfringens*, but no such association existed for soy-derived proteins. Glycine and methionine levels are higher in fish meal than in soya concentrate, and these amino acids are known to stimulate *C. perfringens* growth and phospolipase C production *in vitro* (Ispolatovskaya, 1971; Muhammed *et al.,* 1975; Nakamura *et al.,* 1968; Stevens and Rood, 2000). Wilkie *et al.* (2005) documented a significant correlation of dietary glycine with *C. perfringens* numbers in ileum and cecum of broiler chickens when fed different plant and animal protein based diets. Recently, we reported a positive correlation between dietary protein-bound glycine content and gut *C. perfringens* growth in broiler chickens when gelatin was used as protein source, however, since gelatin also contains high levels of proline, a causative link between glycine and clostridial growth could not be conclusively established (Dahiya *et al.,* 2005). This study was therefore conducted to determine if a causative relationship exists between dietary glycine/proline and intestinal clostridial

growth in broiler chickens. Since crystalline glycine/proline is rapidly absorbed in the duodenum, it is largely unavailable to *C. perfringens* populations in the distal gut. Fat (hydrogenated palm oil) encapsulated glycine/proline was therefore used to slowly release the amino acid along the entire length of the gut. The purpose of the current study was to examine the effect of feeding encapsulated glycine and proline based diets on intestinal *C. perfringens* colonization and NE lesion scores in broiler chickens.

6.3. Materials and Methods

Experimental protocols were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with recommendations of the Canadian Council on Animal Care as specified in the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993).

6.3.1. Animal Management, Experimental Diets, and Design

6.3.1.1. Pilot Study

A pilot study was carried out to determine the effect of fat encapsulation on the release of glycine in the intestinal tract of broiler chickens. A total of thirty six, one-day old male Ross broiler chicks (*Gallus domesticus*) were obtained (Lilydale Hatchery, Wynyard, SK, Canada), housed randomly in four electrically heated battery cages and provided a medicated, ideal protein-balanced (12.0 g/kg lysine) corn-based starter crumble feed (Co-op feeds, Saskatoon, Canada) *ad libitum* for 20 days of age **(Table 6.1)**. The birds were subsequently fasted overnight and refed one of the two experimental diets for 3 h on day 21 (9 birds per cage x 2 cages per diet). The experimental diets contained either 40 g/kg crystalline glycine or 40 g/kg fat encapsulated glycine **(Table 6.2)**. A basal diet (190 g/kg CP, 12.8 MJ/kg ME) was formulated and then appropriate

quantities of either crystalline glycine (Degussa Corporation) or encapsulated glycine (JEFO Nutrition Inc., St. Hyacinthe, Quebec, Canada) were added to provide 40 g/kg of supplemental glycine. The encapsulated glycine contained 342.6 g/kg crude protein and 625.0 g/kg lipid and the diets were formulated to provide equal levels of ME and glycine. After 3 h of feeding experimental diets, the birds were euthanized by cervical dislocation and their intestinal tracts were carefully removed to collect fresh digesta from jejunum, ileum and cecum. Samples were pooled from 3 birds in each cage at each intestinal location, lyophilized and analyzed for amino acid content (Degussa Canada Inc., Burlington, ON).

6.3.1.2. Challenge Study

Two experiments were conducted to study the effect of feeding encapsulated glycine or proline on intestinal *C. perfringens* and lactobacilli colonization, and NE lesion scores in broiler chickens.

6.3.1.2.1. Experiment 1

Seventy two, one-day old conventional male broiler chicks (*Gallus domesticus,* Ross 308) were obtained (Lilydale Hatchery, Wynyard, SK, Canada) and placed randomly into four electrically heated battery cages (18 birds per cage). On day 1 through 14 of the experiment, birds were provided with the same corn-based starter crumble used in pilot study (Table 6.1). On day 14, birds were weighed and randomly re-assigned to one of the 12 battery cages at 6 birds per cage. Two cages were assigned in a randomized complete block design to one of the six ideal protein-balanced experimental diets **(Table 6.3)** up to the end of experiment (day 28). Diets were formulated to contain 7.6 and 10.6 (control), 17.8 and 40.6 (G1P3), 27.8 and 30.6 (G2P2), 37.8 and 20.6

(G3P1), 47.7 and 10.6 (G4P0) and 7.8 and 50.6 (G0P4) g/kg total glycine and proline, respectively, provided primarily by supplementing with encapsulated glycine or proline as required (JEFO Nutrition Inc., St. Hyacinthe, Quebec, Canada). The control diet was formulated to contain 190 g/kg crude protein, and remainder of the diets contained 230 g/kg crude protein. The diets were isocaloric (12.8 MJ/kg ME), contained 11.6 g/kg lysine with other essential amino acid levels formulated to be within 10 per cent of ideal protein ratio. The diets met or exceeded the NRC requirements for broiler chickens for all other nutrients (NRC, 1994).

6.3.1.2.2. Experiment 2

On the basis of results of experiment 1, encapsulated proline was excluded from experiment 2. A total of forty eight, one-day old male Ross broiler chicks (*Gallus domesticus)* were housed randomly into 4 electrically heated battery cages (12 birds per cage) and received the same starter diet as described above for first 14 days of life. On day 14, birds were weighed; randomized and 2 cages of 6 birds each were assigned to one of 4 different ideal protein-balanced experimental diets formulated to contain 7.6 (LG), 21.0 (MG), 34.3 (HG) or 47.7 (UHG) g/kg glycine mainly provided by supplemented encapsulated glycine **(Table 6.4).** The diets were isocaloric (12.8 MJ/kg ME) and contained 11.5 g/kg proline and 11.6 g/kg lysine with other essential amino acid levels formulated to be within 10 per cent of ideal protein ratio. The diets met or exceeded the NRC nutrient requirements for broiler chickens for all other nutrients (NRC, 1994).

The research facility was thoroughly cleaned and disinfected prior to bird placement. The battery cages were arranged in four levels with a wire floor and were equipped with external feed and water troughs. The cages were continuously illuminated

(24 h/day) and located in a room with controlled temperature and humidity. Room temperature was maintained according to industry standards. None of the experimental diets contain antibiotics or coccidiostats and were not pelleted. An indigestible marker, Celite (Celite Corporation, Lompoc, California, USA) was mixed in each diet in both experiments at a concentration of 2%. Throughout the experimental period in each experiment, birds were fed and watered *ad libitum*. Feed consumption and body weight for each cage was recorded for the period day 14-21 and day 21-28 in each experiment for calculation of mortality-corrected feed conversion. Amino acid analysis of the different diets was performed by Degussa Corporation (Degussa Canada Inc., Burlington, ON, Canada).

6.3.2. *Clostridium perfringens* Challenge

The *C. perfringens*-challenge model was based on that developed originally by Dahiya *et al*. (2005). Briefly, an avian *C. perfringens* field strain isolated from a clinical case of NE was obtained from Dr. Manuel Chirino, College of Veterinary Medicine, University of Saskatchewan and characterized by PCR technique as type A toxin producer. The organism was cultured anaerobically on BBL^{TM} Blood Agar Base (Becton, Dickinson and Co., Sparks, MD, USA) containing 5.0% sheep blood and 100 mg/L Neomycin Sulfate (The Upjohn Company, Orangeville, ON, Canada) for 18 h at 37°C, then aseptically inoculated into cooked meat medium (Difco Labs, Detroit, MI), and incubated anaerobically for 8 h at 37°C. All birds were orally challenged in the crop with 0.5 mL on day 1 and 1.0 mL on days 14 to 21 inclusive with this actively growing culture of *C. perfringens* using a 12.0 mL syringe equipped with vinyl tubing (I.D. 0.97mm, O.D.

1.27mm). Bacterial counts were performed on the culture daily prior to inoculation and the numbers ranged from 6.38×10^5 to 7.29×10^6 CFU/mL.

6.3.3. Pathological Parameters

Birds were observed on a pen basis at least once daily for any sign or symptom of NE and all birds that died during the course of experiments were necropsied to determine the cause of death. On day 28, the surviving chickens were euthanized by cervical dislocation, weighed and necropsied. Intestinal tracts were removed immediately and intestinal lesions were scored blindly according to the method of Truscott and Al-Sheikhly (Truscott and Al-Sheikhly, 1977) with slight modifications on a scale 0 to 4 where 0 was apparently normal, no lesion; 0.5 severely congested serosa and mesenteric vessels engorged with blood; 1 thin walled and friable intestines with small red petechiae (> 5); 2 focal necrotic lesions; 3 patches of necrosis (1-2 cm long); and 4 diffused necrosis typical of field cases. Following postmortem examination, a 1.5-2.0 cm long intestinal tissues piece from the ileum with the gross NE lesion was collected in phosphate-buffered formaldehyde solution and processed routinely for paraffin embedding, sectioned at approximately 5 μm, and stained with haematoxylin and eosin.

Table 6.1. Ingredient composition of starter diet used in pilot study (up to day 20 of age) as well as in definitive study (up to day 14 of age)[1].

Ingredients	Inclusion (g/kg)
Soybean meal	409.0
Corn	395.0
Wheat	100.0
Tallow	45.2
Dicalcium phosphate	15.3
Calcium carbonate	12.9
Canola oil	10.0
DL-methionine	2.6
Choline chloride	1.0
Vit/Min. premix[2]	5.0
Salinomycin sodium[3]	0.6
Bacitracin[4]	0.5
Bio-cox 120[5]	0.5

[1] Diet was formulated to contain 315 g/kg crude protein and meet NRC requirements for broiler chickens.

[2] Supplied per kilogram of diet: vitamin A, 3.3 mg; cholecalciferol, 55 µg**;** vitamin E, 30 mg; vitamin K, 0.5 mg; vitamin B12, 0.02 mg; thiamine, 1.5 mg; riboflavin, 6 mg; folic acid, 0.6 mg; biotin, 0.15 mg; niacin, 60 mg; pyridoxine, 5 mg; sodium, 511 mg; iron, 80 mg; maganese, 21.8 mg; selenium, 0.1 mg; iodine, 0.35 mg; zinc, 100 mg.

[3] Phibro Animal Health Ltd.

[4] Alpharma Canada Corp.

[5] Anticoccodial Premix, OzBioPharma Ltd.

Table 6.2. Composition of experimental diets used in pilot study (g/kg as is basis).

Ingredients	Crystalline glycine diet	Encapsulated glycine diet
Corn, yellow grain	500.0	500.0
Barley grain, western	260.8	260.8
Soybean meal dehulled 48%	118.8	118.8
Fish meal, herring 72%	58.7	58.7
Encapsulated glycine	0.0	118.0
Crystalline glycine	40.0	0.0
Canola oil[1]	73.7	0.0
Calcium carbonate	18.2	18.2
Dicalcium phosphate	10.6	10.6
Arginine	12.0	12.0
L-lysine	6.1	6.1
DL-methionine	3.0	3.0
L-tryptophan	2.5	2.5
L-threonine	2.2	2.2
L-isoleucine	0.6	0.6
Vit./Min. premix[2]	5.0	5.0
Choline chloride	1.0	1.0
Analyzed Composition		
Crude Protein	232.5	231.8
Essential amino acids		
Arginine	18.1	18.3
Histidine	3.9	4.0
Isoleucine	6.8	7.2
Leucine	13.9	14.3
Lysine	13.4	13.2
Methionine	5.3	5.7
Methionine + Cystine	8.0	8.4
Phenylalanine	7.6	7.8
Threonine	7.9	7.8
Valine	7.9	7.5
Nonessential amino acids		
Alanine	8.8	9.1
Aspartate	14.0	14.3
Cysteine	2.7	2.7
Glycine	43.9	45.3
Glutamate	28.1	28.5
Serine	7.3	7.3

[1]Canola oil is added in crystalline glycine diet to make the diets isoenergetic because encapsulated glycine contains 342.6 g/kg CP and 625.0 g/kg ether extract.

