THE ROLE OF PROTEIN AND AMINO ACID NUTRITION IN CONTROLLING *CLOSTRIDIUM PERFRINGENS* IN THE GASTROINTESTINAL TRACT OF BROILER CHICKENS

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by

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ABSTRACT

A series of experiments were conducted to examine the effect of dietary protein and amino acids (AA) on intestinal *Clostridium (C.) perfringens* proliferation and necrotic enteritis (NE) in broiler chickens.

The effects of dietary protein level and protein digestibility on the growth performance and carcass characteristics of broilers were evaluated first. Low protein (*LoPro*) diets supported growth performance equal to high protein (*HiPro*) diets when highly digestible protein supplements were incorporated. Birds fed *HiPro* yielded more breast meat than those fed *LoPro* while birds fed diets with highly digestible (*HiDig*) protein supplements accumulated more abdominal fat than those fed diets containing low digestible (*LoDig*) protein ingredients.

The next experiment focused on the development of a feasible experimental model to induce NE experimentally in broilers to investigate potential dietary approaches. Different modes of challenge (oral gavage, in-feed and in-water) with different doses of *C. perfringens* inoculums (2 or 4 ml) were tested using microbiological, pathological and hematological parameters. The findings suggested the possibility to applly any of the evaluated treatments to induce the disease in broilers raised on litter.

The next experiment was conducted to examine the effects of dietary protein level and digestibility on the growth performance and intestinal ecosystem of *C. perfringens* challenged and unchallenged broilers. The results of this experiment again revealed the ability of *LoPro* diets to maintain the growth performance of broilers as with *HiPro* diets, when *HiDig* protein supplements were included. The findings further demonstrated significant reduction in the intestinal *C. perfringens* numbers and severity

of intestinal lesions of infected broilers when *LoPro* diets and *HiDig* protein supplements were fed.

The last experiment was performed to assess the methionine requirement for optimum growth in broiler chickens fed diets supplemented with either DL-methionine (DLM) or 2-hydroxy-4-methylthiobutanoic acid (HMB) under *C. perfringens* infected and uninfected situations. None of the methionine sources affected the growth of *C. perfringens* in the broiler GIT, however, infected birds needed more dietary methionine to achieve maximum growth when DLM, but not HMB, was included in the diet.

Based on the overall findings it can be concluded that the dietary approaches tested in this project may support our efforts in the development of nutritional strategies to minimize the impact of *C. perfringens* on broiler production in an antibiotic free livestock industry.

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DEDICATION

With a great respect and heartfelt gratitude, this work is dedicated to my school Rahula College, Matara – Sri Lanka, and all my past teachers.

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LIST OF ABBREVIATIONS

AA	Amino acid/s
ADFI	Average daily feed intake
ADG	Average daily gain
AGP	Antibiotic growth promoter/s
AI	Auto inducer/s
AID	Apparent ileal digestibility
BW	Body weight
cfu	Colony forming units
CP	Crude protein
DLM	DL-methionine
DM	Dry matter
FCR	Feed conversion ratio
FOS	Fructo oligosaccharides
GIT	Gastrointestinal tract
*h*CP	High crude protein
HiDig	High digestible
HiPro	High protein
HMB	2-hydroxy-4-methylthiobutanoic acid
*l*CP	Low crude protein
LoDig	Low digestible
LoPro	Low protein
MBM	Meat and bone meal
ME	Metabolizable energy

MOS	Mannan oligosaccharides
MR	Molar ratio
NE	Necrotic enteritis
NSP	Non-starch polysaccharides
PCR	Polymerase chain reaction
PER	Protein efficiency ratio
PMN	Polymorphonuclear cells
PPC	Potato protein concentrates
QS	Quorum sensing
RLU	Relative light units
ROS	Reactive oxygen species
SID	Standardized ileal digestible/digestibility
VFA	Volatile fatty acid/s
WBCL	Whole blood chemiluminesence
W-DDGS	Wheat distiller’s dried grains with solubles

1. INTRODUCTION

The gastrointestinal tract (GIT) of farm animals acts as a reservoir for the maintenance and proliferation of gut-specific pathogens capable of inducing diseases in both livestock and humans. Due to the concerns associated with growth performance, bird welfare and poultry product contamination, enteric pathogens are considered an important issue in the poultry industry. Consequently, the inclusion of antibiotic growth promoters (AGP) in poultry rations has been the main strategy for controlling enteric pathogens for many years. Nevertheless, there is an ongoing debate that the continued use of in-feed AGP may result in selection pressure for intestinal microbes that are resistant to certain antimicrobials (JETACAR 1999). In most cases, the drug resistance is encoded on plasmids which may be transferred to other bacteria even in the absence of selection pressure by antibiotics. Hence, the widespread use of antimicrobials, both as therapeutic agents and as AGP, is believed to have contributed to the increased incidence of bacterial resistance to antimicrobials in various human and animal pathogens (WHO 1997; JETACAR 1999). Consequently, use of antibiotics in the livestock industry is closely monitored by all concerned parties; hence, a reduction or total ban on the use of in-feed antibiotics is expected to improve human and animal health, lowering the stress of antibiotic resistance (Ratcliff 2000).

Dietary incorporation of antibiotics in broiler ration formulation has been the commonly employed approach to control the proliferation of *Clostidium* (*C.*) *perfringens,* the causative organism responsible for the disease necrotic enteritis (NE) that leads to reduced productivity and severe economic losses to producers. However, a ban on growth-promoting antibiotic usage in the animal feed industry of some countries has resulted in a re-emergence of NE incidences in broiler chickens (Drouin 1999;

Kaldhusdal and Lovland 2000). Accordingly, with this ban on AGP in these countries and the potential for deterrence in other poultry producing countries, there is an earnest interest in finding alternative strategies to antibiotics to combat this hygienically and economically important pathogen. In this context, there has been increasing investments of both time and money to explore alternatives to keep the growth performance intact, while maintaining the health, safety and acceptability of the resultant product for human consumption (Ewing and Cole 1994).

With a total ban of AGP in animal feed industry, a change in the microbial ecosystem in the GIT of broilers would be inevitable (Knarreborg et al. 2002; Dumonceaux et al. 2006). Nevertheless, our understanding of the microbial ecosystem and its management is enriched by the recent research in this area. Accordingly, this advanced understanding of the microbial ecology of the GIT and associated management practices can potentially be utilized for a sustainable broiler industry, keeping threatening enteric bacterial infections like NE in broiler chickens in check. These approaches must address not only the cost and efficiency of production, but also the impact of health and diseases to both livestock and consumer. Thus, the experiments described herein were undertaken to study the interaction between dietary amino acids (AA) and gut *C. perfringens* populations and concomitant effects on the productivity of broiler chickens.

2. LITERATURE REVIEW

2.1. Necrotic enteritis in broiler chickens

2.1.1. The disease

The disease NE was first reported in Australia (Bennetts 1930), but fully depicted in the United Kingdom in the early nineteen sixties (Parish 1961a, b, c). However, NE did not attract much attention until the recent past (van der Sluis 1997) and now it is a global threat to the entire poultry industry (van der Sluis 2000a, b; Van Immerseel et al. 2004). Broiler chickens of 17 or 18 days of age are most susceptible to this disease and overgrowth of the causative organism, *C. perfringens,* in the small intestine of affected birds leads to mucosal necrosis and acute diarrhea (Fukata et al. 1991; Ross 1999). Furthermore, entering into the portal circulation and biliary system, this pathogen instigates hepatitis and cholangio-hepatitis in broiler chickens (Lovland and Kaldhusdal 1999, 2001; Sasaki et al. 2000). If immediate measures are not taken to treat and control the situation, mortality rates can reach 1% per day within a flock and up to 40% mortality in an affected flock can be expected (Boulianne 1999; Ross 1999). Nevertheless, the sub-clinical type of NE has a greater economic impact due to impaired productivity of a far greater number of birds and increased carcass condemnation at slaughter, due to the concealed nature of the disease (Prescott 1979; Stutz and Lawton 1984; Kaldhusdal and Hofshagen 1992; Lovland and Kaldhusdal 1999, 2001). In addition, the existence of *C. perfringens* in poultry products is a public health concern, as well, due to the potential for food born infections (Van Immerseel et al. 2004). Necrotic enteritis has thus become one of the most important poultry diseases from both a productivity and health standpoint.

2.1.2. The pathogen

William H. Welch first recovered *Bacillus welchii* in 1890 from a person who had died 8 hours before as a result of a variety of complications (Lucey and Hutchins 2004). Based on microscopic characteristics, the organism was described as akin to anthrax bacilli, but having rods with boxed edges and often detected singularly or in pairs rather than chains. Later, the name was changed to *Clostridium welchii* and then again to the current *C. perfringens* (Gatsos 2007). This Gram-positive, rod-shaped, spore-forming and non-motile microorganism is anaerobic in nature and widespread in the environment as well as the GIT of clinically infected and healthy birds (Hatheway 1990; Ficken and Wages 1997). In addition, this microorganism is a common member of the intestinal microflora of most bird species (Asaoka et al. 2004). Previous studies (Hofacre et al. 2003) have categorized *C. perfringens* as an opportunistic pathogen owing to its ability to elicit the onset of NE under some circumstances.

Several bacterial species have been identified as toxin producing, but none produce as many varieties as *C. perfringens* (Hatheway 1990). This pathogen can secrete a variety of toxins and enzymes which may act in a synergistic or additive manner to produce the associated lesions and symptoms in hosts (Awad et al. 1995; Stevens and Bryant 1997; Shimizu et al. 2002). Over a dozen toxins of C. *perfringens* have been documented so far and have been categorized into major and minor groups (Hatheway 1990; Shimizu et al. 2002).

Strains of *C. perfringens* are biologically grouped as A, B, C, D and E, according to the synthesis of four key toxins: α, β, ε and ι (Songer 1996; Petit et al. 1999). Mainly toxinotype A, but to a lesser degree type C are responsible for the clinical and subclinical versions of NE in poultry (Songer and Meer 1996; Engström et al. 2003).

Toxinotypes A and C both are able to synthesize α toxin, but type C produces β toxin besides α toxin (Petit et al. 1999), but the role of β toxin in the avian NE development process has not been fully elucidated yet. Toxinotypes B, D and E are not usually associated with poultry diseases (Van Immerseel et al. 2004).

Even though the synthesis of these four major toxins has been the basis for the categorization of *C. perfringens* into the five toxinotypes, various strains still secrete a number of minor toxins and enzymes such as enterotoxin (CPE), β2 toxin, perfringolysin O (θ-toxin) and collagenase (κ-toxin).

Some *C. perfringens* toxinotypes, predominantly type A, synthesize CPE at the sporulation stage, causing gastroenteritis in humans (Narayan 1982; Ridell et al. 1998; Sarker et al. 1999). This enterotoxin is the only toxin not secreted from vegetative *C. perfringens* cells (Rood 1998). However, only about 10% of *C. perfringens* type A derived from poultry carry the enterotoxin encoding gene, *cpe* (Tschirdewahn et al. 1992), and the position of enterotoxin in the development of NE has not been elucidated.