[2]Composition is the same as shown in Table 6.1.

Table 6.3. Composition of experimental diets fed to broiler chickens from day 14-28 of age in experiment 1 (g/kg as is basis)[1].

	Control	G1P3	G2P2	G3P1	G4P0	G0P4
Corn, yellow, grain	466.4	417.7	418.0	418.3	394.5	417.5
Barley, grain, western	200.0	181.0	180.5	179.9	200.0	181.5
Soybean meal dehulled	171.8	174.2	174.3	174.4	177.0	174.0
Encapsulated glycine	0.0	29.3	58.7	88.1	117.4	0.0
Encapsulated proline	0.0	86.7	57.8	28.9	0.0	115.6
Fish meal, herring	45.1	53.4	53.4	53.4	50.7	53.4
Dicalcium phosphate	21.8	21.7	21.7	21.7	21.8	21.7
Calcium carbonate	12.8	13.5	13.5	13.5	13.6	13.5
Canola oil	67.9	8.5	8.3	8.0	10.8	8.8
Vit/Min. premix[2]	5.0	5.0	5.0	5.0	5.0	5.0
L-lysine	2.5	2.2	2.2	2.2	2.4	2.2
DL-methionine	1.9	1.9	1.9	1.9	2.0	1.9
L-threonine	1.7	1.8	1.8	1.8	1.9	1.8
L-isoleucine	1.0	1.1	1.0	1.0	1.1	1.0
L-arginine	1.0	0.9	0.9	0.9	1.0	0.9
Choline chloride	1.0	1.0	1.0	1.0	1.0	1.0
Analyzed Composition						
Crude Protein	207.7	246.0	249.4	251.4	250.5	240.2
Essential amino acids						
Arginine	12.7	13.0	12.9	12.6	12.8	12.8
Histidine	5.2	5.2	5.1	5.2	5.1	5.1
Isoleucine	9.1	9.5	9.7	9.3	9.2	9.2
Leucine	17.3	16.9	16.7	16.8	16.8	16.8
Lysine	12.5	12.8	12.6	12.4	12.6	12.3
Methionine	5.7	5.9	6.4	5.7	6.1	5.9
Methionine + Cystine	8.8	8.9	9.4	8.7	9.0	8.9
Phenylalanine	9.9	9.8	9.7	9.8	9.7	9.8
Threonine	9.4	9.5	9.6	9.3	9.3	9.4
Valine	9.8	9.8	9.9	9.8	9.8	9.7
Nonessential amino acids						
Alanine	10.8	10.7	10.6	10.6	10.6	10.6
Aspartate	18.3	18.6	18.6	18.4	18.4	18.6
Cysteine	3.1	3.0	3.0	3.0	2.9	3.0
Glycine	8.5	21.7	31.8	41.9	51.0	10.2
Glutamate	36.9	36.1	36.2	36.3	36.3	36.4
Proline	12.8	43.2	35.5	22.7	12.1	56.2
Serine	9.4	9.3	9.2	9.1	9.1	9.4

[1] Diet abbreviations are as follows: Control, 7.6 g/kg glycine and 10.6 g/kg proline with 190 g/kg CP; G1P3, 17.8 g/kg glycine and 40.6 g/kg proline with 230 g/kg CP; G2P2, 27.8 g/kg glycine and 30.6 g/kg proline with 230 g/kg CP; G3P1, 37.8 g/kg glycine and 20.6 g/kg proline with 230 g/kg CP; G4P0, 47.7 g/kg glycine and 10.6 g/kg proline with 230 g/kg CP, G0P4, 7.8 g/kg glycine and 50.6 g/kg proline with 230 g/kg CP.

[2] Composition is same as shown in Table 6.1.

Table 6.4. Composition of experimental diets fed to broiler chicken from day 14-28 of age in experiment 2 (g/kg as is basis)[1].

	LG	MG	HG	UHG
Barley, grain, western	200.0	200.0	200.0	200.0
Corn, yellow, grain	466.4	442.4	418.4	394.5
Fish meal, herring	45.1	47.0	48.8	50.6
Soybean meal dehulled	171.9	173.6	175.3	176.9
Encapsulated glycine	0.0	39.1	78.3	117.4
Canola oil	67.9	48.9	29.9	10.8
Calcium carbonate	12.8	13.1	13.3	13.6
Dicalcium phosphate	21.8	21.8	21.8	21.8
Lysine HCl	2.5	2.5	2.4	2.4
DL-methionine	1.9	1.9	2.0	2.0
L-threonine	1.7	1.8	1.8	1.9
L-arginine	1.0	1.0	1.0	1.0
L-isoleucine	1.0	1.0	1.1	1.1
Vit/Min. premix[2]	5.0	5.0	5.0	5.0
Choline chloride	1.0	1.0	1.0	1.0
Analyzed composition				
Crude Protein	187.0	203.2	218.9	220.8
Essential amino acids				
Arginine	11.4	11.3	11.9	11.7
Histidine	4.5	4.5	4.7	4.5
Isoleucine	8.1	8.0	8.3	8.1
Leucine	15.4	15.3	15.4	15.1
Lysine	11.5	11.9	12.2	11.8
Methionine	5.0	5.4	5.3	5.7
Methionine + Cystine	7.8	8.2	8.1	8.4
Phenylalanine	8.4	8.2	8.4	8.2
Threonine	8.2	8.5	8.5	8.7
Valine	8.6	8.5	8.8	8.6
Nonessential amino acids				
Alanine	9.1	9.2	9.5	9.4
Aspartate	16.3	16.2	17.0	16.5
Cysteine	2.8	2.8	2.7	2.7
Glycine	7.6	22.3	34.6	45.4
Glutamate	32.6	31.9	32.5	31.9
Proline	11.9	11.6	11.0	11.3
Serine	8.3	8.3	8.5	8.3

[1] Diet abbreviations are as follows: LG, low glycine diet i.e. 7.6 g/kg glycine and 190 g/kg CP; MG, medium glycine diet i.e. 21.0 g/kg glycine and 203 g/kg CP; HG, high glycine diet i.e. 34.3 g/kg glycine and 217 g/kg CP; UHG, ultra high glycine diet i.e. 47.7 g/kg glycine and 230 g/kg CP.

[2] Composition is same as shown in Table 6.1.

6.3.4. Quantification of *C. perfringens* and Lactobacilli

In both experiments, the fresh intestinal contents from ileum (Meckel's diverticulum to 1 cm proximal to ileocecal junction) and ceca were collected aseptically into sterilized plastic dram vials and mixed well. Using sterile spatula, the subsamples were transferred into pre-weighed 15 mL sterile plastic tubes containing 1mL 0.1% sterile peptone buffer with 5 g/L cysteine hydrochloride. The ceca samples were directly collected into the pre-weighed 15 mL sterile tubes. The samples were immediately placed and kept on ice until plated within 3 h of collection. The samples were weighed and diluted in peptone water to an initial 10^{-1} dilution. Ten-fold dilutions were spread in duplicate using an automated spiral plater (Autoplate, Spiral Biotech Inc., Bethesda MD, USA) on BBL Blood Agar Base (VWR International, Mississauga ON) containing 50 mL/L sheep blood and 100 mg/L neomycin sulfate (The Upjohn Company, Orangeville, ON, Canada) for the enumeration of *C. perfringens* and MRS agar (Becton, Dickinson and Co., Sparks, MD, USA) for the enumeration of lactobacilli. The blood agar/neomycin plates were incubated anaerobically for 24 h at 37°C while MRS agar plates were incubated anaerobically for 48 h at 37°C. The α- and β- hemolytic colonies on blood agar/neomycin plates were counted as *C. perfringens* with presumptive colonies being randomly picked, Gram stained, plated on Mannitol Yolk Polymixin agar (Oxoid Inc., Napean, ON, Canada) and examined microscopically to confirm them as *C. perfringens.* Bacterial counts were expressed as the Log_{10} CFU/g of intestinal contents.

6.3.5. Samples for Amino Acids Digestibility Determination

In both experiments, the remainder of the ileal contents of 3 birds from each pen was pooled, lyophilized and analyzed for amino acid content (Degussa Canada Inc.,

Burlington, ON). The acid insoluble ash in experimental diets and freeze dried ileal digesta samples was determined using method of MaCarthy *et al.* (1974). Amino acid digestibilities were calculated using the formula:

$$\frac{\text{(Amino acid/acid insoluble ash) diet - (Amino acid/acid insoluble ash) digesta}}{\text{(Amino acid/acid insoluble ash) diet}}$$

6.3.6. Whole Blood Chemiluminescence Assay

In experiment 2, three mL peripheral blood was collected into 4 mL sodium heparin tubes (Vacutainer®, BD) from all birds before killing on day 28 from the brachial vein. Hanks' balanced salt solution (HBSS, Sigma) was used for the dilution of blood and for preparing working solutions of various reagents used during the assay. Zymosan A from *Saccharomyces cerevisiae* (Sigma) was prepared as described by (Marnila *et al.,* 1995), and was used non-opsonized as a stimulator of cells, whereas Lucigenin (N, N'-Dimethyl-9, 9'-biacridium dinitrate; Sigma) was used as a light enhancer.

Briefly, blood was first diluted in HBSS and added to clear Polysorp microplates (Nunc, Denmark). Working solution of zymosan was added to the diluted blood samples followed by addition of lucigenin, both within 2 min before starting test run in a Novostar (BMG Labtech) luminometer. The final reaction volume was 150 µL in each well, with final blood dilutions of 1:15, final zymosan dilution of 0.3 mg/mL and a final lucigenin concentration of 0.3 mM. Light emission was recorded over a 2-3 h period, at 4 min intervals and 1.5 sec/reading at 39 ºC. Plates were shaken for 5 sec before each interval. Light emission results are presented as counts per min (light units measured per min). Background light emission was subtracted.

6.3.7. Statistical Analyses

In pilot study, the effects of two diets (crystalline versus encapsulated glycine based) on the concentration of glycine in intestinal contents of birds were compared using one-way analysis of variance and differences between means were considered significant when $P < 0.05$. In experiment 1 and 2, each cage was considered an experimental unit. Bacterial counts, growth performance and lesion scores were analyzed using General Linear Model procedure of SPSS (v.12.0, SPSS Inc, Chicago IL, USA). Treatment means were compared using the Ryan-Einot-Gabriel-Welch multiple F test, and were considered significantly different when $P < 0.05$.

6.4. Results

6.4.1. Effect of Encapsulation on Amino Acid Level in Intestinal Contents

The analyzed amino acid content of the experimental diets used in the pilot study was similar between diets including similar glycine content whether provided in crystalline or encapsulated form (Table 6.2). Glycine concentration was significantly higher ($P < 0.05$) in jejunum and ileum of birds fed encapsulated glycine (12.6 and 10.5 g/kg) compared to crystalline glycine (9.8 and 6.2 g/kg) whereas there was no significant difference in glycine concentration in cecum **(Figure 6.1).** The rests of amino acids were in similar concentrations in both diets at all locations in the intestinal tract of these birds (data not shown). For diets used in the challenge experiment, analyzed dietary glycine and proline content were in reasonable concordance with supplemented levels and analyzed content of all other amino acids were similar (Tables 6.3 and 6.4). In agreement with results of the pilot study, glycine and proline content in ileal digesta collected during the challenge studies varied directly with the amount of encapsulated amino acid

supplemented in the diet **(Table 6.5)**. In contrast, ileal content of all other amino acids was similar. The ileal digestibilities of various amino amino acids in both experiments are shown in **Table 6.6.**

6.4.2. Performance Data

The experimental diets were readily accepted in both challenge experiments and there were no significant differences in feed consumption either during day 14-21 or day 21-28 of age. In both experiments, diets did not affect average daily gain (ADG) and feed conversion (FC) at the end of 14-21 day period, whereas dietary treatments had a significant effect on ADG and FC at the end of 21-28 day period. In experiment 1, ADG tended to be lower in the 230 g/kg CP diets supplemented with encapsulated amino acids (**Table 6.7**) compared with the 190 g/kg CP unsupplemented control diet. This response was significant ($P < 0.05$) in birds receiving G3P1 and G4P0 diets. Conversely, FC was significantly increased in all supplemented 230 g/kg CP diets. In experiment 2, ADG and FC was significantly lower ($P < 0.05$) in birds provided with UHG diets.

6.4.3. Quantification of Lactobacilli and *C. perfringens*

Bacterial counts in ileum and cecum of chickens on day 28 of the two challenge experiments are presented in **Figures 6.2 and 6.3**. The number of lactobacilli and *C. perfringens* increased from the proximal to the distal part of the intestine with the highest counts measured in the ceca. In both experiments, we observed a decrease in number of lactobacilli and increase in *C. perfringens* with increased dietary glycine concentration.

Significantly higher ($P < 0.05$) *C. perfringens* numbers were observed in the ileum and cecum of day-28-old chickens fed either G3P1 or G4P0 diets compared with those given either control or G0P4 diets in experiment 1 **(Figure 6.2.B).** Additionally, *C.*

perfringens numbers were not significantly different in birds fed lowest glycine diets with either 190 g/kg (control) or 230 g/kg (G0P4) crude protein which indicates that *C. perfringens* growth was not influenced by dietary crude protein or proline concentrations. In experiment 2, *C. perfringens* counts in ileum increased from 3.12 to 4.52 $\log_{10}$ CFU/g of intestinal contents as dietary glycine concentration increased from 7.6 to 47.7 g/kg, and the differences were significant ($P < 0.05$) among the birds which were fed either LG or MG diets and those fed either HG or UHG diets **(Figure 6.3.B)**. The birds which were fed HG and UHG diets contained significantly elevated counts of *C. perfringens* in ceca compared to those fed LG diets. It should be noted that there was more than 2 log difference in cecal *C. perfringens* numbers when dietary glycine concentrations were increased from 7.6 to 47.7 g/kg.

In experiment 1, a significant decline ($P < 0.05$) in the lactobacilli counts were observed in ileum of chickens fed G4P0 diet compared to rest of the dietary treatments while in cecum, the lactobacilli growth was significantly lower in birds fed G1P3 diet than those fed either control or G0P4 diets **(Figure 6.2.A).** However, there was a no significant difference in the lactobacilli numbers either in ileum or cecum of birds receiving either control or G0P4 diets i.e. low glycine diets (7.6 or 7.8 g/kg glycine) with low and high dietary crude proteins (either 190 or 230 g/kg). In experiment 2, the lactobacilli numbers were significantly higher ($P < 0.05$) in ileum as well as cecum of chickens which were given LG diets than those with other levels of dietary glycine tested **(Figure 6.3.A)**. Also there was no significant difference in lactobacilli growth in ileum and cecum among birds fed MG, HG or UHG diets.

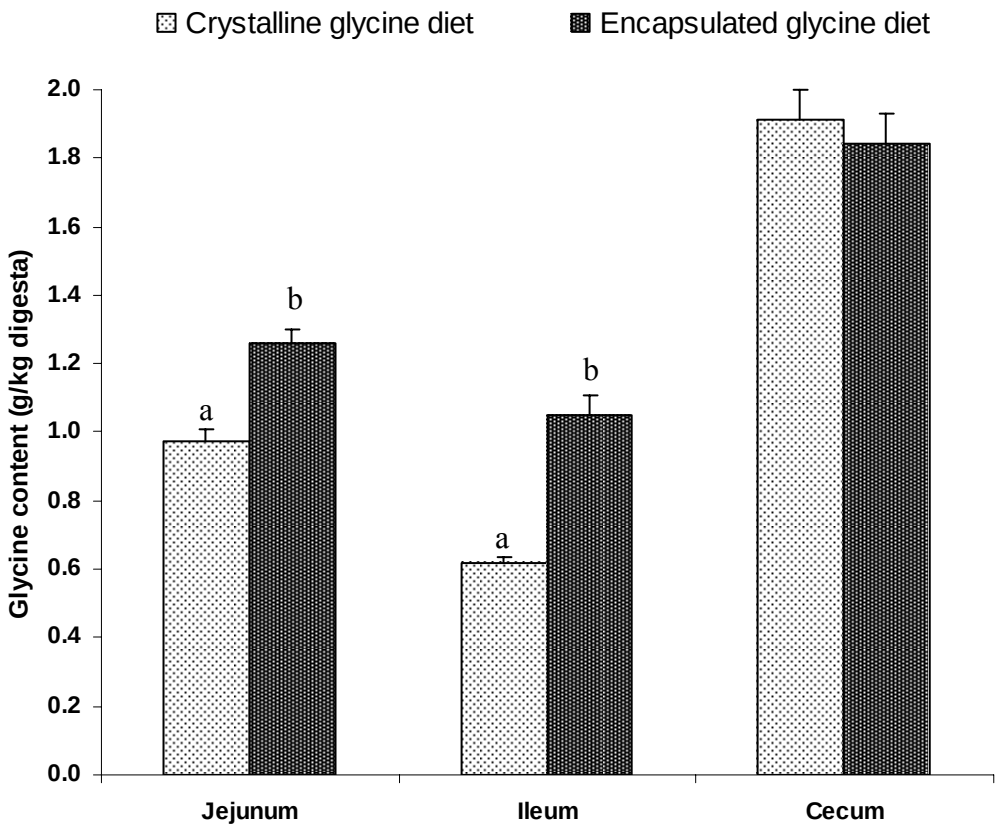


Figure 6.1. Glycine concentration (g/kg) of digesta at different intestinal locations in chickens fed diets supplemented with either crystalline or fat encapsulated glycine in the pilot study.

Table 6.5. Protein and amino acid contents (g/kg) of ileal digesta in experiments 1 and 2.

	Experiment 1[1]						Experiment 2[2]			
	Control	**G1P3**	**G2P2**	**G3P1**	**G4P0**	**G0P4**	**LG**	**MG**	**HG**	**UHG**
Crude Protein	106.9	109.1	122.3	133.3	113.8	133.4	122.5	117.1	116.1	111.2
Essential amino acids										
Arginine	4.9	4.8	5.3	5.6	4.8	5.8	4.9	4.6	4.3	4.2
Histidine	2.5	2.5	2.8	3.0	2.6	3.0	2.6	2.6	2.4	2.3
Isoleucine	3.8	4.0	4.5	4.9	4.2	5.1	4.3	4.1	3.8	3.8
Leucine	6.7	7.0	8.0	8.6	7.5	9.1	8.1	7.9	7.4	7.0
Lysine	4.7	5.0	5.6	5.7	4.9	6.2	5.0	4.8	4.6	4.4
Methionine	1.4	1.5	1.8	1.8	1.5	2.0	1.5	1.5	1.4	1.7
Meth.+ Cyst.	3.4	3.5	4.0	4.2	3.6	4.4	3.7	3.8	3.5	3.8
Phenylalanine	3.8	4.0	4.6	4.9	4.2	5.1	5.1	4.9	4.6	4.5
Threonine	5.1	5.1	5.7	5.9	5.2	6.1	5.4	5.3	5.0	5.0
Valine	4.7	5.0	5.4	6.0	5.2	6.2	5.3	5.2	4.8	4.8
Nonessential amino acids										
Alanine	4.7	5.0	5.7	5.9	5.2	6.4	5.2	5.1	4.7	4.6
Aspartate	10.3	10.4	11.5	12.4	10.6	12.4	11.3	10.5	10.1	9.9
Cysteine	2.0	2.1	2.3	2.4	2.1	2.3	2.2	2.3	2.1	2.1
Glycine	5.7	8.3	11.5	16.2	21.4	7.9	6.0	7.8	14.7	20.5
Glutamate	12.1	12.5	14.4	15.8	13.7	16.0	14.9	13.9	13.1	12.4
Proline	5.8	15.6	12.3	7.8	5.7	19.4	7.0	6.8	6.6	6.2
Serine	5.1	5.1	5.7	5.9	5.1	6.1	5.5	5.6	5.1	5.0

[1] Diet abbreviations are as follows: Control, 7.6 g/kg glycine and 10.6 g/kg proline with 190 g/kg CP; G1P3, 17.8 g/kg glycine and 40.6 g/kg proline with 230 g/kg CP; G2P2, 27.8 g/kg glycine and 30.6 g/kg proline with 230 g/kg CP; G3P1, 37.8 g/kg glycine and 20.6 g/kg proline with 230 g/kg CP; G4P0, 47.7 g/kg glycine and 10.6 g/kg proline with 230 g/kg CP, G0P4, 7.8 g/kg glycine and 50.6 g/kg proline with 230 g/kg CP.

[2] Diet abbreviations are as follows: LG, low glycine diet i.e. 7.6 g/kg glycine and 190 g/kg CP; MG, medium glycine diet i.e. 21.0 g/kg glycine and 203 g/kg CP; HG, high glycine diet i.e. 34.3 g/kg glycine and 217 g/kg CP; UHG, ultra high glycine diet i.e. 47.7 g/kg glycine and 230 g/kg CP.

Table 6.6. Ileal digestibilities (%) of various amino acids in experiments 1 and 2.