Clostridium perfringens produce β2 toxin that may play a role in pathogenecity of NE (Petit et al. 1999). However, Tschirdewahn et al. (1992) did not observe a correlation between β2 toxin production and incidence of NE. Similar observations were reported by a number of subsequent studies (Chalmers et al. 2007, 2008a, 2008b; Crespo et al. 2007; Thompson et al. 2006), providing clear evidence that β2 toxin is not a critical factor for inducing NE in broiler chickens. A recently identified pore-forming toxin with partial homology to beta-toxin, NetB, has demonstrated to play a significant role in the pathogenesis of NE in broiler chickens (Keyburn et al. 2008). This study demonstrated that a *netB* mutant was incapable of producing NE in an experimental disease model and that virulence was re-established with the complementation of the mutation by a

functional *netB* gene. Nevertheless, 22% of the NE-causing strains of *C. perfringens* tested in this study were negative for *netB*, indicating the existence of NetB might not be vital for all *C. perfringens* isolates to elicit NE in broiler chickens. Moreover, Chalmers et al. (2008b) did not detect *netB* in all isolates from chickens affected with NE. These findings suggest that NetB toxin appeared to be a critical, but not an essential, virulent agent in the induction of avian NE caused by *C. perfringens.* In an earlier study with chickens, Keyburn et al. (2006) did not observe a positive correlation between *in vitro* θ-toxin production of *C. perfringens* and the severity of NE. Thus, the majority of toxins secreted by this pathogen do not appear to be associated with the development of clinical NE. The characteristics of identified toxins synthesized and secreted by *C. perfringens* are shown in Table 2.1.

2.1.3. Pathogenesis

Clostridium perfringens is typically resident in the GIT of avian species, including chickens (Gazdzinski and Julian 1992; Branton et al. 1997) and can be recovered in high numbers from almost every section of the GIT of presumably healthy birds (Ficken and Wages 1997; Engberg et al. 2002; Pedersen et al. 2003). Despite the fact that 75 – 95% of birds are colonized by *C. perfringens*, the mere existence of the organism in the GIT does not inevitably causes NE in broiler chickens (Shane et al. 1984; Kaldhusdal et al. 1999; Craven et al. 2001; LaRagione and Woodward 2003). This can be attributed either to the total absence of NE-producing strains of C. *perfringens* in the GIT of birds or maintenance of dominance by non-NE strains over the NE-producing strains in the normal intestinal flora. However, the domination of non-NE strains suppressing the growth of NE-producing isolates of *C. perfringens* in the normal intestinal flora is

Table 2.1. Characteristics of identified toxins of *C. perfringens.*

Toxin type	Produced by	Biological activity
Major toxins		
α-toxin	All types	Phospholipase C, lecithinase and sphingomyelinase activity, hemolytic, cytolytic (platelets, leukocytes), dermonecrotic
β-toxin	Types B and C	Cytolytic to human leukaemia 60 cell line, increases capillary permeability, increases blood pressure, decreases heart rate, neurotoxic
ε-toxin	Types B and D	Protoxin – requires trypsin activation, induces neurotransmitter release resulting in hippocampal damage, able to pass blood/brain barrier, cytotoxic for Madin-Darby canine kidney cell line, increases vascular permeability thus enhancing its own uptake in intestinal wall, causes swollen hyperaemic kidneys, edemic lungs, excess pericardial fluid
ι-toxin	Type E	Binary toxin: Ia and Ib, protoxin requiring trypsin for activation, Ia; ADP-ribosylating enzyme, Ib; binding and translocation, increases vascular permeability, dermonecrotic on intradermal and lethal on i.v injection
NetB toxin	Type A	Pore forming
Minor toxins		
θ – toxin	All types	Perfringolysin-O, haemolysin, cytolysin, O_2-labile
κ – toxin	All types	Collagenase/gelatinase activity
μ – toxin	Types A, B, C and D	Hyaluronidase activity
λ – toxin	Types B, D and E	Protease activity
ν – toxin	All types	DNase activity
δ – toxin	Types B and C	Haemolysin activity
α – clostripain like protease	Type A (not yet tested for other types)	Cysteine protease activity
Neuraminidase/Sialidase	All types	N-Acetylneuraminic acid glycohydrolase activity

Sources: Hatheway (1990), Shimizu et al. (2002), Gatsos (2007) and Keyburn et al. (2008)

dubious, because Barbara et al. (2008) reported that normal flora strains do not inhibit each other or any NE-producing strains of *C. perfringens* residing in chicken GIT. On the other hand, if NE-producing strains are present, even in undetectable small numbers, in the GIT of healthy birds, it is against the Koch's postulate that a pathogenic strain should not be present in healthy individuals.

In general, NE-associated lesions cannot be observed in birds inoculated experimentally with *C. perfringens* strains from the normal flora. However, taking selective advantage under appropriate conditions, NE-producing pathogenic strains of *C. perfringens* expel non-pathogenic strains from the intestinal tract of affected birds (Barbara et al. 2008). The pathogenic strains of *C. perfringens* suppress the proliferation of other NE-causing strains, in addition to growth inhibition and displacement of non-NE strains, a feature not detected in the normal flora strains (Barbara et al. 2008). Later, using 26 non-NE strains and 24 NE-producing strains in an *in vitro* experiment, Timbermont et al. (2009) reported that 58% of the non-NE strains and 17% of NE-producing strains were not successful in suppressing the proliferation of other *C. perfringens* isolates. As per the results from pulsed-field gel electrophoresis, normal birds harbor a variety of *C. perfringens* clones, but birds with NE carry only one or two clones (Engström et al. 2003; Nauerby et al. 2003). Using *C. perfringens* isolates from broiler chickens without clinical problems and from birds suffering from NE, Gholamiandekhordi et al. (2006) reported similar observations. These findings suggest that NE in poultry is caused only by certain strains of *C. perfringens* bearing specific characteristics and NE-producing strains can selectively exclude the non-pathogenic strains. The reason for the selective proliferation of a clone in case of NE in poultry is still obscure. However, this phenomenon can, entirely or in part, be attributed to factors

such as better adhesion capabilities, faster proliferation, and synthesis of certain toxins and/or substances that selectively suppress the growth of the non-NE strains and other NE-producing strains of *C. perfringens* (Barbara et al. 2008; Timbermont et al. 2009).

2.1.4. *Clostridium perfringens* virulence factors

Upon inoculation of *C. perfringens*-derived α - toxin, NE-associated lesions and mortality were observed in regular (Al-Sheikhly and Truscott 1977a,b) as well as gnotobiotic (Fukata et al. 1988) chickens. Besides, Fukata et al. (1988) observed the neutralization of the particular effect of α - toxin by anti-α - toxin serum. These and subsequent studies led the researchers to conclude that, being the main toxin produced by *C. perfringens*, α - toxin must be the key virulent determinant of NE in broilers. Accordingly, α - toxin attracted much attention in the pathogenesis of avian NE and studies conducted over three decades have been the principal testimonies to validate the suggested role of α - toxin as the most potent virulence agent in the pathological process of NE in broiler chickens.

Being a phospholipase C sphingomyelinase, α - toxin breaks down membrane phospholipids and thus, disrupts cellular structure (Naylor et al. 1998; Titball et al. 2000). Diacylglycerol is formed as a result of membranous lecithin hydrolysis, leading to the activation of protein kinase C, which in turn stimulates the arachidonic acid pathway. This prompts the production of a variety of inflammatory mediators (Titball 1993; Bunting et al. 1997). Some physical conditions initiated due to the effects of these mediators can cause the death of the host.

The expression of the α - toxin gene has been considered as one of the fundamental steps in the initiation of NE. Differential gene expression can be attributed to the variances in the level of α - toxin, although definitive proof that NE is associated with an upregulation of *C. perfringens* α - toxin has not been demonstrated. A two component regulatory system, VirR/VirS, controls the genes of extracellular toxin synthesis of this pathogen (Ba-Thein et al. 1996). Acting as a sensor histidine kinase, VirS phosphyorylates VirR in response to a local signal. Upon phosphorylation, VirR regulates transcription of specific genes, including *cpa,* as seen in other two component regulatory systems (Rood and Cole 1991). In their review, McDevitt et al. (2006) speculate that expression of *cpa* by *C. perfringens* is down-regulated in the normal healthy gut, and in response to an environmental signal, is upregulated to initiate enteric disease. Nevertheless, based on the observations reported by Barbara et al. (2008), *C. perfringens* strains of the normal intestinal flora are incapable of executing the clinical disease.

The nature of the environmental signal is less clear, although evidence suggests that quorum sensing (QS) may regulate toxin production by *C. perfringens* (Novak and Fratamico 2004). Many bacteria coordinate gene expression within as well as between species by synthesizing, secreting, detecting and responding to signaling molecules. These molecules are named as auto inducers (AI), the extracellular concentration of which is correlated to the bacterial cell density (Neaslon 1977; Neaslon and Hastings 1979). This phenomenon of QS is employed by certain bacteria including *C. perfringens*. Each bacterial species synthesizes and reacts to a specific AI (Lu et al. 2005). For instance, Gram-negative QS bacteria commonly utilize homoserine lactones, while various oligopeptides act as AI for Gram-positive organisms. A novel AI, termed

AI-2, has been discovered and shown to comprise a furanosyl borate diester (Chen et al. 2002). The gene *luxS* regulates the synthesis of AI-2 from S-adenosyl methionine (SAM: Xavier and Bassler 2003); hence all bacterial species with *luxS* synthesis AI-2 using the same biosynthetic pathway. Accordingly, this system has been suggested to be a universal signal for inter-species cell-cell communication (Mok et al. 2003). Some studies found the regulation of a range of 'niche-specific' genes by AI-2 in a wide variety of bacterial species (Surette et al. 1999; Miller and Bassler 2001). In *C. perfringens*, regulatory functions like the control of toxin production are associated with the *luxS* gene (Ohtani et al. 2002). Secreted AI-2 binds to luxP, a periplasmic binding protein, which, through a phosphorelay system of kinase activations, ultimately leads to changes in expression of the α-toxin gene, *cpa*.

Based on these observations, *C. perfringens* appears to be regulated in a QS manner, using either oligopeptides or AI-2, or both to regulate expression of the *cpa* gene, and thereby the production and secretion of α-toxin. Oligopeptide AI are species specific whereas AI-2 can act as an inter-species inducer owing to its universal characteristics. This brings up an interesting possibility regarding the initiation of NE in poultry. Synthesis of the oligopeptide AI is a response to focal population density of *C. perfringens*, however the response to AI-2 could be due to a critical population of a different bacterial species. Accordingly, interspecies communication and interaction in the GIT may be attributed to the initiation of NE in broiler chickens. In this scenario, cell-cell interaction would be a novel concept for the induction of enteric diseases.

Despite numerous research efforts to justify α - toxin as the key virulent determinant in the pathogenesis of NE, the validity of this concept has been challenged by a number of studies. Using *C. perfringens* isolates derived from clinically healthy

birds and from birds diagnosed as undergoing NE, Gholamiandekhordi et al. (2006) demonstrated that *in vitro* α - toxin production of *C. perfringens* was not positively correlated with the clinical outcome. Similarly, Keyburn et al. (2006) did not observe a correlation between severity of NE and *in vitro* α - toxin production. One can argue that, under *in vitro* circumstances it is very difficult to provide the exact conditions which mimic the conditions of the predilection sites of the respective organism. Therefore, interactions between the members of intestinal microflora and between the causative organism and the host, and the surrounding environment are limited, which in turn can lead to the lack of the QS regulation of the pathogen. However, another study found that *in vivo* α - toxin level as well, was not correlated with the intestinal lesion score of affected birds (Wilkie et al. 2006). In contrast, some studies reported a direct correlation between *in vitro* α - toxin production and, *C. perfringens* count and the clinical disease status of broilers (Hofshagen and Stenwig 1992; Dahiya 2007). With these conflicting results, it seems clear that production and level of α - toxin cannot be used as indicators to differentiate the strains from NE and strains from clinically healthy chickens.

Keyburn et al. (2006) was able to induce NE in broiler chickens using defined chromosomal *cpa* mutants of a pathogenic *C. perfringens* strain isolated from poultry. Microscopic and macroscopic lesion comparison did not show noticeable differences between regular and mutant challenged groups. This study provides definitive evidence that α - toxin is not a key pathogenic determinant in the development of NE in broilers.