	Experiment 1[1]						Experiment 2[2]			
	Control	**G1P3**	**G2P2**	**G3P1**	**G4P0**	**G0P4**	**LG**	**MG**	**HG**	**UHG**
Crude Protein	106.9	109.1	122.3	133.3	113.8	133.4	122.5	117.1	116.1	111.2
Essential amino acids										
Arginine	87.28	86.63	84.12	81.12	85.39	83.29	86.17	85.63	85.29	85.42
Histidine	84.15	82.32	78.48	75.60	80.24	78.23	81.04	80.10	79.12	79.13
Isoleucine	86.18	84.58	82.18	77.95	82.20	79.55	82.84	82.11	81.47	81.30
Leucine	87.20	84.92	81.39	78.42	82.70	80.06	82.96	81.81	80.62	81.21
Lysine	87.48	85.85	82.66	80.48	85.00	81.50	85.80	85.76	84.91	84.94
Methionine	91.82	90.74	89.38	87.02	90.31	87.40	90.25	90.41	89.31	87.96
Meth.+ Cyst.	87.04	85.58	83.47	79.83	84.50	82.06	84.48	83.62	82.52	81.73
Phenylalanine	87.38	85.14	81.58	78.86	83.12	81.08	80.17	79.02	78.08	77.71
Threonine	81.93	80.36	76.84	73.40	78.43	76.28	78.69	78.00	76.44	77.02
Valine	83.94	81.43	78.72	74.22	79.34	76.54	79.97	78.62	78.04	77.46
Nonessential amino acids										
Alanine	85.43	83.07	79.30	76.46	81.08	77.92	81.43	80.63	79.98	80.34
Aspartate	81.35	79.60	75.98	71.56	77.67	75.48	77.57	77.35	75.90	75.89
Cysteine	78.51	75.12	70.54	66.52	72.27	71.55	74.17	70.84	68.15	68.59
Glycine	77.57	89.47	92.95	93.74	94.36	71.48	74.44	87.72	91.81	92.76
Glutamate	89.08	87.39	84.58	81.62	85.37	83.91	85.12	84.67	83.75	84.37
Proline	84.91	95.30	92.07	87.43	81.66	95.72	81.02	79.42	75.91	77.84
Serine	82.11	80.23	76.04	72.70	78.39	76.28	78.46	76.52	75.72	75.79

[1] Diet abbreviations are as follows: Control, 7.6 g/kg glycine and 10.6 g/kg proline with 190 g/kg CP; G1P3, 17.8 g/kg glycine and 40.6 g/kg proline with 230 g/kg CP; G2P2, 27.8 g/kg glycine and 30.6 g/kg proline with 230 g/kg CP; G3P1, 37.8 g/kg glycine and 20.6 g/kg proline with 230 g/kg CP; G4P0, 47.7 g/kg glycine and 10.6 g/kg proline with 230 g/kg CP, G0P4, 7.8 g/kg glycine and 50.6 g/kg proline with 230 g/kg CP.

[2] Diet abbreviations are as follows: LG, low glycine diet i.e. 7.6 g/kg glycine and 190 g/kg CP; MG, medium glycine diet i.e. 21.0 g/kg glycine and 203 g/kg CP; HG, high glycine diet i.e. 34.3 g/kg glycine and 217 g/kg CP; UHG, ultra high glycine diet i.e. 47.7 g/kg glycine and 230 g/kg CP.

Table 6.7. Average daily feed intake (ADFI, g/day), average daily gain (ADG, g/day) and feed:gain ratio (FC, feed intake/ weight gain, g/g) of birds during days 14-21 and 21-28 of the main experiments.

	Days 14-21			Days 21-28		
Experimental diets	ADFI	ADG	FC	ADFI	ADG	FC
Experiment 1[1]						
Control	76.4	40.1	1.9	82.7	52.2[a]	1.6[a]
G1P3	66.9	35.8	1.9	89.9	40.7[ab]	2.2[b]
G2P2	68.1	36.2	1.9	94.0	44.4[ab]	2.1[b]
G3P1	68.2	33.3	2.1	91.3	39.5[b]	2.3[b]
G4P0	70.4	29.5	2.4	86.6	40.4[b]	2.1[b]
G0P4	67.9	35.8	1.8	86.8	41.2[ab]	2.1[b]
Pooled SEM	1.15	1.08	0.06	1.80	1.48	0.07
P value	0.11	0.54	0.72	0.63	0.04	0.007
Experiment 2[2]						
LG	77.7	43.0	1.8	92.4	55.2[a]	1.7[a]
MG	72.0	38.9	1.8	87.7	49.3[a]	1.8[a]
HG	75.8	39.9	1.9	96.5	50.9[a]	1.9[a]
UHG	68.4	33.5	2.0	90.4	42.6[b]	2.1[b]
Pooled SEM	1.64	1.26	0.06	2.16	1.94	0.08
P value	0.19	0.21	0.73	0.77	0.03	0.01

[abc] Means with the different superscripts within a column (within a trial) differ significantly ($P < 0.05$)

[1] Diet abbreviations are as follows: Control, 7.6 g/kg glycine and 10.6 g/kg proline with 190 g/kg CP; G1P3, 17.8 g/kg glycine and 40.6 g/kg proline with 230 g/kg CP; G2P2, 27.8 g/kg glycine and 30.6 g/kg proline with 230 g/kg CP; G3P1, 37.8 g/kg glycine and 20.6 g/kg proline with 230 g/kg CP; G4P0, 47.7 g/kg glycine and 10.6 g/kg proline with 230 g/kg CP, G0P4, 7.8 g/kg glycine and 50.6 g/kg proline with 230 g/kg CP.

[2] Diet abbreviations are as follows: LG, low glycine diet i.e. 7.6 g/kg glycine and 190 g/kg CP; MG, medium glycine diet i.e. 21.0 g/kg glycine and 203 g/kg CP; HG, high glycine diet i.e. 34.3 g/kg glycine and 217 g/kg CP; UHG, ultra high glycine diet i.e. 47.7 g/kg glycine and 230 g/kg CP.

6.4.4. Clinical Symptoms and Necrotic Enteritis Lesions

Subsequent to challenge with *C. perfringens* some of the birds were dull, depressed and had abnormally wet droppings for first 3-5 days (i.e. after day 14) in both experiments. During the course of this study, 3 birds died in experiment 1 (two from G3P1 while one from G1P3 groups) and 4 birds died in experiment 2 (three from UHG while one from MG groups) of causes unrelated to *C. perfringens* challenge. Most dead birds were in good body condition and did not have any detectable gross lesions of NE either in intestine or any other organ with the exception of occasional petechial hemorrhages in distal jejunum and proximal ileum. Surviving birds had no apparent signs of morbidity 7-10 days post-challenge.

The mean NE lesion scores of chickens fed different experimental diets and killed on day 28 are presented in **Figure 6.4.** Some birds had very thin and friable intestinal wall with congested mucosa and mesenteric vessels engorged with blood having focal hemorrhagic lesions in various intestinal regions. In experiment 2, intestines of at least two birds were grossly hemorrhagic throughout with blood-stained fluid in the lumen. However, typical field type lesions specific to NE were not observed in any of the birds in either experiment. In experiment 1, there was no significant difference ($P > 0.05$) in the intestinal lesion scores in chickens among various dietary treatments although there was a tendency of increasing the NE lesions with increase in dietary glycine concentration with maximum score in birds fed G3P1 diet. In experiment 2, intestinal lesion scores were significantly higher ($P < 0.05$) in birds which received UHG diet than those fed either LG or MG diets; no significant difference in the lesion score was observed with increase in dietary glycine concentration from 34.3 to 47.7 g/kg.

6.4.5. Histological Examination

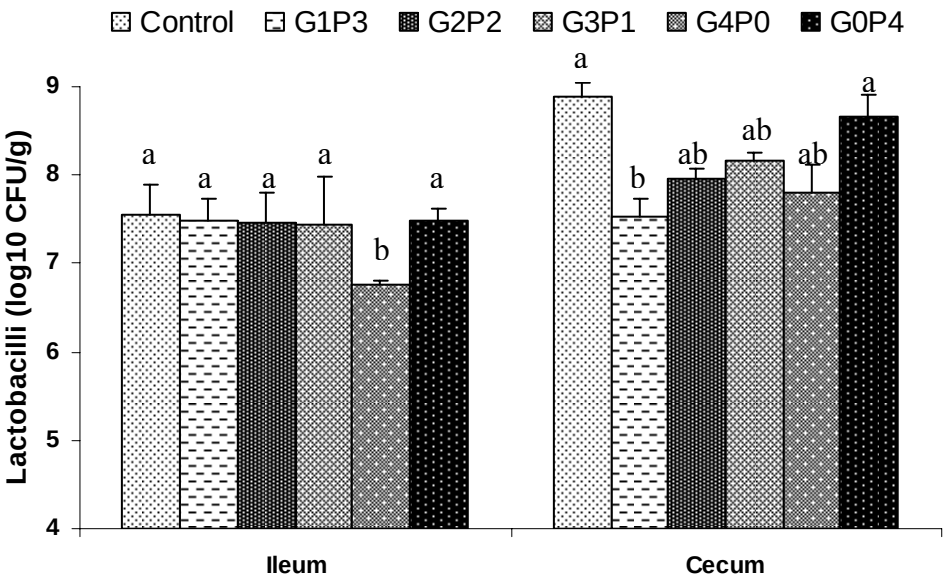
Histological examination of formalin fixed intestinal tissues from 28-day-old broiler chickens euthanized in both experiments revealed no frank lesions of NE except slight edema and diffuse hemorrhages in lamina propria in some sections. There was no evidence of gram-positive rod-shaped organisms attached to intestinal mucosa. Polymorphonuclear cell infiltration in the lamina propria was not seen in any of the sections. There was no evidence of coccidial oocysts in any sections examined.

6.4.6. Whole Blood Chemiluniscence Response

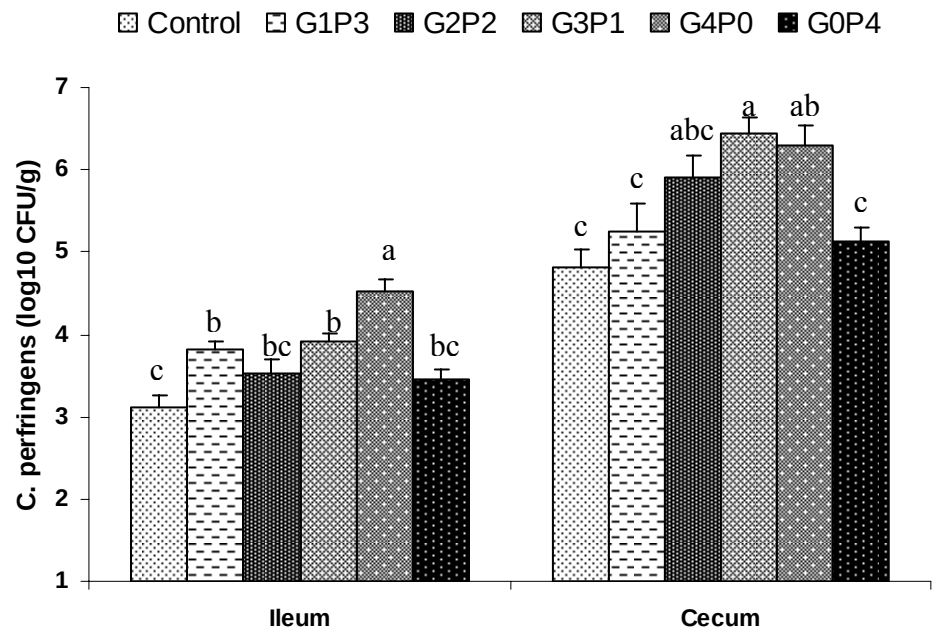
The WBCL assay was done only in experiment 2 and the results are presented in **Figure 6.5**. The chemiluniscence response increased significantly ($P < 0.05$) in birds fed HG and UHG diets compared to LG diets.

6.5. Discussion

Glycine is considered as a semi-essential amino acid in young broiler chicks and metabolic pathways involving glycine include the synthesis of proteins, purines, porphyrin moiety of heme groups, glutathione, creatine, uric acid, glycocholic acid and hippuric acid. Two experiments reported in this study support the hypothesis that the dietary glycine concentration is an important determinant of *C. perfringens* growth in the intestinal tract of broiler chickens and thus can predispose birds to clostridial enteritis. It was also suggested that *C. perfringens* growth is not significantly influenced by dietary proline or crude protein levels tested in this study.

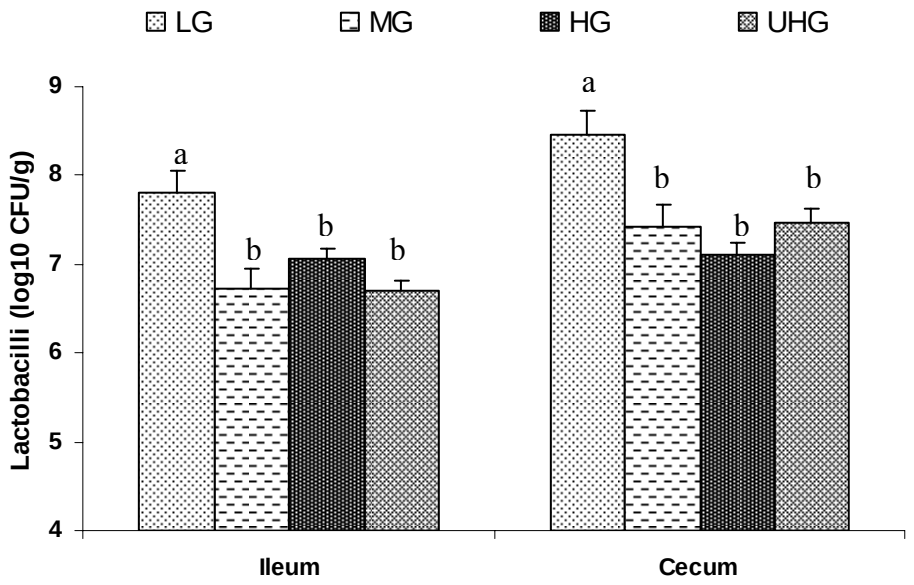


A

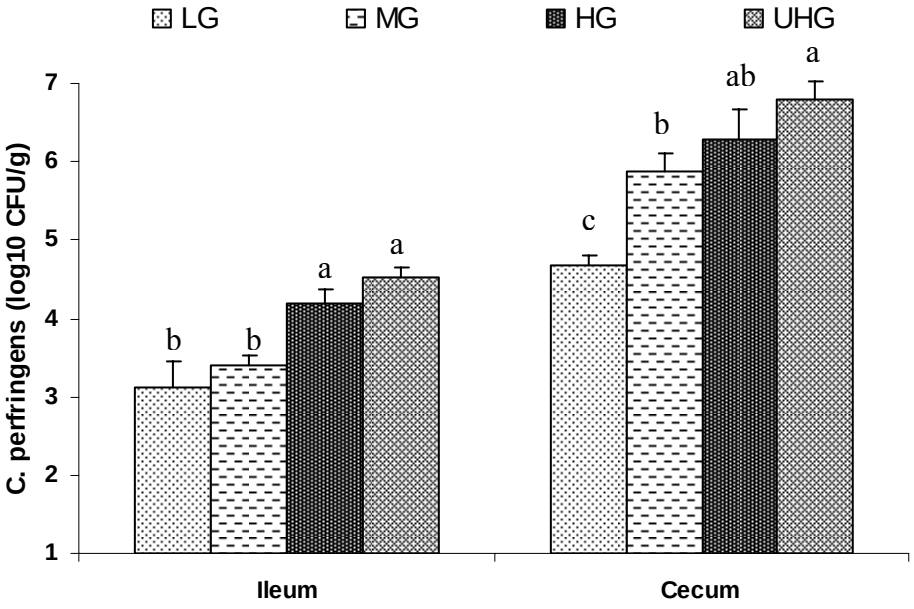


B

Figure 6.2. Mean Lactobacilli (A) and *C. perfringens* (B) populations in ileum and cecum of broiler chickens on day 28 of experiment 1. Bars represent mean ± SEM.



A



B

Figure 6.3. Mean Lactobacilli (A) and *C. perfringens* (B) populations in ileum and cecum of broiler chickens on day 28 of experiment 2. Bars represent mean ± SEM.

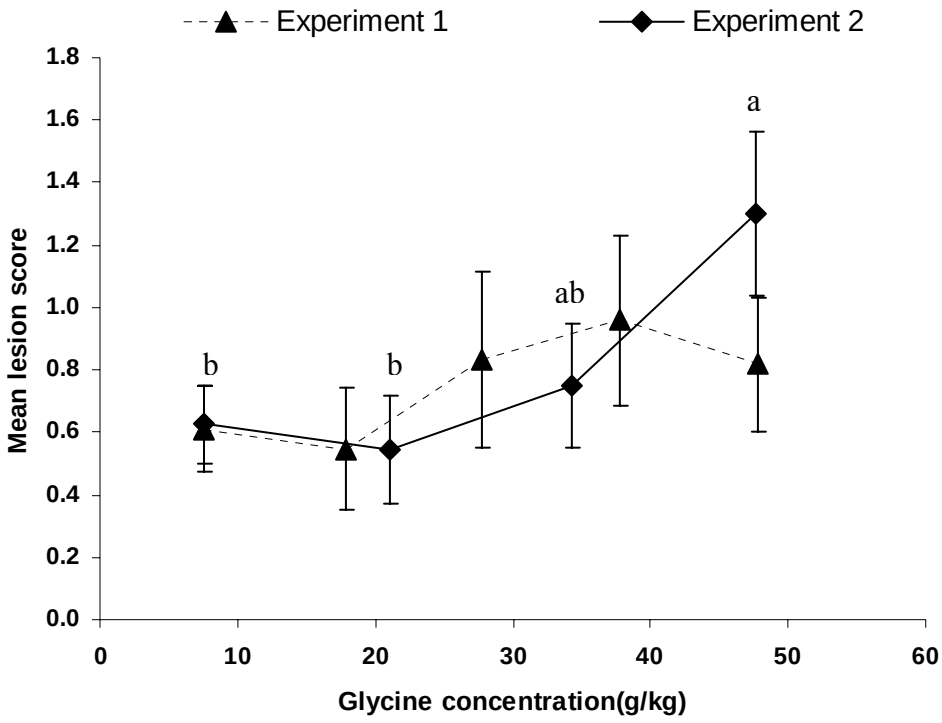


Figure 6.4. Mean necrotic enteritis lesion scores in 28-day-old broiler chickens given experimental diets (day 14 to 28) containing different levels of encapsulated glycine and proline in experiments 1 and 2.

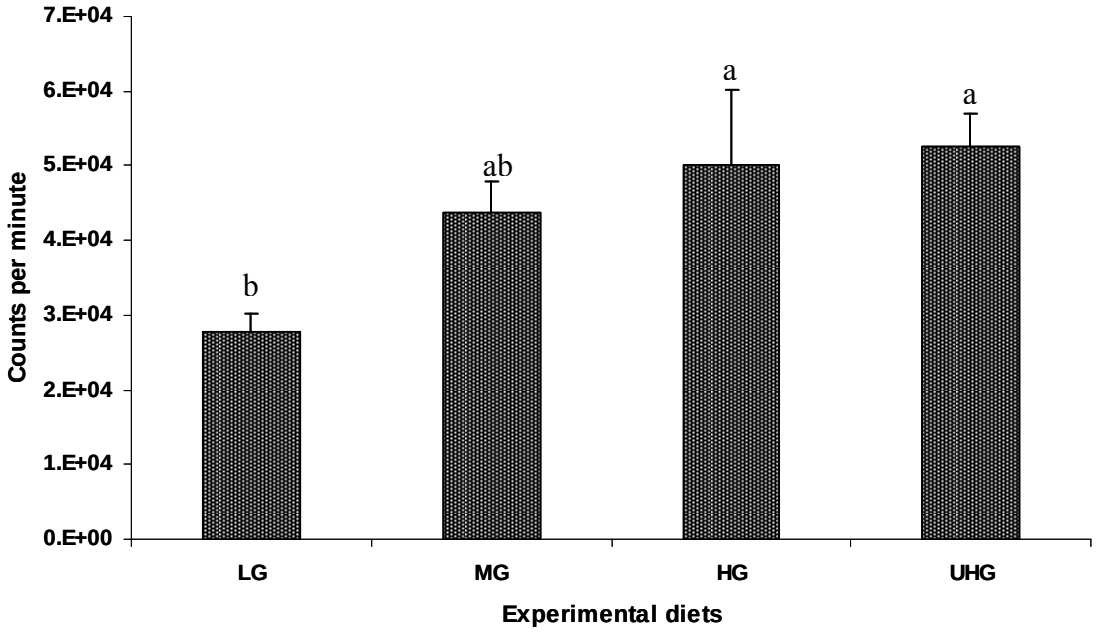


Figure 6.5. Whole blood chemiluminescence (WBCL) response of broiler chickens in experiment 2. Lucigenin-dependent, non-opsonized zymosan-stimulated WBCL response was measured on day 28 of age. Peak WBCL responses (cpm) are shown. Diet abbreviations are as follows: LG, low glycine diet i.e. 7.6 g/kg glycine and 190 g/kg CP; MG, medium glycine diet i.e. 21.0 g/kg glycine and 203 g/kg CP; HG, high glycine diet i.e. 34.3 g/kg glycine and 217 g/kg CP; UHG, ultra high glycine diet i.e. 47.7 g/kg glycine and 230 g/kg CP.

Previous studies in our laboratory have demonstrated that diets high in glycine added as animal proteins such as fish meal or gelatin can result in increased populations of *C. perfringens* (Dahiya *et al.,* 2005; Drew *et al.,* 2004). However, the addition of 40 g/kg of crystalline glycine to the diet had no effect on *C. perfringens* populations in broiler chickens (Wilkie *et al.,* 2005). Several studies have reported that crystalline amino acids are absorbed very rapidly in the small intestine (Drew *et al.*, 2003; Lingens and Molnar, 1996; Maenz and Engele-Schaan, 1996). This rapid absorption might prevent the added glycine from reaching the distal small intestine making it unavailable to enteric bacteria including *C. perfringens*.

In the pilot study, we demonstrated that lipid encapsulation increased glycine concentration in jejunum and ileum of birds and effect confirmed in the challenge experiments. This suggests that the crystalline glycine was rapidly absorbed in the upper gastrointestinal tract whereas fat encapsulated glycine was released slowly along the length of intestine and would therefore be more available to enteric bacteria. Lipid encapsulation of amino acids has previously been reported for use in aquaculture larval feeds to prevent leaching of amino acids after immersion in water (Onal and Langdon, 2004; Lopez *et al.,* 1994). However, to our knowledge, lipid encapsulated amino acids have not been evaluated in poultry.

Average daily feed intake during day 14-21 and day 21-28 was not significantly different among various dietary treatments in both experiments indicating that palatability was not an issue with the experimental diets. Average daily gain and feed conversion ratio were not significantly altered during the period 14-21 day in both experiments while dietary treatments had a significant effect on these performance parameters during 21-28

day. This might be due to the fact that although orally challenged from day 14-21, *C. perfringens* counts did not substantially increase in the intestine until the period 21-28 day. Commercial outbreaks of NE are more common around 28 day of age and may be related to maturation of the intestinal microbiota establishing conditions more conducive to *C. perfringens* colonization. As evidenced by intestinal lesion scores and high intestinal colonization of *C. perfringens*, most of the birds in the present study had a subclinical form of NE as documented by Lovland and Kaldhusdal (2001). Decreased growth rate and poor feed conversion efficiency have already been reported in broilers having high numbers of *C. perfringens* in the intestinal tract (Dahiya *et al.,* 2005; Kaldhusdal and Hofshagen, 1992; Stutz, 1983). Another possible explanation of lower ADG and poor FE in birds receiving high glycine diets in both experiments is the poor digestibility of the encapsulated material.