Consequently, further work in the same lab reported the isolation and characterization of a novel pore-forming toxin, NetB, from avian C. *perfringens* strains (Keyburn et al. 2008). This study demonstrated that *netB* mutant was non-pathogenic in a chicken disease model and the virulence was re-established with the complementation

of the mutation by the regular *netB* gene. However, all NE-producing strains tested in this study were not essentially *netB* positive. In addition, findings of later studies (Chalmers et al. 2008b; Martin and Smyth 2009) revealed that NetB was not an essential virulent factor in the pathogenesis of NE in poultry.

As previously described, α - toxin, being a phospholipase, promotes disorientation of the cellular membrane thus instigates tissue necrosis in the intestinal mucosal epithelium (Taguchi and Ikezawa 1976; Hofshagen and Stenwig 1992). Nevertheless, a new etiological point for the development of NE in broiler chickens was revealed by Olkowski et al. (2008), based on ultra-structural and molecular changes in the intestinal tissue. The authors first discovered the secretion of several potent collagenolytic enzymes by *C. perfringens* strains isolated from field outbreaks of NE and then observed high levels of a number of collagenolytic enzymes in the intestinal tissues of the broilers challenged with field isolates of pathogenic *C. perfringens* strains. Using immuno-histochemistry, the study confirmed the expression of a collagenolytic enzyme at high levels in mucosal tissue affected with NE. This reveals a pathogen-host interaction in the pathological process of NE in broiler chickens, wherein *C. perfringens* appears to persuade the host's collagenases to elicit intestinal tissue damage. This study demonstrated that the baso-lateral membranes of mucosal epithelial cells were disrupted by collagenolytic enzymes whereas the apical membranes appeared unharmed even in progressive phases of the disease. These findings suggest that the damage caused by α - toxin to the intestinal epithelial cell membrane, is not an integral part in the pathogenic process of NE.

Alpha toxin derived from *C. perfringens* strains from chickens displaying clinical signs of NE demonstrates high homology to the α - toxin from mammalian strains which mediate gas gangrene (Sheedy et al. 2004). Despite this similarity (> 98% identity), contradictions in terms of immune response are associated with the role of α - toxin in the induction of mammalian gas gangrene and NE in broiler chickens. Substantial leukostasis with little inflammatory infiltration can be observed in the affected tissues in gas gangrene (Flores-Diaz and Alape-Giron 2003). In contrast, extensive immune cell infiltration can be detected in affected tissues in NE, revealing the differences in etiologic processes of the two diseases, or possibly differences in host immune function and/or response.

2.2. Experimental induction of necrotic enteritis

Several approaches to reproduce NE in broiler chickens have been documented in the literature. Nevertheless, no widely recognized disease model exists to induce NE experimentally in broiler chickens (Kaldhusdal et al. 1999). Several methods have been described in the literature but little information about their repeatability is available (Williams et al. 2003). Such methods are listed in Table 2.2. However, numerous efforts made to reproduce NE experimentally in different facilities have resulted in inconsistent findings, ranging from no clinical signs or lesions to typical clinical symptoms and lesions in most of the challenged birds. In fact, recent attempts to induce NE experimentally in broiler chickens were less successful than earlier experimental work. This can be attributed to the significant changes that took place over the subsequent time period in the major determinants of pathogenesis of the disease, the virulence of the pathogen, the host resistance and the respective environment. Accordingly, it appears

Table 2.2. Different methods employed for the experimental induction of necrotic enteritis in broiler chickens.

Method	Reference
Rearing of birds on *C. perfringens* infected poultry-house litter	Maxey and Page 1977; Wicker et al. 1977; Hamdy et al. 1983; Cowen et al. 1987; Kaldhusdal et al. 2001
Oral administration of *C. perfringens*	
in feed	Long and Truscott 1976; Prescott 1979
in drinking water	Jansson et al. 1990; Takeda et al. 1995
by gavage	Bernier et al 1977; Baba et al. 1997; Répérant and Humbert 2002; Wilkie et al. 2005; Dahiya et al. 2005, 2007a,b
Intravenous administration of *C. perfringens*	Bernier and Filion 1971; Bernier et al. 1977
Inoculation of *C. perfringens*	
into the canuulated duodenum	Truscott and Al-Sheikhly 1977
into the cloaca	Williams et al. 2003
Infusion of crude toxins of *C. perfringens* into the duodenum	Truscott and Al-Sheikhly 1977

that current broiler strains are less susceptible to NE compared to past broiler genotypes (Olkowski et al. 2006). Here, the possible efforts made by primary breeding companies in eliminating undesirable traits may have contributed to the improved resistance of the current broiler genotypes. In addition, changes in the pathogen that may have occurred over the last three decades could have prompted a reduction in the degree of pathogenecity.

2.3. *Clostidium perfringens* and broiler growth performance

The clinical version of NE leads to increased mortality owing to the acute effect of toxins produced by *C. perfringens* (Kaldhusdal and Lovland 2000). However, in the sub-clinical form, damage caused by the toxins to the intestinal tissue and associated organs (liver) leads to inferior growth performance of birds in an affected broiler flock (Elwinger et al. 1992; Hofshagen and Kaldhusdal 1992; Lovland and Kaldhusdal 1999; Kaldhusdal et al. 2001; Hofacre et al. 2003). Knarreborg et al. 2002, reported the expression of bile salt hydrolase activity in *C. perfringens* recovered from the chicken gut, which, may impair fat digestion by reducing the emulsification of dietary lipids. Accordingly, this pathogen may negatively affect broiler growth performance by de-conjugating bile acids and hindering the absorption of dietary lipids and fat-soluble vitamins. Efficient fat digestion and absorption is vital for optimum broiler growth performance, because the lipid fraction of the diet contributes towards a considerable share of dietary energy and other important nutrients. Moreover, bile salts are antibacterial in nature (Inagaki et al. 2006), thus the hydrolysis of bile salts by *C. perfringens* may lead to impaired broiler growth performance, due to increased intestinal microbial activity.

The real economic cost caused by *C. perfringens* to the poultry industry is hard to assess. The cost of control methods for all infectious diseases in poultry in the USA has previously been estimated as almost $3 billion per annum (Shane and Van der Sluis 2002). About a decade ago the cost of the sub-clinical form of NE alone believed to be $0.05 per bird in the US with an annual global loss of almost $2.6 billion (Van der Sluis 2000a). Nevertheless, the economic impact of sub-clinical NE may be much higher in current production situations. There is little data available on the economic cost of NE to countries within the EU, however, highly infected broiler flocks have brought the profits down by 33% when compared to flocks with low levels of the disease (Lovland and Kaldhusdal 2001). Accordingly, economic cost of NE is difficult to estimate but both clinical and sub-clinical forms of the disease have a significant impact on the economics of current broiler production.

2.4. Control using antibiotics

Antibiotics are used in animal production for a variety of purposes, including therapy, disease prevention and improvement of productivity. In addition, AGP enhance the food safety by reducing food-borne pathogens in the GIT of poultry (Stanley et al. 1994; Verstegen and Williams 2002). Prophylactic use of certain antibiotics in feed as growth promoters for an extended period of time is a general practice in the poultry industry. The exact mechanism by which AGP exert their performance enhancing effect is still obscure. The observed effects are assumed to be the result of 1) prevention of sub-clinical bacterial infections, 2) reduction of bacterial metabolites and toxins in the GIT, 3) reduced competition for nutrients by gastrointestinal microorganisms, 4) optimization of nutrient absorption and utilization by promoting the gut health or 5) reduction of

microbial metabolism of essential nutrients (François 1962; Visek 1978b; Yokoyama et al. 1982; Gaskins et al. 2002). Use of AGP in broiler rations accounts for 3–5% improvement in growth as well as feed conversion ratio (FCR; Thomke and Elwinger 1998). Based on the assessment of the Animal Health Institute of America, without the use of AGP in poultry rations, the USA would require an additional 452 million chickens to reach the levels of production achieved with AGP (AHI 1998).

The use of most AGP results in a significant decrease in the occurrence of NE in poultry, compared to non-medicated counterparts (Dahiya et al. 2006). Traditionally, main NE prevention strategies have taken the form of in-feed AGP such as virginiamycin, bacitracin, tylosin, lincomycin, avoparcin and penicillin (Maxey and Page 1977; George et al. 1982; Stutz and Lawton 1984; Hofshagen and Kaldhusdal 1992; Ficken and Wages 1997; Watkins et al. 1997). Nevertheless, use of AGP applies selection pressure on bacteria to acquire resistance via genetic mutations (Walsh 2000). This acquired resistance may be transferred horizontally and vertically across bacterial strains (Davies 1994; Shoemaker et al. 2001), particularly a cross-resistance between the AGP virginiamycin and the human antibacterial agent quinopristin/dalfopristin (Wegener et al. 1999) and the cross-resistance of the AGP avoparcin with human antibiotic vancomycin in *Enterococcus faecium* (Aarestrup et al. 2000). In this scenario, broiler producers have come under increasing pressure to reduce or eliminate in-feed antibiotics and to curtail prophylactic application of these antimicrobials despite their evident benefits. Consequently, national policies have lead to a ban of AGP in the poultry industry, first in Scandinavian countries followed by the European Union. While these products are still allowed in feeds in North America, recently President Obama has announced that his administration will pursue the banning of the routine use of

antibiotics in animal production (New York Times, July 13, 2009). A total ban on usage of antimicrobials in livestock industry has been estimated to cost about US$ 1.2–2.5 billion annually to US consumers (Gill and Best 1998). Thus, a considerable interest exists in the development of alternative non-antibiotic means for the control of gut-specific pathogens, particularly, *C. perfringens* in broilers.

2.5. Alternative strategies

Pathogen reduction, nutritional approaches and biologicals such as vaccines and probiotics are considered to be the three main basic areas in controlling NE in broilers raised without in-feed antibiotics (Revington 2002). Pathogen reduction can be accomplished through the introduction of comprehensive biosecurity and hygienic practices. Surprisingly, there are no recent reports of the effectiveness of this approach in managing NE. Effective vaccines against *C. perfringens* have proved difficult to produce and there are currently no commercial vaccines for the prevention of NE in broilers. Research in this area appears promising, however. Both recombinant live oral (Thompson et al. 2006; Zekarias et al. 2008; Kulkarni et al. 2010), and recombinant subunit (Jiang et al. 2009) vaccines have been investigated as methods for preventing clinical NE. Probiotics have also been investigated with some success (Hofacre et al. 1998; Craven et al. 2000; McReynolds et al. 2009). These approaches appear promising and commercial products may become available in the next decade. Several alternative strategies for the control of NE in broilers have been discussed in a recent review by Dahiya et al. (2006), including (1) direct-fed microbials (undefined microbial cultures and probiotics), (2) prebiotics, (3) organic acids, (4) plant products and essential oils, (5) feed enzymes, (6) hen egg antibodies, (7) vaccination, (8) anticoccidial vaccination, (9)

bacteriophages and (10) diet formulation and ingredient selection. To date none of these strategies have yielded control equivalent to in-feed antibiotics for the control of NE in broilers.

Currently, there is a widespread interest in using antimicrobials from natural sources as replacements or alternatives to conventional antibiotics. These include lysozyme from hen egg white, lactoferrin and lactoferricin B from milk and protamine from fish, molecules with well characterized antimicrobial activity and which are available commercially. These antimicrobials have a wide spectrum of activity and are effective against Gram-positive bacteria including *C. perfringens* (Bellamy et al. 1992; Teraguchi et al. 1995; Truelstrup et al. 2001; Masschalck and Michiels 2003). Nevertheless, attempts to control NE with these antimicrobials have not been successful so far due to the fact that a loss in activity during transit through the digestive tract, leading to insufficient delivery to affected sites and to the target organism. Therefore, experiments are underway to develop potential delivery system for these antimicrobials.