In the present study, we observed an increase in the intestinal populations of *C. perfringens* and decrease in lactobacilli when a high concentration of encapsulated glycine was used. However, dietary crude protein or proline level did not significantly influence these bacteria in the current study. This observation suggests that birds fed diets containing high levels of glycine will be at greater risk for a clinical outbreak of NE, increased carcass condemnations during processing and an increased risk of infection in humans.

Dietary components such as fish meal, wheat, barley and rye have been widely accepted as predisposing factors for NE (Kaldhusdal and Hofshagen, 1992; Kaldhusdal and Skjerve, 1996; Riddell and Kong, 1992). High protein diets, particularly those containing proteins of animal origin have been reported to increase the incidence of NE

and have been used successfully to induce the disease experimentally (Truscott and Al-Sheikhly, 1977; Wilkie *et al.,* 2005). A number of studies have demonstrated that source and level of protein in diets had a profound affect on the number of *C. perfringens* in intestinal contents in chickens (Dahiya *et al*., 2005; Drew *et al*., 2004; Wilkie *et al*., 2005), pigs (Mansson and Smith, 1962) and dogs (Zentek *et al.,* 1998). Kaldhusdal and Skjerve (1996) conducted a longitudinal study of NE and feeding practices in Norway over a 20-year period and documented an increased incidence of NE with the use of rendered animal products. Because animal-source proteins contain from 2 to 4 times as much glycine as a percentage of crude protein compared to plant based proteins, glycine content may be one factor in animal protein-based diets that could be responsible for over proliferation of *C. perfringens* in the intestinal tract of animals.

Previously Drew *et al.* (2004) documented a significant increase in *C. perfringens* populations in ileum and cecum of broiler chickens fed fish meal-based diets with 400 g/kg CP. However, there was no difference in *C. perfringens* growth in birds that received soy protein concentrate-based diets containing 230, 315 or 400 g/kg crude protein. Amino acid analysis of the diets suggested that glycine was in relative excess in 400 g/kg CP diet than 400 g/kg CP soy protein concentrate diet. Wilkie *et al.* (2005) compared the effect of various protein sources (animal vs. plants) on intestinal colonization of *C. perfringens* in broiler chickens and reported a significant correlation between dietary glycine concentration and the number of *C. perfringens* in both ileum and cecum. Correlations between all other dietary amino acids and *C. perfringens* populations were not significant. They also observed a significant correlation between the glycine content of ileal digesta and *C. perfringens* numbers in both ileum and cecum.

Earlier we observed a significant quadratic response for *C. perfringens* growth in cecum of 28-day-old broiler chickens with maximum response at 33.0, 38.9 and 35.1 g/kg dietary glycine concentration with r^2 values of 0.95, 0.99 and 0.95 in three different experiments. Also number of lactobacilli in cecum declined significantly with increasing levels of glycine in these experiments (Dahiya *et al.,* 2005). Although glycine levels and *C. perfringens* populations were strongly correlated in this study, but gelatin was used in the experimental diets which also contains high levels of proline and results of these experiments were confounded by this factor.

In the present study, the number of lactobacilli decreased with increasing dietary glycine concentration, and this effect was clearer in experiment 2 than experiment 1. There was almost one log decrease in lactobacilli counts (both in ileum and cecum) in birds fed either the lowest and highest levels of dietary glycine. Interestingly, the effect of various dietary treatments on intestinal lactobacilli populations was opposite to that on *C. perfringens* populations which is consistent with earlier findings in which protein-bound glycine was fed (Dahiya *et al*., 2005; Wilkie *et al.,* 2005). One possible explanation is that high dietary glycine concentration might cause a reduction in lactic acid producing bacteria (mainly *Lactobacillus, Streptococci*, and *Staphylococci*) which are predominant in the intestines of normal healthy chickens and generally considered to be protective against colonization of pathogenic micro-organisms. Fukata *et al.* (1991) reported that pathogenic effect of *C. perfringens* could be reduced by feeding chicks a monoflora of *Lactobacillus acidophilus* or *Streptococcus faecalis*. The probiotic strains of lactobacilli which are able to colonize the human intestinal tract induce epithelial cells to secret mucins that diminish enteric pathogens binding to mucosal epithelial cells

(Johansson *et al*., 1993). An antagonistic effect of lactobacilli against *Helicobacter pylori* (Aiba *et al.,* 1998), *Salmonella* spp. (Gill *et al.,* 2001), *E.coli* (Mangell *et al*., 2002) and *Listeria monocytogenes* (de Waard *et al.,* 2002) has been demonstrated in murine model. The antagonistic activity may be mediated either directly by the production of inhibitory substances such as lactic acid, hydrogen peroxide and bacteriocins or by competing with the pathogen for binding sites on the epithelial cell surface.

The exact mechanism(s) by which glycine promotes the intestinal growth of *C. perfringens* in broiler chickens is unclear. Titball *et al.* (1999) documented that growth of *C. perfringens* and production of α toxin is influenced by various amino acids. Glycine accelerated the *C. perfringens* growth (Ispolatovskaya, 1971) while α toxin production required the presence of glycine-containing peptides in defined media (Nakamura *et al*., 1968; Stevens and Rood, 2000). Putrefactive clostridia and anaerobic Gram-positive cocci have been considered to be important amino acid fermenting bacteria (Mead, 1971). Earlier in an *in vitro* study of amino acid fermenting bacteria in human large intestine, it was reported that all clostridia were able to attack single amino acids, with pairs of amino acids not being essential, although *Clostridium bifermentans* and *C. indolis* were enhanced when provided with Stickland amino acid pairs (Smith and Macfarlane, 1998). It is well documented phenomenon that some strict anaerobic bacteria have a unique energy conserving mechanism and they catalyze with glycine as substrate using an internal Stickland reaction in which glycine serves as electron donor during oxidation by a glycine cleavage system or as electron acceptor being reduced by glycine reductase. The glycine reductase system is present in several clostridia (*C. sticklandii, C. difficile, C. litorale* and *Eubacterium acidaminophilum*) and catalyzes the reductive deamination of

glycine to acetylphosphate and ammonia with the generation of adenosine 5'-triphosphate from adenosine 5'-diphosphate and orthophophate (Costilow, 1977; Andreesen, 1994). However, *C. perfringens* is not a protein fermentor and this suggests that the effect of higher levels of dietary glycine on intestinal *C. perfringens* populations might be indirect. As *C. perfringens* is a strong mucolytic bacterium, it enjoys a growth advantage over several other bacteria in the intestinal tract (Deplancke *et al*., 2002). High glycine diets may somehow enhance mucus production and secretion through up regulation of mucin gene, thus providing intestinal mucus constantly. Another possibility is that the protein/amino acids are serving as energy and/or nitrogen source for other microflora in the intestinal tract that in turn modify the intestinal micro-environment in a way which promotes clostridial overgrowth or stimulates toxin production in the intestinal lumen. This may occur by providing positive selection pressure on those gastrointestinal microbes which can directly ferment amino acids for carbon and nitrogen, in doing so make available to *C. perfringens* nutritional resources that would be otherwise unavailable in highly competitive environment of intestinal tract. Alternatively, the high glycine diets might affect the host in such a manner that the gastrointestinal milieu is made more favorable for *C. perfringens* proliferation i.e. induction of an inflammatory response, which in turn leads to leakage of serum proteins into the gut lumen. This leakage of serum protein may ultimately provide the environmental stimulus required for an expansion of *C. perfringens* population, and perhaps even the up regulation of virulence factors (Shane *et al*., 1985). Clearly, further research is required to elucidate the exact mechanism by which glycine increases *C. perfringens* populations in the intestinal tract of broiler chickens

In the current study, despite inoculation of very high doses (6.38×10^5 to 7.29×10^6 CFU/mL) it was not possible to induce NE-specific mortality. Previous controlled studies of chickens challenged with *C. perfringens* have also failed to induce mortality or other signs of NE, even though high *C. perfringens* colonization was reported in the intestinal tract of the birds (Cowen *et al*., 1987; Drew *et al*., 2004; Wilkie *et al*., 2005). In several of the previously described models of clinical NE, birds were co-infected with *Eimeria* spp. along with *C. perfringens* (Al-Sheikhly and Al-Saieg, 1980; Shane *et al*., 1985; Triscott and Al-Sheikhly, 1977), but the model described in the present study is designed for sub clinical form of the disease because lesions are more sensitive disease indicator than mortality, and sub clinical NE is more frequent than clinical disease in broiler chickens as described by Kaldhusdal *et al.* (1999). Since *C. perfringens* is the etiological agent of NE it seems reasonable to assume that increased numbers of this organism in the gut are associated with an increased predisposition to clinical NE.

In agreement with our previous findings, with an increase in dietary glycine concentration there was a corresponding increase in NE lesion scores although the differences were only significant in experiment 2, between birds fed UHG and LG or MG diets. The demonstration of a relationship between NE lesion scores, performance data and *C. perfringens* numbers is an important feature of both experiments. There was no bird with full blown field type lesion of NE (i.e. with score of 4) in any of the experiments, although there were two birds with score of three in experiment 2. Lesion scores were very similar in birds fed either control or G0P4 diets indicating that dietary crude protein or proline concentrations had no effect on *C. perfringens* growth and NE lesions in these birds. In the present study, even clinically healthy birds were highly

colonized with *C. perfringens* which confirms the sub clinical nature of the disease because high numbers of *C. perfringens* with macroscopically visible, focal necrotic lesions in the small intestine is a strong indicator of an occurrence of NE (Kaldhusdal and Hofshagen, 1992). Previously, *C. perfringens* has also been isolated from the bile ducts and livers of affected birds and is associated with fibrosing hepatitis, which results in liver abnormalities and condemnation of processed birds. It would be interesting to enumerate *C. perfringens* in liver tissue and examine the liver for any lesions in subsequent studies.

The WBCL response was significantly elevated in HG and UHG groups compared to LG treatment. There was a nice relationship between the WBCL response, intestinal *C. perfringens* populations and NE lesions in the current study. Elevated WBCL responses are generally correlated with increased PMN numbers in blood in different species including chickens (Nagahata *et al.,* 1991; Tono-Oka *et al.,* 1983). Leukocytosis and heterophilia are often associated with infectious agents such as bacteria in birds, and the magnitude of heterophilia usually indicates the magnitude or severity of the initiating inflammatory process. Although the role of heterophils in fighting against bacterial infection usually involves infiltration of heterophils at the site of invasion, heterophil infiltration in the lesions was not observed in our experiments. This may be due to the fact that α toxin prevents the influx of PMNs at the site of infection (Bryant et al., 1993; Stevens et al., 1997). To our knowledge, this is the first report using the WBCL assay as a measure of innate immune reponses in domestic chickens exposed to various experimental treatments. Specifically, we used this assay to gain information about

phagocyte function in the blood of chickens on different experimental diets and challenged with *C. perfringens*.

In contrast to some earlier findings, the microscopic lesions in intestine were not conclusive of NE in the present study (Long *et al*., 1974; Shane *et al.,* 1985). Presence of slight edema and hemorrhages in lamina propria were observed in few birds. However, desquamated epithelial cells and polymorphonuclear cells (PMN) were not detected in any of the sections. Previously, an absence of PMN cells at the site of *C. perfringens* infection had been demonstrated, and it was postulated that higher *in situ* concentration of *C. perfringens* toxins, especially α toxin, may inhibit PMN influx (Stevens *et al*., 1997). So in spite of high numbers of *C. perfringens* in the intestinal tract of these birds, the clinical disease could not be produced. It is possible that some other triggering factors might be required to produce a full blown disease through secretion of α toxin. An experimental study investigating the effect of purified α toxin on intestinal epithelium is needed in order to provide evidence of a casual relationship between α toxin and NE changes.

In conclusion, dietary glycine concentration plays an important role in altering *C. perfringens* and lactobacilli populations in the intestinal tract of broiler chickens, whereas proline concentration or crude protein level as such has no significant effect on this important group of bacteria. Although there were very high numbers of *C. perfringens* in the intestinal tract of these birds, still the disease typical of field cases could not be produced, which again demonstrates a complex multi-factorial epizootiology of NE. Nevertheless high numbers of *C. perfringens* in the intestine are a strong indicator of an occurrence of clostridial enteritis. Poor feed conversion efficiency and reduced body

weight gain in birds with high *C. perfringens* colonization signifies the economic importance of this disease for the poultry producers. Balancing feed composition is probably the most cost-effective prevention and control measure of NE. Knowledge of specific dietary components, such as amino acid profile of different feed proteins and their propensity to cause disease, may aid in the formulation of broiler diets that reduce the risk of clostridial enteritis in post-antibiotic era. The subclinical model of NE in broilers as presented here is suitable to evaluate the effects of feed additives and diet composition upon the occurrence of NE in broilers.

6.6. Conclusions

In conclusion, dietary glycine concentration plays an important role in altering *C. perfringens* and lactobacilli populations in the intestinal tract of broiler chickens, whereas proline concentration or crude protein level as such has no significant effect on this important group of bacteria. Although there were very high numbers of *C. perfringens* in the intestinal tract of these birds, still the disease typical of field cases could not be produced, which again demonstrates a complex multi-factorial epizootiology of NE. Nevertheless high numbers of *C. perfringens* in the intestine are a strong indicator of an occurrence of clostridial enteritis. Poor feed conversion efficiency and reduced body weight gain in birds with high *C. perfringens* colonization signifies the economic importance of this disease for the poultry producers. Balancing feed composition is probably the most cost-effective prevention and control measure of NE. Knowledge of specific dietary components, such as amino acid profile of different feed proteins and their propensity to cause disease, may aid in the formulation of broiler diets that reduce the risk of clostridial enteritis in post-antibiotic era. The subclinical model of NE in

broilers as presented here is suitable to evaluate the effects of feed additives and diet composition upon the occurrence of NE in broilers.

7.0. OVERALL CONCLUSIONS

NE is a disease of poultry that has a high economic and animal welfare cost and has become increasingly prevalent in the world especially in the European Union due to removal of AGPs and coccidiostats from diet formulations. Currently, a large research effort is going on through out the world to develop cost-effective alternatives to AGPs in poultry feed. However, no single satisfactory non-antibiotic measure against *C. perfringens* has been identified so far. Strategies to control NE in the absence of AGPs, without resorting to the use of prophylactic or therapeutic treatment, have mainly centered upon dietary and management practices.

In order to explore new methods of controlling NE it is essential to be able to experimentally induce the disease in a controlled and reproducible manner that will allow testing of various therapeutic agents, feed additives, vaccines or other approaches. Nearly without exception, all current NE models employ a polymicrobial challenge using coccidia along with *C. perfringens*, the main etiological agent. However, to date, there is no generally accepted robust disease model for NE in poultry. In our model, the birds were not only challenged with a high dose of *C. perfringens* on day-of-hatch, but they were also orally gavaged for an additional seven days from day 14 through 20. This challenge model was based on work done by both others and ourselves and resulted in a reliable model for intestinal *C. perfringens* colonization. However, this model did not produce the disease typical of field cases although there were varying degrees of intestinal lesions of NE in most experiments. Additionally the microscopic lesions in the intestinal tissues were not conclusive of NE which illustrates a weakness of our challenge

model. Hence more comprehensive research is needed in this direction to develop a reliable and reproducible model of NE in poultry.

Chemical composition of the diet related to cereal grain selection, protein source and amino acid profile might influence disease propensity. Thus, a consideration of dietary chemical composition might be helpful in formulation of poultry diets to reduce the incidence of NE. The chicken gastrointestinal tract may contain more than 650 bacterial species and they derive most of their energy for reproduction and growth from dietary compounds which are either not absorbed by the host or absorbed so slowly that bacteria can successfully compete for them. As different bacterial species have different substrate preferences and growth requirements, the chemical composition of the digesta largely determines the composition of microbial community in the chicken GIT. As a result, the microbial community structure is very much dependent upon the diet as the ultimate source of substrates for metabolism. This suggests that one of the most effective method to manage the enteric pathogens in the absence of AGPs is through appropriate ingredient selection and diet formulation that will influence the competitiveness of harmful and beneficial bacteria by changing the gut dynamics.

The amino acid composition and digestibility of dietary proteins has a profound impact on the incidence and severity of NE through altering the *C. perfringens* growth and/or α toxin synthesis and release. *C. perfringens* lacks the ability to produce at least 13 amino acids out of 20 essential amino acids, and its growth is therefore enhanced in an environment rich with amino acids. Putrefactive clostridia and anaerobic gram-positive cocci have been considered to be important amino acid fermenting bacteria. Diets that contain relatively high concentrations of protein, as well as those with imbalanced

profiles of amino acids, have reduced digestibility in the upper GIT. Larger concentrations of these compounds are found in the lower GIT which act as substrates for the microflora, thus encouraging the proliferation of enteric pathogens including *C. perfringens*. It is worth noting that the amino acids are not always associated with *C. perfringens* growth. In one of our studies, we observed a significantly reduced population of *C. perfringens* in the intestinal tract of broiler chickens when fed high concentrations of dietary methionine. The antibacterial effect against *C. perfringens* was not different among two commonly used methionine sources (DL-Met and MHA-FA). So there is a possibility of controlling NE in broiler chickens using low crude protein diets supplemented with relatively high concentrations of methionine. A further assessment of the putative associations between these methionine sources and NE is of considerable interest. Further studies to examine the impact of these two methionine sources on a wider group of bacteria in the GIT using PCR-based techniques would be an interesting addition to the present study.

The present studies demonstrated that the presence of glycine in the intestinal environment provides a stimulus for *C. perfringens* growth, thus increasing the risk of NE. It is still unclear whether glycine has a direct effect on *C. perfringens* or it affects indirectly through altering various other bacterial species in the GIT of birds. Some strict anaerobic bacteria have a unique energy conserving mechanism and they catalyze with glycine as substrate using an internal Stickland reaction. The glycine reductase system is present in several clostridia such as *C. sticklandii, C. difficile, C. litorale* and *Eubacterium acidaminophilum.* As *C. perfringens* is not a protein fermentor, the effect of higher levels of dietary glycine on intestinal *C. perfringens* populations might thus be

indirect. *C. perfringens* is a strong mucolytic bacterium, so it enjoys a growth advantage over several other bacteria in the intestinal tract. High glycine diets may somehow enhance mucus production and secretion through up regulation of mucin gene, thus providing intestinal mucus constantly. So further studies are needed to determine the exact mechanism(s) how glycine affects this important group of bacteria.

We also demonstrated a reduced lactobacilli population in birds fed high dietary glycine. Again it is hard to speculate whether it is lactobacilli populations that decrease first, resulting in *C. perfringens* overgrowth or vice versa. A better understanding of the dynamics of bacterial populations shifts due to dietary glycine concentrations are essential to developing a better understanding of the etiology of NE. However, it is possible to conclude that it is important to keep the glycine concentration to a minimum required level while formulating broiler diets to reduce the risk of NE, especially in adult birds.

In the present studies, we enumerated only some specific groups of intestinal bacteria using culture based methods. However, given the complexity of the intestinal microbiota, it would be extremely useful to use culture-independent approaches such as PCR with denaturing gradient gel electrophoresis that would allow the study populations shifts in a large number of intestinal bacterial species which may be involved in the development of clinical NE.

A number of unresolved questions remain in determining the role of diet in predisposing broiler chickens to NE. What are the associated changes in intestinal morphology, gene expression and nutrient uptake that are associated with these dietary factors? Since *C. perfringens* metabolizes nutrients in the gut that were not absorbed by

the bird, what is the effect of reducing nutrient availability to the bacteria by increasing nutrient digestibility or lowering levels of dietary crude protein in broiler diets? How does the protein content of the diet interact with other dietary ingredients such as small grains? The answers to these questions may provide poultry producers with new tools for the reduction of NE in broiler chickens with the use of antibiotics.

New research should focus on reducing the risk of NE through control of *C. perfringens* in the immediate environment, removal of coccidiosis as a risk factor, and elimination of predisposing dietary factors. An improved understanding of the host, the microbes, molecular and cellular mechanisms of bacteria-host interactions, activation of innate and adaptive immune response in poultry and toxin-induced signal transduction across the intestinal epithelium is of prime importance. Further work is required to identify the initiating events of pathogenesis of NE. It is necessary to characterize various molecular and biochemical events that signal the cellular cascade that eventually results in clinical disease.

We are entering a new era of poultry nutrition where antibiotics and coccidiostats are becoming increasingly unavailable to the poultry industry. Thus new methods to control enteric pathogens must be developed. An improved understanding of the relationship between feed chemistry and enteric bacteria will help us in formulating broiler diets that might reduce the risk of various enteric pathogens including *C. perfringens.*

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9.0. APPENDICES

9.1. Appendix A

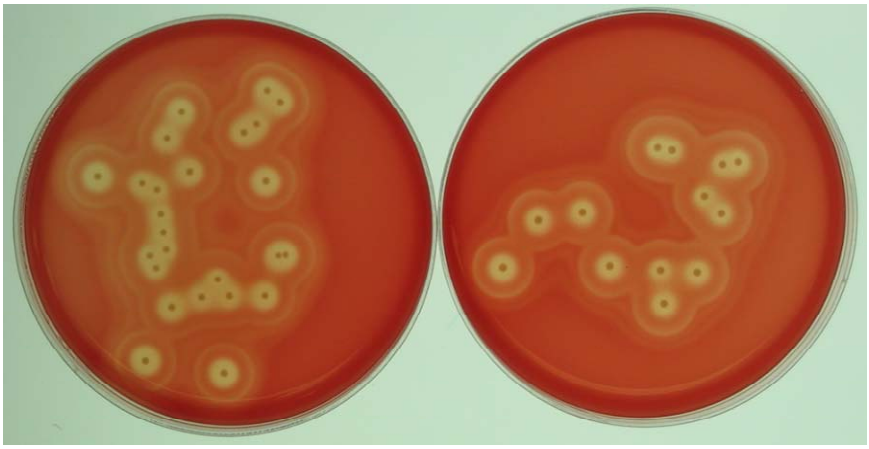

Figure 9.1. Macroscopic appearance of *C. perfringens* colonies on Blood Agar Base containing 50 mL/L sheep blood and 0.01% neomycin sulfate, and incubated anaerobically for 24 h at 37°C. The α- and β-hemolytic zones are clearly visible.