2.6. Predisposing factors

Many factors have been documented as predisposing poultry to the development of clinical signs and lesions of NE. These identified predisposing factors fall into two broad categories; management- and diet-associated. Still, the documented list of risk factors is most likely far from complete. Moreover, the contribution of the predisposing factors towards the overgrowth of the pathogenic strains and hence the subsequent progression of disease is poorly elucidated and experimental results have been conflicting. For instance, Olkowski et al. (2006) did not observe an increase risk of NE in broiler chickens with either dietary- or management-associated predisposing factors when the

perceived factors were introduced individually. Therefore, the likelihood of developing NE in a poultry flock possibly depends on interactions between these various factors, which further bolster the concept of multifactorial pathogenesis of NE.

Management-related predisposing factors include concurrent or prior infections with enteropathogenic agents, coccidia in particular (Cowen et al. 1987; Baba et al. 1992a,b; Williams et al. 2003), stress factors such as transportation, high stocking density, litter conditions, sudden changes in the diet, sudden environmental changes and immunecompromising agents like cyclophosphamide (Truscott and Al-Sheikhly 1977; Cowen et al. 1987; Stuve et al. 1992; Kaldhusdal et al. 1999; Lovland et al. 2003), and absence of in-feed AGP. In addition, introduction of a new strain of *C. perfringens* via a vector such as house-hold flies is another important management-associated predisposing factor since the consumption of flies or fly excretory deposits can cause NE in broiler chickens (Dhillon et al. 2004). Nevertheless, diet related risk factors of NE have seized much attention, especially with the withdrawal of antibiotics from broiler diet, due to their substantial role in the pathogenesis of NE, thus will be discussed widely herein.

2.6.1. Cereal type

Cereal grains normally represent the largest proportion of poultry diets giving them a large potential to influence gut function and intestinal microbiota. Many studies have reported a lower prevalence and intensity of NE in broilers fed corn-based diets than in birds fed diets based on other cereal grains, such as wheat, barley, rye or oats (Branton et al. 1987; Kaldhusdal and Hofshagen 1992; Riddell and Kong 1992; Kaldhusdal and Skjerve 1996; Branton et al. 1997; Annett et al. 2002; Kocher 2003). Feeding *C.*

perfringens-inoculated feed for periods of 1 to 5d, starting at 14 to 18d of age, Riddell and Kong (1992) compared NE-associated mortality in broiler chickens fed corn, wheat, rye or barley based diets. They reported a mortality rate ranging from 26 to 35% in birds fed wheat, rye or barley-based diets compared to a mortality rate reached only 12.5% in birds fed the corn-based diet.

Kaldhusdal and Skjerve (1996) evaluated feed formulation data for a period of 20 years and reported two considerable episodes of NE in Norway during years with high inclusion of wheat and barley in broiler formulations. They reported a significant direct relationship between the development of NE and the ratio of wheat plus barley to corn fed in the country. Using broiler chickens infected with *C. perfringens*, Craven (2000) reported an increase in the pathogen numbers in the distal gut and droppings of birds fed a diet containing 50% rye, from $\log_{10}$ 3.4 to $\log_{10}$ 7.1 – 7.9 with the progression of age from 2 to 21d. Conversely, *C. perfringens* numbers in all segments of the GIT of birds fed a corn-based diet remained constant during this time period. In a more recent study, Jia et al. (2009a) inoculated broilers fed corn- or wheat-based diets with *C. perfringens* at d 13 of age for 16h and observed significantly higher pathogen counts and intestinal lesion scores in broilers fed wheat-based diet than in birds fed corn-based diet. However, NE-associated mortality was not different between the two treatment groups. In another study in the same lab (Jia et al. 2009b), broilers fed wheat/soybean meal and wheat/flaxseed meal were inoculated with *C. perfringens* at d 14 of age for 16h and the final body weight (BW), intestinal *C. perfringens* numbers, NE lesion scores and mortality were compared. Pathogen numbers, intestinal lesion score and NE-associated mortality were higher in birds fed wheat/flaxseed meal than in birds fed wheat/soybean meal. Flaxseed contains approximately 280 g kg^{-1} carbohydrates, with around one third

of the fibre being water-soluble mucilage (Oomah et al. 1995). Mucilage has been reported to cause the proliferation of intestinal microflora (Alzueto et al. 2003; Langhout et al. 1999) and may contribute to increased NE-associated mortality in broilers.

While the observation that diets based on corn decrease the development of NE has been widely reported, the mechanism(s) underlying this reduced incidence have not extensively been evaluated. Several hypotheses have been suggested. One hypothesis suggests that the cell wall- associated non-starch polysaccharides (NSP) such as arabinoxylans (wheat, rye and triticale) and β-glucans (barley and oats) may increase the enteric bacterial proliferation and toxin production by reducing the mixing of intestinal contents and creating a barrier between nutrients and the intestinal epithelium (Choct et al. 1996; Langhout et al. 1999; Hubener et al. 2002). This may result in decreased efficiency of nutrient digestion and absorption by the host and increased intestinal transit time, which in turn increases nutrient availability to enteric pathogens. Moreover, digesta viscosity may induce intestinal mucus secretion by the host (Larsen et al. 1993; Langhout et al. 1999; Piel et al. 2005) possibly leading to increased proliferation of mucolytic bacteria in the GIT (Langhout et al. 1999). Deplancke et al. (2002) reported a significant acidomucolytic ability of *C. perfringens* and observed a faster proliferation of the pathogen on a medium that contain mucin. Consequently, Collier et al. (2003) suggested that the growth of this pathogen in the GIT might be supported by high mucus secretion linked with diet and intestinal infections. Corn contains negligible levels of NSPs (Choct et al. 1996) and this may explain why it has lesser effect on broiler growth performance and intestinal *C. perfringens* numbers compared to other main cereal grains (Chesson 2001).

Other hypotheses have also been put forth to explain the mechanism associated with reduced effects of corn-based diets on NE in broilers. Riddell and Kong (1992) compared the NE-associated mortality of broilers fed a corn-based diet with or without an added carbohydrate source (glucose) and observed an increase in mortality rate upon inclusion of glucose to the diet. They hypothesized that compared to carbohydrates in other main cereal grains, lower availability of those in corn for microbes may limit the intestinal microbial activity. Annett et al. (2002) hypothesized that certain unknown constituents present in wheat and barley may impair the activity of host digestive enzymes, hindering the degradation of α-toxin thus triggering the intestinal *C. perfringens* proliferation and/or α-toxin synthesis. They conducted an *in vitro* experiment in which supernatants originating from either digested or non-digested broiler rations based on corn, wheat or barley were mixed with thioglycollate broth. No significant differences were observed in *C. perfringens* growth among non-digested diets. However, *C. perfringens* growth in the digested corn diet was significantly lower than in the respective barley and wheat diets. These findings suggest that digestive enzymes play role in NE pathogenesis by releasing or activating one or more elements from corn to suppress or from wheat and barley to stimulate the *C. perfringens* proliferation in the avian GIT. In contrast, Branton et al. (1996) observed a suppression of *C. perfringens* proliferation in thioglycollate broth upon addition of wheat extracts with compared to deionized water. However, prior digestion of the wheat was not used in this experiment and the release of nutrients and other inhibitory factors may have been absent due to the lack of enzymatic breakdown. Williams (1992) correlated the nutritional characteristics of cereal types to one of the predisposing factors of NE to explain the different proliferation rates of *C. perfringens* in birds fed corn- and wheat-

base diets. The author hypothesized that the higher vitamin A and E concentrations in corn than in wheat might reduce the pathogenicity of *E. tenella* and higher niacin and riboflavin concentrations in wheat compared to corn may increase its' pathogenicity.

Growing *C. perfringens* in supernatants derived from wheat and corn, Christianson (2011) demonstrated higher pathogen proliferation in wheat supernatant than in corn supernatant. However, the alpha toxin production per cfu was significantly lower when the pathogen was grown in wheat supernatant compared to corn supernatant. Nevertheless, since no indication on the toxin concentration in each supernatant used in this study, it would be hard to correlate these observations to *C. perfringens* pathogenicity.

2.6.2. Exogenous enzymes

As previously stated, increased digesta viscosity created by some NSP components of cereal grains negatively affect the digestive process of the host animal and has a significant influence on the intestinal microbial activity. In this context, utilization of exogenous carbohydrases may be beneficial in decreasing intestinal viscosity and limiting the amount of nutrients available to intestinal microbes including *C. perfringens* (Choct et al. 1999; Bedford 2001; Bedford and Apajalahti 2001). Unfortunately, due to the differences in challenge models, types of feed and the characteristics of supplementary enzymes, the documented findings seem to be inconsistent and thus hard to interpret. Evidence suggests that when supplemented with xylanase-based enzyme preparations, wheat-based diets are able to reduce intestinal bacterial counts by 60% in broiler chickens (Bedford and Apajalahti 2001). These findings were in agreement with Hubener et al. (2002). However, feeding a wheat-based diet supplemented with

pentosanase, Riddell and Kong (1992) did not observe an effect on NE-associated mortality but this study lacked information on parameters like *C. perfringens* counts and intestinal lesion scores. Jia et al. (2009a) used a similar disease model to challenge broiler chickens fed diets based on either wheat or corn supplemented with a carbohydrase enzyme cocktail mainly containing cellulase, pectinase, xylanase, glucanase, mannanase and galactanase. The authors did not observe any effect of supplementary enzyme on the proliferation of *C. perfringens*, intestinal lesion scores or NE-related mortality. However, Choct et al. (2006) demonstrated a reduction in cecal *C. perfringens* counts in apparently healthy broilers fed xylanase-supplemented wheat-based diets. Challenging broilers with both *C. perfringens* and *Eimeria*, Jackson et al. (2003) reported increased growth performance and reduced intestinal lesion scores in birds fed β-mannanase supplemented corn-based diets. The negative effects of β-mannans may be due to their potent stimulatory effect on inflammation and resultant cytokine production (Zhang and Tizard 1996; Duncan et al. 2002).

In a study conducted at the University of Manitoba, broiler chickens were offered wheat/soybean meal-based and flaxseed meal containing feeds with or without a supplementary carbohydrase enzyme cocktail. Intestinal sections excised from broilers were ligated and inoculated with *C. perfringens* spores. The findings of this *in vitro* study revealed a significant reduction in the proliferation of *C. perfringens* in digesta contents of both treatment groups by 50 and 67%, respectively, in the presence of supplementary enzymes, the flaxseed fed group showing a more distinct outcome (Jia et al. 2009b). They reported a significant reduction in digesta viscosity and *C. perfringens* counts in broiler chickens fed a multicarbohydrase enzyme supplemented diet based on wheat/flaxseed meal.