9.2. Appendix A

This *in vitro* experiment was conducted to find out the growth medium which supports the α toxin production best so that we can use the same medium for growing *C. perfringens* for challenging the birds orally because the α toxin is the main virulence factor responsible for NE in chickens. We studied three commonly used media for *C. perfringens* growth at two different concentrations (full strength, 100% and half strength, 50%). 1. Brain heart infusion broth (BHI), 2. Cooked meat medium (CMM), and 3. Brewer's thioglycolate broth (TGB)

Six, 250 mL culture flasks, each containing 100 mL of respective media (BHI, CMM and TGB) were used in this *in vitro* experiment. Culture flasks were pre-reduced overnight in anaerobic glovebox and the experiment was conducted anaerobically in the glovebox at 37˚C. 500 µL actively growing culture of *C. perfringens* type A was inoculated in each flask. At times 0, 2, 4, 6, 8, 12, 24, and 48 h, 500 µL culture were removed from each flask and centrifuged. The supernatant was stored quickly at -80°C. The α toxin (PC-PLC) activity was estimated using Amplex Red Phosphatidylcholine-Specific Phospholipase C Assay Kit (Molecular Probes; Cedarlane Lab. Ltd.) according to the manufacture's instructions.

We observed a much higher α toxin production in cooked meat media compared to brain heart infusion broth and thioglycolate broth **(Figure 9.2A)**. Also there was not a significant difference in α toxin production between full strength (100%) and half strength (50%) cooked meat media and the production was maximum at h 8 in 50% CMM.

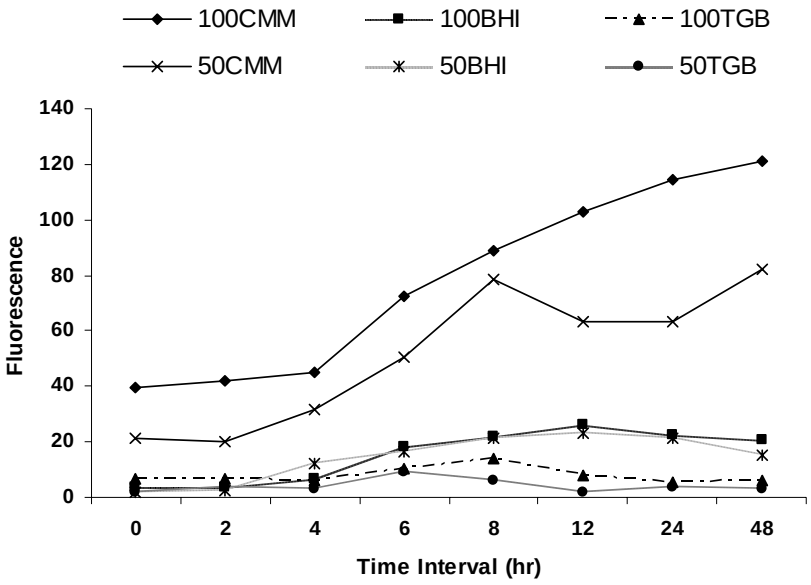

Figure 9.2A. Relative Phospholipase C production by *C. perfringens* in different media (CMM, cooked meat medium; BHI, brain heart infusion agar; TGB, thioglycolate broth) at different intervals of time.

9.2. Appendix B

This experiment was conducted to determine the relationship between *C. perfringens* populations and α toxin production *in vitro.*

Six, 250 mL culture flasks, each containing 100 mL of cooked meat media in three different concentrations i.e. 100, 50 and 10%, were used in this *in vitro* experiment i.e. there were two flasks for each concentration. Culture flasks were pre-reduced overnight in anaerobic glovebox and the experiment was conducted anaerobically in the glovebox at 37˚C. 500 µL of actively growing culture of *C. perfringens* type A were inoculated in three flasks (one from each concentration). At times 0, 2, 4, 6, 8, 12, 24, and 48 h, 1mL culture was removed aseptically from each flask and divided in two aliquots. One aliquot (50µL) was diluted to10^{-2} and 10^{-4} dilutions and plated on $BA^{Neomycine}$ plates in duplicates and plates were incubated at 37°C anaerobically. Second aliquot was centrifuged; supernatant was collected and stored immediately at -80°C for α toxin estimation using Amplex Red Phosphatidylcholine-Specific Phospholipase C Assay Kit (Molecular Probes, Cedarlane Lab. Ltd.).

We observed that there was a peak of α toxin at h 6 and 8 in 100 and 50% cooked meat media, respectively. Also there was an exponential growth of *C. perfringens* up to 6 h in full strength media and it enters into the stationary phase. The exponential growth was achieved in first 2 and 4 h, respectively in 10 and 50% cooked meat media (**Figure 9.2B**).

Conclusions

From the above two *in vitro* experiments, we concluded that it is the cooked meat media which supports α toxin production best. There was a direct correlation between *C.*

perfringens count and α toxin production *in vitro*, which may not be true *in vivo*. We also concluded that *C. perfringens* count and α toxin production was at its peak between 6 to 8 h in cooked meat media. So, we decided to use 6-8 h cooked meat media broth culture of *C. perfringens* for oral gavaging in our subsequent experiments so that we can challenge the birds with high number of actively growing *C. perfringens* and also a high amount of α toxin.

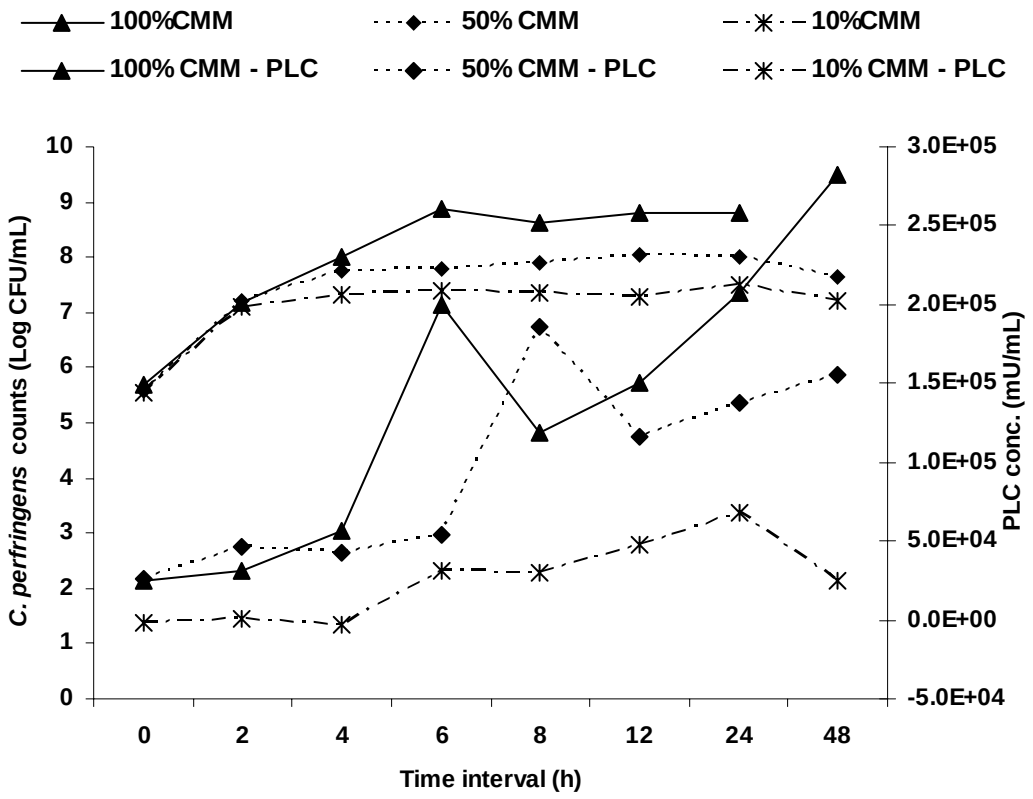

Figure 9.2B. *C. perfringens* growth and phospholipase C production in various concentrations of cooked meat medium at different time intervals.

9.3. Appendix A

Supplemental image for chapter 5**.**

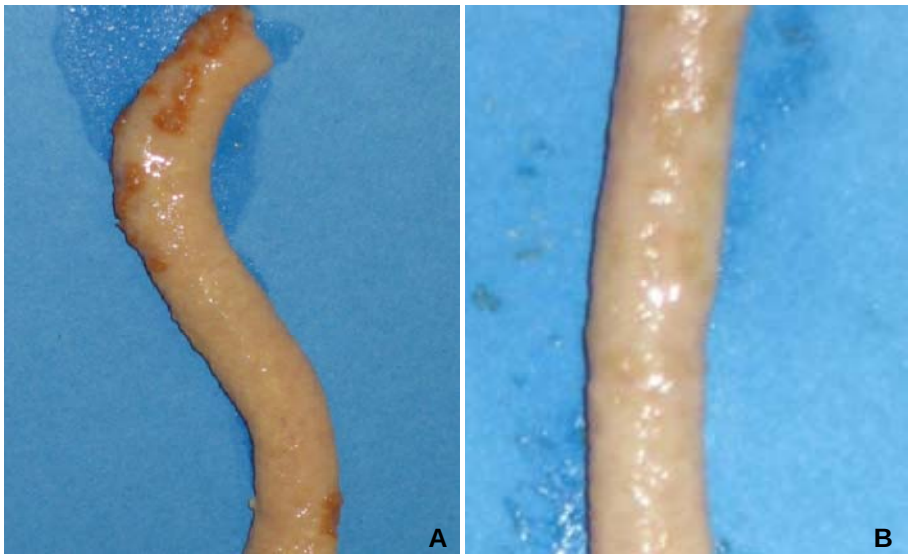

Figure 9.3A. Photographs showing mucosal surface of terminal ileum of 28-day-old broiler chickens challenged with *Clostridium perfringens* type A from 14-20 days of age. Mucosa is seen necrosed and covered with brownish pseudo-membrane (A and B).

9.3. Appendix B

Supplemental image for chapter 5.

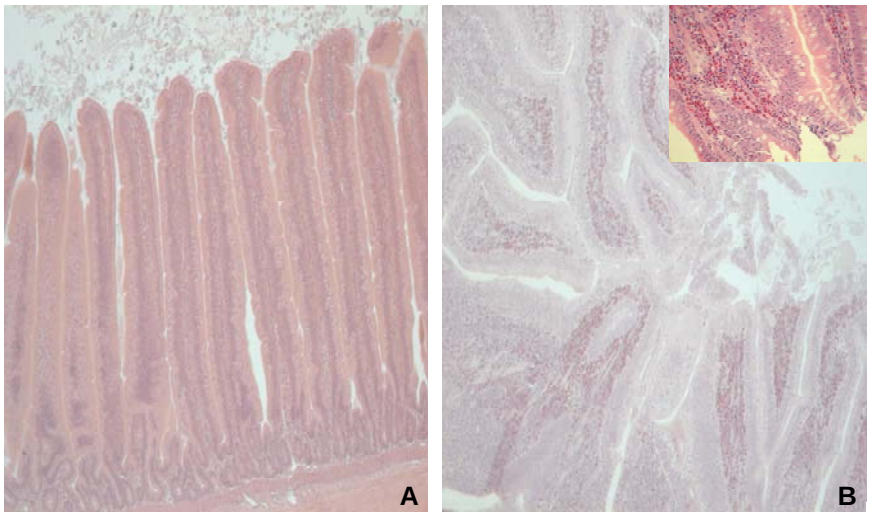

Figure 9.3B. Photomicrographs of intestinal mucosa in normal healthy birds (A) and birds that developed lesions following the oral challenge with *C. perfringens* type A (B). Original magnification: 50X. Severe congestion and/or hemorrhages were commonly present through out the lamina propria in challenged birds. Images are of formalin-fixed, H & E-stained tissue using an Axiostar *plus* Zeiss microscope (Carl Zeiss Vision GmbH, Germany) fitted with Axiocam MRc camera.