Although a number of hypotheses have been presented to describe the mode of action of exogenous enzymes, the exact mechanism behind the role of these enzymes on the proliferation of *C. perfringens* and induction of NE remains unclear. Bedford (2000) categorized the effects of exogenous microbial enzymes on intestinal microflora into two phases: ileal and cecal. In the ileal phase, enzymes increase the efficiency of nutrient utilization of host and limit the amount of nutrients available to microbes. In the ceca, enzymes de-polymerize complex carbohydrates into soluble, poorly absorbed sugars, mainly oligosaccharides (de Silva et al. 1983). These oligomers may act as prebiotics with the selective support of the proliferation and/or activity of favorable bacteria (Gibson and Roberfroid 1995; Monsan and Paul 1995), and suppressing the proliferation of certain enteric pathogens including *C. perfringens* (Fukata et al. 1991; Gibson and Roberfroid 1995; La Ragione and Woodward 2003). Some previous studies reported a stimulatory effect of supplementary enzymes on intestinal proliferation of lactic acid-producing bacteria in broiler GIT (Vahjen et al. 1998; Engberg et al. 2004). Further, feeding diets containing a supplementary carbohydrase enzyme cocktail, Kiarie et al. (2007) reported higher lactobacilli counts and lactic acid levels in the piglet ileum. In fact, using lactic acid-producing bacterial strains such as *Lactobacillus acidophilus* and *Streptococcus faecalis*, Fukata et al. (1991) has demonstrated promising results in containing *C. perfringens* growth in broiler GIT. The ability of a lactic acid bacterial culture in the reduction of *C. perfringens*-related broiler mortality was not affected by either presence or absence of mannonoligosaccharides (MOS: Hofacre et al. 2003). However, spraying a commercially available microbial enzyme preparation with xylanase and β- glucanase enzyme activities on isocaloric and isonitrogenous diets based on major cereal grains used in poultry feeds (barley, corn, wheat and sorghum),

Shakouri et al. (2009) did not observe an effect of enzyme supplementation either on lactobacilli or *C. perfringens* numbers either in ileum or cecum. In conclusion, the discrepancies in responses in birds to supplementary exogenous enzymes can primarily be attributed to the diverse characteristics of cereal grains used in feed formulation, the content of water soluble NSP and the origin and nature of microbial enzymes used.

2.6.3. Non-nutritional ingredients

A number of what can be termed non-nutritional ingredients has been investigated as means to control *C. perfringens* and NE in broilers. Prebiotics are non-nutritional carbohydrates that selectively promote the growth, activity or both of potentially beneficial commensal bacterial species in the GIT (Gibson and Roberfroid 1995). Prebiotics are primarily oligosaccharides which resist degradation by host intestinal enzymes. Most of these oligomers are fermentable in nature thus serve as substrates for beneficial microbes of the intestinal tract (Blaut 2002). Non-fermentable prebiotic oligosaccharides such as fructoologosaccharides (FOS) and MOS provide an alternative attachment site for the pathogen rather than the intestinal mucosa hence eliminating them from the GIT of the host (CFNP 2002). Available information on the role of prebiotics on the growth and activity of *C. perfringens*, present a complex and often contradictory picture. Studies with mammals have demonstrated a significant decrease in *C. perfringens* numbers in the GIT of rats, cats and dogs fed diets containing FOS, than in those fed diets without FOS (Gallaher et al. 1996; Sparkes et al. 1998; Swanson et al. 2002). On the other hand, Hofacre et al. (2003) did not observe a significant effect on NE-associated mortality in broilers fed either FOS or MOS. Similarly, in a study with

quail, oligofructose did not demonstrate any effect on *C. perfringens* in the GIT (Butel et al. 2002).

The antibacterial effects of a variety of herb and spice products (Besra et al. 2002; Fabio et al. 2003; Wilkinson et al. 2003) and essential oil components (Briozzo et al. 1989; Cosentino et al. 1999; Dorman and Deans 2000; Losa and Kohler 2001; Fabio et al. 2003; Mitsch et al. 2002, 2004) have been assessed for their ability to control intestinal pathogens. However, few of these studies have investigated the use of these products for the control of *C. perfringens*. An exception to this is the use of muscadine pomace, a by-product from grape- (*Vitis rotundifolia*) based juice and wine industries, to control NE in chickens. This by-product has a high antioxidant capacity (Pastrana-Bonilla et al. 2003; Talcott and Lee 2002), is rich in polyphenolic compounds (Lee et al. 2005; Yilmaz and Toledo 2004) and possesses marked topical and systemic anti-inflammatory properties (Greenspan et al. 2005). McDougald et al. (2008) reported that supplementation of a corn/soybean meal-based commercial type diet with muscadine pomace at 0.5 and 2% inclusion levels increased growth performance along with lower intestinal lesion scores and mortality in broilers co-infected with *Eimeria* (*acervulina* and *maxima*) and *C. perfringens*. Moreover, another experiment in the same study demonstrated an increase in resistance to coccidiosis in birds fed same muscadine pomace-supplemented diets. Accordingly, the reduced pathogenesis of *C. perfringens* can primarily be attributed to the negative effects of this by-product on both enteric pathogens. The available information suggests some beneficial properties of natural products in controlling NE in broiler chickens. Nevertheless, more studies are needed in this area to apply the utilization of these products in commercial feeding practices.

2.6.5. Feed processing and feeding

While ingredients and nutrients have significant effects on gut *C. perfringens* populations and clinical NE, the manufacturing of feed can also modulate these effects. Feeding broilers with mash and pellet form diets, Engberg et al. (2002) observed higher *C. perfringens* and lactobacilli counts in the GIT of birds fed mash diet compared to those fed pellets. In addition, microbial fermentation in the cecum as reflected by VFA production was significantly higher in birds fed the pelleted diet. Branton et al. (1987) observed a reduction in NE-related mortality when broilers were fed coarsely ground wheat (28.9%) compared to finely ground wheat fed counterparts (18.1%). Coarse grinding of cereals is beneficial in terms of controlling gut pathogens compared to fine grinding (Engberg et al. 2002). When birds are fed finely ground rations the normal gastric reflux does not take place and consequently, more proteins reach the lower intestinal tract where these proteins are subjected to microbial fermentation. Highly processed feeds may cause gizzard atrophy and malfunction, hindering the grinding capabilities of the organ (Engberg et al. 2002). Whole wheat incorporation into the diet optimizes the gizzard activity with a resulting increase in nutrient digestibility and utilization (Hetland et al. 2003). Broilers fed whole wheat demonstrated lower gizzard pH and lesser *C. perfringens* numbers in the intestinal contents compared to finely ground wheat fed counterparts (Engberg et al. 2004; Bjerrum et al. 2005). Whitehead (2002) observed an increase in both healthiness and feed efficiency in broiler chickens reared under meal feeding and feed restriction during the early phase of the cycle. Finally, meal feeding results in higher nutrient utilization by broilers compared to *ad libitum* feeding which in turn restricts nutrient availability to the gut bacteria. While no experiments have been reported the effect of meal feeding on intestinal *C. perfringens*

populations, this approach appears promising and future studies in this area would be a welcome addition to the literature.

2.7. Dietary protein, amino acids and necrotic enteritis

Among other well-identified dietary factors, the source and level of dietary protein have a distinct effect on the growth of *C. perfringens* in the GIT. The protein fraction in a broiler diet is variable as local and world prices determine the source and quality of protein(s) to be included (Palliyeguru et al. 2010). Amino acid balance and crude protein (CP) content have a significant effect on the *C. perfringens* numbers in the GIT of broiler chickens (Kaldhusdal 2000; Drew et al. 2004) as well as mammals such as pigs (Mansson and Smith 1962; Mansson and Olhagen 1967) and dogs (Zentek 1998). Studies with broiler chickens have revealed that birds fed high protein diets, particularly diets high in animal-derived proteins are predisposed to clinical NE (Kaldhusdal 2000; Kocher 2003; Drew et al. 2004; Wilkie et al. 2005). Kaldhusdal and Skjerve (1996) reported evidence of a link between animal-derived proteins and NE. In a longitudinal study, they examined the relationship between NE and feeding systems for a more than 20 year period in Norway, and reported an increase in the occurrence of NE in broiler chickens during years when animal-derived proteins were used at higher levels. This correlation while suggestive did not prove a cause and effect relationship between NE and animal-derived proteins. However, existing evidence correlates the proliferation and/or α toxin production of *C. perfringens* to some specific AA (Titball et al. 1999). Methionine was reported to be required for sporulation and to be highly stimulatory for growth, although not an essential nutrient for *C. perfringens* (Muhammed et al. 1975). However, *in vitro* experiments conducted in our laboratory reported a significant

reduction in the proliferation of this microorganism upon incubation of mixed bacterial culture for 24 hours in minimal salt media with supplementary DL-methionine (DLM) added at a concentration of 10 mg/mL, compared to the unsupplemented media (Wilkie et al. 2005; Wilkie 2006). Moreover, Dahiya et al. (2007b) observed significantly lower *C. perfringens* growth in the GIT of broiler chickens fed either of the two commercially available methionine forms: DLM and 2-hydroxy-4-methylthiobutanoic acid (HMB). Several studies have reported the stimulatory properties of dietary glycine on the proliferation of *C. perfringens* in broiler GIT, thus suggesting an increased possibility in the development of NE (Ispolatovskaya 1971; Nakamura et al. 1978; Dahiya et al. 2005; Wilkie et al. 2005). In support of this observation, the presence of peptides with glycine in them is a requirement for *C. perfringens* to produce α toxin in chemically defined media (Nakamura et al. 1978; Stevens and Rood 2000). As a percentage of CP, proteins originating from animal sources, such as meat and bone meal (MBM), contain two to four fold higher levels of glycine compared to plant-derived proteins (AminoDat 2001). Therefore, glycine in excessive amounts in diets containing animal-derived proteins can promote the growth of *C. perfringens* in the GIT of broilers. In support of this notion, Wilkie et al. (2005) tested diets containing 40% CP formulated using seven different ingredients as the primary sources of protein namely, fish meal, MBM, feather meal, potato protein concentrate (PPC), pea protein concentrate, soy protein concentrate or corn gluten meal. The ileal and cecal *C. perfringens* numbers in broilers fed diets containing animal-derived protein sources were significantly higher than those in birds fed the control diet or diets with plant-derived proteins, except for the diet containing PPC. In addition, a significant positive correlation was observed between the dietary glycine level and both ileal and cecal *C. perfringens* numbers. Potato protein concentrate

contains a relatively higher level of glycine as a percent of CP (18.6 g/kg), so differs from most plant protein sources (Wilkie et al. 2005). However the CP level and test ingredient inclusion rates were much higher in the diets formulated for this study than would be used in commercial type diets. Furthermore, a correlation does not establish a causal relationship between the glycine level in the diet and *C. perfringens* numbers in the intestine. Palliyeguru et al. (2010) formulated three nutritionally complete corn-based iso-caloric and iso-nitrogenous diets containing PPC (15.8%), fish meal (19.3%) and a blend of soy protein (soybean meal and full-fat soy: 17.8 and 12.0%, respectively), to evaluate the effects of type of protein on the development of sub-clinical NE in broiler chickens. These diets were fed to broilers raised on litter composed of new wood savings and litter material from a previous poultry flock in 4:1 ratio. The authors found that birds fed PPC had higher values for proximal small intestinal necrotic lesions, distal ileal lesion score, liver lesions, and α - toxin antibody level and also had reduced growth performance in comparison to birds fed the other protein sources. Nevertheless, no treatment effect was observed on *C. perfringens* numbers in proximal jejunal mucosal scraping. The glycine content in PPC (0.94%) and soy protein (0.90%) containing diets used in this experiment were not significantly different. Similarly, the methionine content (0.49%) was same for both dietary treatments. Accordingly, these findings, on one hand, are rather in agreement with the previously testified effects of dietary gylcine and methionine on *C. perfringens* growth and colonization. However, on the other hand, *C. perfringens* numbers in distal segments of the GIT were not determined, thus hard to correlate and interpret the findings because, if determined, the numbers could have been higher in birds fed PPC, owing to the NE- associated pathological, immunological and growth changes with that particular dietary treatment. The increased severity of the

disease and substandard growth performance of birds fed PPC can be attributed either to possible virulence enhancement of the pathogen by some component present in PPC or to increased proliferation and activity of *C. perfringens* in the distal GIT, which was not determined or to other factors associated with PPC itself or the diet containing PPC. The estimated glycoalkaloid level of PPC seems to be much higher (257 mg/kg: Lokra et al. 2008) than the general safety limit (200 mg/kg) for humans (Smith et al. 1996). Solanine, the major heat-stable glycoalkaloid present in PPC, along with deleterious compounds derived from aromatic AA, which are present in higher amounts in PPC containing diet, might have initiated intestinal epithelial damage, encouraging *C. perfringens* growth and activity in the GIT (Smith et al. 1996; Smith and Macfarlane 1997). In addition, the authors identified other factors, such as higher trypsin inhibitor activity as well as lower lipid content in PPC containing diet, as stimulatory to the proliferation of *C. perfringens*. Trypsin inhibitors suppress the protein digestibility, increasing the amount of protein that reaches the distal GIT. Low lipid consumption reduces bile acid synthesis and secretion, diminishing the bile salt-associated antibacterial activity. Both these factors may contribute towards an increase growth and colonization of *C. perfringens* in birds fed the diet containing PPC. Drew et al. (2004) evaluated the impact of protein source (fish meal vs. soy protein concentrate, SPC) and level (23, 31.5 or 40 % CP) on *C. perfringens* growth in the broiler intestinal tract and revealed a significantly higher *C. perfringens* numbers in broilers fed the diet containing fish meal at 40% CP. Both ileal and cecal *C. perfringens* numbers were increased as the amount of CP went up from 23 to 40% in broilers fed diets containing fish meal, however not in broilers fed diets with SPC. The numbers were low in broilers fed diets containing SPC at all CP levels. According to the results of feed analysis, dietary

concentrations of both glycine and methionine were higher in fish meal-based diets compared to SPC-based diets. To further verify the influence of glycine on the growth of *C. perfringens*, Dahiya et al. (2007a) incorporated crystalline and protected forms of glycine into the experimental diets and observed stimulatory effect of this AA on *C. perfringens* growth only in broilers fed diets with the protected glycine. This observation can be attributed to the fact that crystalline form is considered to be 100% true digestible (Izquierdo et al. 1988; Chung and Baker 1992) and rapidly absorbed in the upper part of the GIT (Lingens and Molnar 1996; Maenz and Engele-Schaan 1996a; Drew et al. 2003), making it unavailable to the microbes residing in the distal part, while glycine incorporated in the encapsulated preparation is released slowly so reaches the lower GIT, increasing the availability to microbes. Nevertheless, more research is warranted in this area to further elicit the role glycine plays in the proliferation of *C. perfringens* in the broiler intestinal tract.

2.7.1. Low protein diets

In view of the above discussion, diets containing low levels of CP can be speculated as a potential nutritional strategy to control *C. perfringens* growth in the GIT of broilers, in post-in-feed antibiotic era. Commercially available synthetic forms of AA have created a great opportunity for nutritionists to formulate diets that are lower in CP. These synthetic AA are included in poultry rations in order to substitute a portion of such AA supplied by protein rich, comparatively expensive raw materials. Accordingly, the advantages of synthetic AA incorporated-low CP (*l*CP) diets include reduced N excretion by precisely formulating diets to meet the bird's AA requirement and giving the nutritionists more flexibility in ingredient selection (Bercovici and Fuller 1995). As a

result, more environmentally friendly diets can be formulated at a lower cost. In fact, Rostagno et al. (1995) demonstrated superior growth and production performance and considerable economic benefits, when relatively inexpensive feed ingredients were incorporated in the formulation of *l*CP - AA supplemented broiler diets. However, despite great efforts to understand *l*CP diets in broilers, the greatest point of concern is that growth and carcass performance seem to be impaired when broilers are fed *l*CP diets even if formulated to meet all known nutrient requirements. This area has been reviewed in depth by Payne (2006) evaluating a total of 59 datasets, which spans over 15 years worth of research. A majority of datasets reviewed by Payne (2006) suggests that growth performance is reduced when broilers are fed *l*CP diets. In their review, Aftab et al. (2006) proposed a number of potential factors to describe the negative effects of *l*CP diets on growth performance of broilers, including: (1) alterations in potassium or the electrolyte balance in the diet; (2) deficiency of the non-protein nitrogen for the synthesis of non-essential AA; (3) possible reduction in broiler feed intake; (4) change in essential : non-essential AA ratio; (5) limited production of non-essential AA to fulfill the demand of fast-growing broilers; (6) efficiency of AA utilization from a synthetic product compared to intact dietary protein, for the synthesis of body protein; (7) deficiency of certain essential AA; (8) correlation between the dietary metabolizable energy (ME) and the net energy of *l*CP vs. high CP (*h*CP) diets. The effects of *l*CP diets on broiler growth and production performance in different age groups, as reported by previous studies, are outlined in Table 2.3. When attempting to bring the CP level down to formulate *l*CP (AA supplemented) diets, two approaches have been documented in the literature; maintaining either a constant ME:CP ratio or a constant energy level. The *l*CP diets formulated to be isocaloric (increasing the ME:CP ratio) increase feed intake with a

Table 2.3. Effect of low crude protein diets on performance of broilers (After Aftab et al. 2006).

CP*i*[a] (CPAA)[b]%	Control CP %	CPAA (Control)	ME/CP	Age (days)	Gain	FE	Breast Yield	Abdominal Fat	Reference
					CPAA/Control CP group				
16.2 (16.6)	22.2	0.75	193	0-18	0.99	1.05*	-	-	Dean et al. (2006)
18.3	22.9	0.80	171	8-21	1.03	0.96	0.97[e]	0.95[f]	Fatufe and Rodehutscord (2005)
22.0	24.0	0.92	145	0-21	0.95	0.95	-	-	Jiang et al. (2005)
20.0	23.0	0.87	160	7-17	1.01	1.01	-	1.06	Sterling et al. (2005)[c]
20.3 (21.5)	22.5	0.96	149	0-21	0.93	0.97	-	1.07	Si et al. (2004)
17.3	19.3	0.90	155	0-21	1.06	1.01	-	-	Aftab et al. (2004a)[d]
15.3	17.2	0.89	180	21-42	1.04	0.99	-	-	Aftab et al. (2004b)[d]
17.6 (18.3)	23.4	0.78	175	7-21	0.97	0.94*	0.95*[e]	1.46*[f]	Bregendahl et al. (2002)
20.5	21.9	0.94	151	0-21	1.00	1.00	-	-	Cauwenberghe and Burnham (2001)
18.6	20.7	0.90	169	22-35	0.96	1.00	-	-	Cauwenberghe and Burnham (2001)
16.0	17.6	0.91	202	35-49	1.00	1.00	0.99	-	Cauwenberghe and Burnham (2001)
18.0[g] /18.7[h]	20.4/21.5	0.88/0.87	174	29-42	0.94	0.92	-	-	Jeroch and Pack (1995)
20.0 (20.8)	24.2	0.86	154	0-21	1.01	0.98	-	-	Moran et al. (1992)
17.0 (17.8)	20.5	0.87	180	21-42	0.97	0.97	-	1.17	Moran et al. (1992)
19.0 (21.4)	23.0	0.93	150	0-21	1.00	0.98	-	1.16[f]	Han et al. (1992)
16.0 (18.5)	20.0	0.93	173	21-42	1.00	0.96	-	-	Han et al. (1992)

[a] CP*i* - CP from intact proteins.

[b] CPAA - CP including crystalline amino acid nitrogen.

[c] 0.9% lysine in *l*CP and control diets.

[d] low-ME (2700–2750 kcal ME per kg) diets.

[e] CP,% whole body composition.

[f] Fat,% whole body composition.

[g] experiment 1.

[h] experiment 2.

* significantly ($P < 0.05$) differed from the control.

simultaneous increase in the deposition of abdominal fat (Fancher and Jensen 1989a, b; Rosebrough and Steele 1990; Rosebrough and McMurtry 1993; Aletor et al. 2000; Dozier et al. 2006). However, according to Hidalgo et al. (2004) and Kamran et al. (2008), no differences in the yield of abdominal fat were seen in chickens fed *l*CP diets if a constant ME:CP ratio was maintained. On the contrary, lowering the protein content in isocaloric diets (increasing the ME:CP ratio) optimizes the utilization of dietary protein in broilers, as indicated by protein efficiency ratio (PER: determined as grams of weight gain per gram of protein intake: Cheng et al. 1997; Aletor et al. 2000). Yet, the PER is reduced linearly in broilers during the grower, finisher and overall growth periods with the reduction of the protein content in diets which maintain a constant ME:CP ratio (Kamran et al. 2008). The authors link this observation to the significantly reduced growth rate in birds fed *l*CP and low ME diets, despite the consumption of the same amount of protein with increased feed intake. Similarly, the breast meat yield is reduced when broilers are fed isocaloric diets, reducing the CP content (Dozier et al. 2006). Demonstrating enhanced performance in broilers fed glycine supplemented *l*CP diets, Parr and Summers (1991), Jiang et al. (2005) and Dean et al. (2006) indicated that the NRC recommended glycine level (1994) was not sufficient for broilers fed *l*CP diets. Despite the prompt synthesis of glycine by the broilers, the rate of synthesis might not be quick enough to fulfill the demand for muscle growth and nitrogen excretion in rapidly growing birds (Parr and Summers 1991; Jiang et al. 2001, 2005; Corzo et al. 2004; Dean et al. 2006), thus should be supplied in the diet. Nevertheless, due to the stimulatory nature of this specific AA on *C. perfringens* growth in the GIT of broilers as previously described, the possibility of exceeding NRC (1994) suggested requirement is limited in the absence of AGP in the diet. However, based on the findings of Dahiya et

al. (2007a), *l*CP broiler diets can be supplemented with crystalline glycine, averting the risk of accelerated *C. perfringens* proliferation.

2.7.2. High protein diets

The level of protein in the diet can directly or indirectly influence the growth performance of broiler chickens. In addition, AA themselves can have adverse effects on growth and production performance, in the form of dietary imbalances or antagonisms. According to the definition, an imbalance is an alteration to the proper ratio of dietary AA with subsequent reduction in feed intake and growth, whereas an antagonism is a negative interaction between structurally similar AA (D'Mello 2003). Both imbalance and antagonism reduce the efficacy of AA utilization: nevertheless, formulating broiler rations based on the ideal protein concept, both these undesirable factors can easily be avoided, with the assurance of proper ratio of essential AA in the diet (Baker 1994; NRC 1994; Emmert and Baker 1997; Mack et al. 1999; Lemme 2003a,b). When diets are formulated based on ideal protein concept, the CP and AA requirements for broiler chickens to maximize growth performance and feed efficiency may be higher than current NRC requirement (Baker and Han 1994; Mack et al. 1999). Hoehler et al. (2001) reported that *h*CP diets balanced according to ideal protein ratios may be commercially feasible. However, there is a potential for negative interaction between the diets with excess protein and NE. As discussed above, previous studies have demonstrated the intestinal overgrowth of *C. perfringens* in broilers fed diets containing high concentrations of protein, increasing the potential for NE in broiler chickens (Drew et al. 2004; Wilkie et al. 2005).

Although digestion by endogenous enzymes and absorption from the small intestine are very effective, a constant supply of nutrients to the lower GIT occurs in the form of undigested dietary components, host enzymes, mucoproteins, serum albumin, amides and desquamated intestinal epithelial cells. The amount and composition of substances reaching the lower GIT can be readily modified by the diet. For instance, diets containing protein in relatively high amounts, and diets with imbalanced patterns of AA, result in low AA digestibility and utilization in the proximal GIT thus higher amounts of AA accumulate in the lower GIT as undigested proteins (Lan et al. 2004; McDevitt et al. 2006). This accumulation, together with low fermentable carbohydrate sources lead to more proteolytic fermentation (Piva et al. 1995). Accordingly, some of the intestinal VFA are derived from proteins which are apparently the major source of mainly branched-chain VFA such as *iso*butyrate, *iso*valerate and *iso*caproate, formed by the metabolism of branched-chain AA (valine, leucine, isoleucine; Macfarlane et al. 1992). In addition, certain bacteria are capable of metabolizing AA, producing potentially toxic metabolites which can negatively affect the intestinal cell turnover and broiler growth performance (Ewing and Cole 1994; Macfarlane and Macfarlane 1995; Gaskins 2001; van der Klis and Jansman 2002). Ammonia, volatile phenols and indoles, and a number of amines represent such toxic metabolites and these metabolites can negatively impact the overall health thus growth performance of livestock animals (Yokayama et al. 1982; Russell et al. 1983; Macfarlane et al. 1992 Gaskins 2001; van der Klis and Jansman 2002; Juskiewicz et al. 2004). Available information suggests that high concentrations of ammonia, generated by bacterial urease and deamination of AA from both dietary and endogenous protein (Visek 1978a; Wrong and Vince 1984; Pond and Yen 1987), readily passes across the gut wall thus gaining access to other tissues of

the body (Rowland 1992). This ammonia tends to depress growth of animals as a result of cell toxicity and subsequent increase in intestinal epithelial cell turnover and mucin degradation followed by an increase in synthesis and secretion of mucin (Lin and Visek 1991). In addition, ammonia has been reported to be negatively correlated to reduced villus height as well (Nousiainen 1991). Furthermore, elimination of the produced and absorbed ammonia requires about 7% of the total energy expenditure in monogastrics and ruminants (Eisemann and Nienaber 1990). Sub acute concentrations of ammonia can affect the metabolism of the host, resulting in reduced performance (Piva et al. 1995). Microbially generated amines, such as histamine and cadaverine can alter the amount of acid and bicarbonate added from the gut mucosa and rate of transit through the gut (Calloway 1968). Accordingly, Porter and Kenworthy (1969) have attributed these amines to the occurrence of diarrhea in weanling pigs as a result of increased peristalsis of the alimentary tract. Growth performance can also be negatively affected by phenols and indoles derived from the microbial metabolism of aromatic AA (Yokoyama et al. 1982). In addition, the presence of nitrogenous degradation products tends to elevate the distal gut pH due to their relatively high pKa values thereby counteracting the bacterial generated acetic and lactic acid associated pH changes and potentially increasing intestinal *C. perfringens* proliferation (Juskiewicz et al. 2004; Lan et al. 2005).

2.7.3. Amino acid digestibility and availability

The supply of AA as protein supplements and in synthetic form represents a significant cost of production in poultry feed industry. Accordingly, optimum utilization of these expensive nutrients is one of the vital aspects in poultry feed formulation. In fact, the existing knowledge on AA availability is a key approach to improve utilization. The

concept of AA digestibility is recognized as a sensitive indicator of AA availability in dietary ingredients for poultry (Parsons 1986; Johns et al. 1986; Albino et al. 1992), because AA availability has been defined as the portion of dietary AA which is in a state suitable for digestion, absorption and utilization (Batterham 1992). Accordingly, AA of poorly digestible protein sources are less available to birds and are concentrated in the lower GIT making them available for gut-specific pathogens (Williams et al. 2001; Lan et al. 2004; McDevitt et al. 2006). This provides a good source of nutrients for the proliferation of pathogenic microbes in the gut and affects gut health of broilers especially when antibiotic free diets are fed. As a result, feeding these types of protein can negatively affect the growth performance of broilers, as well.

2.8. Digestible amino acid values in broiler ration formulation

Reliance upon total AA content of the diet is considered inappropriate in poultry feed formulation. Poultry ration formulation on digestible AA basis is becoming important, because according to available information broiler chickens fed diets formulated on digestible AA basis have demonstrated superior growth and production performance. One of the current debates is concerned with the relative merits of ileal and fecal/excreta digestibility as a basis for feed formulation. Digestibility coefficients of AA assessed at the terminal ileal level of monogastrics give sensible estimations for AA availability. One study evaluated differences in digestibility coefficients using terminal ileal digesta and excreta of broiler chickens and demonstrated that digestibility coefficients measured in the ileal digesta are more precise than those measured in the excreta for AA availability values (Ravindran et al. 1999). Formulating broiler rations based on ilial digestible AA values, Rostagno et al. (1995) reported a better prediction of dietary

protein quality and broiler performance than rations formulated on total AA. Nevertheless, ileal digestible AA values are used only by a few feed industry nutritionists at present to formulate commercial poultry rations (Bryden and Li 2004). The main concerns for this reluctance include: (a) inconsistency in available data from different sources on digestible AA values, owing to variation in ingredients, strain of birds and method of evaluation; (b) lack of knowledge of the batch related variations in AA digestibility coefficients; and (c) scarcity of available information on broiler growth performance to diets formulated based on digestible AA values (Bryden and Li 2004). In fact, in a study with grower finisher pigs to evaluate the effect of the basis for feed formulation (total, apparent ileal or apparent fecal digestibility values of AA) on the weight gain, Wiseman et al. (1991) reported no advantage of using ileal rather than faecal, apparent digestible AA values in terms of accuracy of diet formulation. Yet, much more pronounced reduction in weight gain was observed when total AA were used in feed formulation.

2.9. Summary

With the intensification of the poultry production systems, the risk of both clinical and sub-clinical types of diseases has increased to a considerable level. In an attempt to minimize the problems associated with enteric pathogens, use of antibiotics in-feed has commonly been practiced at both a therapeutic level to combat diseases and at a sub-therapeutic level as growth promoters, since their discovery more than five decades ago. Nevertheless, various public health concerns associated with animal product consumption has resulted an increasing consumer pressure to limit the use of in-feed antibiotics. On the account of these concerns, some countries have already banned the

use of AGP in animal feed industry, which in turn has paved the way for an increased prevalence of NE in broiler chickens. This development has created a new insight in the research area which evaluates alternatives to AGP to improve both animal performance and enteric disease control. In this scenario, nutritional strategies have attracted much attention owing to their potential role in mitigating conditions that are favorable for the proliferation of *C. perfringens*, because a number of dietary factors that predispose birds to NE have already been identified. In fact, risk factors associated with all major components of the diet, namely carbohydrate, protein and fat, have extensively been discussed in the literature. Consequently, considerable amounts of time and research funds are being invested to explore ways to alleviate these risk factors and to manipulate the intestinal ecosystem to maintain the health, safety and consumer acceptability of the resultant product, without sacrificing the growth performance of broiler chickens, in a post in-feed antibiotic era. Nevertheless, to date, despite all these efforts, the non-antibiotic measures introduced to control both clinical as well as sub-clinical forms of NE in broilers are not 100% satisfactory in every situation, and a deficiency stands against the existing knowledge of the issues which play an important role in the progression of this economically important infection.

Based on the above observations, the following objectives were developed for the experiments described in this thesis:

- To evaluate the effects of dietary protein level and protein digestibility on growth performance and carcass characteristics of broiler chickens fed diets formulated based on ileal digestible protein and AA values.
- To develop a feasible experimental model for NE in broiler chickens raised on litter in the absence of coccidial co-infection

- Using the developed experimental model, to investigate the effects of dietary protein level and protein digestibility on the proliferation of *C. perfringens* in the GIT of broiler chickens fed diets formulated based on ileal digestible protein and AA values.
- To determine the dose response of *C. perfringens*-challenged and -unchallenged broiler chickens fed different methionine precursors in diets formulated based on ileal digestible protein and AA values.

3. EFFECTS OF PROTEIN LEVEL AND DIGESTIBILITY ON THE GROWTH AND CARCASS CHARACTERISTICS OF BROILER CHICKENS

This chapter was published in the Poultry Science journal. The full citation is: Widyaratne, G. P. and Drew, M. D. 2011. Effects of protein level and digestibility on the grrowth and carcass characteristics of broiler chickens. Poult. Sci. 90: 595 - 603. This study was conducted to evaluate the effects of low protein diets formulated based on digestible AA values on broiler production, before testing the particular diets under C. perfringens challenged situations.

3.1. Abstract

A study was conducted to evaluate the effects of dietary protein level and protein digestibility on the growth performance and carcass characteristics of broilers from 1 to 35d of age. Broiler chickens (N=320) were fed four different ideal protein-balanced (based on digestible AA), isocaloric diets in a 2 x 2 factorial design with two levels of ileal digestible protein (*HiPro*: 20 and 18% and *LoPro* 18 and 16% on days 1-14 and 15-35 respectively) and two levels of protein digestibility (*HiDig* and *LoDig*; approximately 85% and 80% CP digestibility respectively). The *HiDig* diets were formulated using soybean meal and fishmeal while the *LoDig* diets used wheat distillers grains and solubles (W-DDGS) and MBM as the primary protein sources. The standardized ileal digestibility (SID) and contents of AA in the W-DDGS and MBM were measured prior to the experiment to improve the accuracy of the diet formulations. The interaction between protein level and digestibility was significant for most of the AA for SID and, for digestible contents of all AA; birds fed *HiPro:HiDig* had significantly higher SID values and birds fed *LoPro:HiDig* had the lowest values for digestible AA content.

During the starter phase, the protein level x digestibility interaction was significant for average daily gain (ADG); with *LoPro:HiDig* and *LoPro:LoDig* fed birds showing the highest and lowest ADG, respectively. During the grower phase and the overall experimental period, the interaction between protein digestibility and level was significant for average daily feed intake (ADFI); that of birds fed the *LoPro:LoDig* diet was significantly lower than those fed diets with *HiDig* proteins, during the grower phase and lower than those fed the other three diets, during the overall experimental period. Total breast meat yield was significantly higher in birds fed the *HiPro* than those fed *LoPro* diets while birds fed *HiDig* diets had significantly more abdominal fat than those fed *LoDig* diets. The results suggest that low protein diets can support growth performance equal to high protein diets when highly digestible ingredients are used. However, breast meat yield goes down with the reduction of dietary protein level, regardless of ingredient digestibility.

3.2. Introduction

A considerable amount of research has been done to evaluate the effects of *l*CP–AA supplemented diets on the growth performance and carcass characteristics of broiler chickens. However, conflicting results from some research does not allow a clear conclusion on the effects of these diets in practical broiler production. Evaluating the impact of *l*CP-AA supplemented diets, some studies have reported inferior performance on broiler productivity (Edmonds et al. 1985; Fancher and Jensen 1989a,b,c; Holsheimer and Janssen 1991; Bregendahl et al. 2002; Kamran et al. 2008; Namroud et al. 2008), while others have reported no effect on performance when *l*CP diets are fed (Parr and Summers 1991; Deschepper and De Groote 1995; Moran and Stilborn 1996; Yamazaki

et al. 1996, 1998; Aletor et al. 2000; Kamran et al. 2010). The increased accumulation of abdominal fat in birds fed low protein (*LoPro*) diets was the only consistent observation reported in these studies. Nevertheless, the preponderance of information suggests that the rate and efficiency of growth is reduced, and carcass composition is inferior when dietary CP level is reduced by more than 3%, even when all known nutrient requirements are met (Fancher and Jensen 1989a,b,c; Aletor et al. 2000; Bregendahl et al. 2002; Sterling et al. 2005; Waldroup et al. 2005).

Diet formulation based on digestible nutrients is clearly superior to formulation based on total nutrients (Dari et al. 2005). However, this approach does not take into account the fact that the indigestible portion of the feed remains in the intestinal tract and can affect the growth performance of broilers by acting as a substrate for intestinal bacteria (Hill et al. 2005; Dahiya et al. 2006; Pieper et al. 2008) and slowing down the rate of passage of digesta. Thus, the amount and chemical content of unabsorbed nutrients might play a significant role in nutrient utilization in otherwise nutritionally similar diets. Based on these observations, it was hypothesized that diets formulated using poorly digestible ingredients will decrease the growth performance and reduce the carcass quality of broiler chickens, and that this effect will be exacerbated in *LoPro* diets. The current study was performed to test this hypothesis.

3.3. Materials and methods

Two experiments were conducted at the Animal Care Unit in the Western College of Veterinary Medicine, University of Saskatchewan. Experimental protocols for both experiments 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).

One-day-old male broiler chicks (Ross 308; Lilydale Hatchery, Wynyard, SK, Canada) were used in both experiments and standard management procedures were followed. Ambient temperatures gave optimal comfort for the birds with controlled temperature and humidity. The lighting schedule used was intended not to restrict early growth and consisted of 23 h light and 1 h dark from day 1 to 14 and, 20 h light and 4 h dark from day 15 onwards. Birds had free access to feed and water, throughout both experiments.

3.3.1. Experiment 1

Exp. 1 was performed to determine the apparent ileal digestibility (AID) and SID of the distillers dried grains and solubles derived from wheat (W-DDGS) and MBM derived from bovine materials. Compared to soybean meal and fish meal, the nutrient content and digestibility are not consistent in W-DDGS and MBM due to many factors. For example, factors including variety of wheat used for fermentation, method of fermentation, duration of the fermentation and drying processes, drying temperature, and the amount of solubles added to distiller's dried grains may have substantial effect on the consistency of the product (Carpenter 1970; Olentine 1986; Spiehs et al. 2002; Belyea et al. 2004; Bandegan et al. 2009). Similarly, species origin and ratio, processing conditions (temperature and pressure), processing time and ash content may affect the nutritional characteristics of MBM (Johnson and Parsons 1997; Wang and Parsons 1998; Karakas et al. 2001). Therefore, to improve the accuracy of diet formulation AA digestibility co-efficients of these two ingredients were measured.

A total of 72 broiler chickens were housed randomly in six electrically heated battery cages for a three week pre-experimental period during which they were fed a medicated, ideal protein-balanced (13.38 MJ/kg of ME; 1.2% Lys) corn-based starter crumble diet (Federated Coop Feeds, Saskatoon, SK, Canada; Table 3.1). At the end of the 3-week pre-experimental period, the birds (970.83 ± 18.57 g) were weighed and re-randomized into 12 battery cages. Four cages of six birds each were assigned to each of the three experimental diets (a reference diet and two test diets; Table 3.2) in mash form. The test diets were produced by mixing 60% of the reference diet and 40% of the ingredients (W-DDGS; Mohawk Canada, Lloydminster, AB, Canada and MBM; Saskatoon Processors, SK, Canada) together. The reference diet contained 0.40% chromic oxide as an indigestible marker. The birds were killed by cervical dislocation on d 28 of the experiment. Intestinal tracts were removed immediately and the contents of the distal half of the ileum (from Meckel's diverticulum to 2 cm proximal to the ileocecal junction) were collected. The collected digesta of the six birds within a cage were pooled at the sampling time and mixed with 4% formic acid. The pooled digesta samples were stored in a freezer maintained at –20°C until analyzed.

3.3.2. Experiment 2

A total of 320 birds (43.81± 1.07 g) were randomly distributed in 32 floor pens upon arrival at the research facility. Each pen (1 m^2) was equipped with a pan feeder, a manual drinker and fresh wood shavings. The birds were subjected to a 2-phase feeding regimen consisting of starter (1 to 14d: 12.75 MJ/kg) and grower (15 to 35d: 13.17 MJ/kg) phases. Eight pens of birds were randomly assigned to one of four dietary treatment groups in a 2 x 2 factorial arrangement of treatments with two AID protein

Table 3.1. Ingredient composition of the starter diet used from 0 to 21d of age in Exp. 1.

Ingredient	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
Sodium chloride	0.30
Choline chloride	0.10
Vitamin-mineral premix[1]	0.50
BMD® 110G[2]	0.05
Bio-Cox® 120G[3]	0.05

[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.

[2]Bacitracin (as methylene disalicylate) 110g/kg (Alpharma Inc. Fort Lee, NJ).

[3]Salinomycin sodium 120g/kg (Alpharma Inc. Fort Lee, NJ).

Table 3.2. Ingredient and nutrient composition of the reference diet used in Exp.1.

Ingredient	(%)
Corn	54.00
Soybean meal	40.00
Test ingredient	0.00
Canola oil	0.97
Choline chloride	0.10
Vitamin – mineral premix[1]	0.50
Dicalcium phosphate	2.12
Limestone	1.53
Sodium chloride	0.38
Chromic oxide	0.40
Calculated ME (MJ/kg) and nutrients (%)	
ME	12.00
Crude protein	18.09
Calcium	1.09
Available phosphorus	0.52
Lysine[2]	1.04
Methionine+Cystine[2]	0.69
Linoleic acid	1.51
Sodium	0.18

[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.

[2]Standardized ileal digestible: based on values obtained from AminoDat 3.0 Platinum version (Evonik-Degussa GmbH, Hanau, Germany).

levels; high (*HiPro*: 20 and 18% in starter and grower phases, respectively) and low (*LoPro*: 18 and 16% in starter and grower phases, respectively) and two ileal protein digestibilities; high (*HiDig*: approximately 85%) and low (*LoDig*: approximately 80%). Celite (10 g/kg; Celite 545, Celite Corporation, World Minerals Co., Lompoc, CA, USA) was added as a filler to balance the diets for all nutrient specifications. The ideal protein-balanced diets were formulated to have the same standardized ileal digestible (SID) AA concentrations for the reference AA, lysine. The SID AA contents were calculated in accordance with dietary ME level in each growth phase, using QuickChick 1.0 program (Evonik-Degussa GmbH, Hanau, Germany), as per values determined in Exp. 1 for W-DDGS and MBM and values obtained for all other ingredients from AminoDat 3.0 Platinum version (Evonik- Degussa GmbH, Hanau, Germany). The *HiDig* diets were formulated with soybean meal and Nova Scotia herring meal (Shur-Gain Aquaculture, Truro, Nova Scotia, Canada) as the major protein sources while the *LoDig* diets were formulated using W-DDGS and MBM as the main protein sources (Tables 3.3 and 3.4).

Birds and feeders were weighed by pen at the initiation of the experiment (d 0), and weekly thereafter, (d 7, 14, 21, 28, and 35). Birds were monitored twice a day (8:30 and 15:30) and in the case of mortality, the weight of the dead bird/s and remaining feed in the respective feeder/s were recorded immediately.

At 35 d of age, all birds were killed by cervical dislocation and carcasses were weighed individually. Intestinal digesta samples of four randomly selected birds from each pen were collected, pooled and stored as previously described. Upon manual evisceration, weights of the abdominal fat pads and boneless, skinless breast fillet and tenders (*Pectoralis major* and *P. minor* muscles) were recorded. As defined by Fancher

Table 3.3. Ingredient and nutrient composition of starter diets[1] used in Exp. 2.

Ingredient (%)	*LoPro*:*LoDig*	*LoPro*:*HiDig*	*HiPro*:*LoDig*	*HiPro*:*HiDig*
Wheat	20.00	20.00	30.00	30.00
Corn	22.55	28.47	2.64	7.40
Barley	5.51	1.40	11.50	4.77
Peas	7.73	5.83	15.00	15.00
Soybean meal	7.50	17.00	6.43	13.75
Canola meal	12.00	1.56	8.10	2.27
W-DDGS[2]	4.00	-	6.00	-
Wheat middlings	-	7.13	-	8.00
Meat and bone meal	3.06	-	4.15	-
Fish meal	3.31	4.00	-	0.50
Blood meal	-	-	3.35	3.88
Canola oil	5.71	5.00	6.00	6.00
Dicalcium phosphate	0.85	1.25	0.95	1.62
Calcium carbonate	0.50	1.03	0.61	1.22
Sodium chloride	0.38	0.46	0.31	0.40
Vitamin/mineral premix[3]	0.50	0.50	0.50	0.50
Choline chloride	0.10	0.10	0.10	0.10
L-Lysine.HCL	0.40	0.32	0.32	0.24
DL-Methionine	0.25	0.27	0.29	0.31
L-Threonine	0.12	0.09	0.09	0.07
Arginine	0.13	0.10	0.07	0.07
Isoleucine	0.12	0.08	0.16	0.15
Valine	0.13	0.14	0.05	0.07
Glycine	0.10	0.22	0.05	0.19
AVIZYME® 1302[4]	0.05	0.05	0.05	0.05
Celite	5.00	5.00	3.27	3.45
Calculated ME (MJ/kg) and nutrients (%)				
ME	12.75	12.75	12.75	12.75
Total protein	22.5	21.0	24.5	23.0
AID protein	18.0	18.0	20.0	20.0
Neutral detergent fiber	9.6	9.5	9.5	9.5

Calcium	0.94	0.94	0.94	0.94
Available Phosphorus	0.42	0.42	0.42	0.42
Linoleic acid	3.46	3.22	3.26	3.35
Sodium	0.20	0.20	0.20	0.19
AA (%) - analyzed and (calculated digestible[5])				
Arginine	1.47 (1.28)	1.44 (1.28)	1.52 (1.28)	1.47 (1.29)
Isoleucine	0.99 (0.84)	0.94 (0.84)	0.98 (0.84)	0.95 (0.85)
Leucine	1.71 (1.33)	1.71 (1.33)	1.88 (1.35)	1.89 (1.35)
Lysine	1.44 (1.24)	1.40 (1.24)	1.47 (1.24)	1.51 (1.24)
Methionine	0.64 (0.57)	0.64 (0.57)	0.62 (0.57)	0.65 (0.57)
Methionine+Cystine	1.04 (0.90)	0.98 (0.90)	1.00 (0.90)	0.98 (0.90)
Threonine	0.93 (0.78)	0.87 (0.78)	0.90 (0.78)	0.85 (0.79)
Valine	1.19 (0.98)	1.15 (0.98)	1.27 (0.98)	1.29 (0.99)
Tryptophan	(0.19)	(0.20)	(0.21)	(0.21)
Protein digestibility (calculated%)	80.0	85.7	81.6	87.0

[1] *LoPro*: low protein level, *HiPro*: high protein level, *LoDig*: low protein digestibility, *HiDig*: high protein digestibility.

[2]Wheat distiller's dried grains with solubles.

[3]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.

[4]Xylanase 5000 U/g; Protease 1600 U/g (Halchemix Canada Inc.).

[5]Standardized ileal digestible: based on values determined in Exp. 1 for W-DDGS and MBM and values obtained for all other ingredients from AminoDat 3.0 Platinum version (Evonik-Degussa GmbH, Hanau, Germany).

Table 3.4. Ingredient and nutrient composition of grower diets[1] used in Exp. 2.

Ingredient (%)	*LoPro*:*LoDig*	*LoPro*:*HiDig*	*HiPro*:*LoDig*	*HiPro*:*HiDig*
Wheat	33.58	33.75	74.50	70.00
Corn	14.39	18.67	-	-
Barley	12.29	8.31	-	-
Peas	4.96	0.72	0.64	4.65
Soybean meal	-	8.45	-	-
Canola meal	14.72	4.48	3.28	6.50
W-DDGS[2]	4.50	-	6.50	-
Wheat middlings	-	9.50	-	3.87
Meat and bone meal	4.25	-	8.50	-
Fish meal	-	2.75	-	2.18
Blood meal	-	-	0.23	2.47
Canola oil	6.00	6.00	2.70	3.93
Dicalcium phosphate	0.88	1.31	0.33	1.52
Calcium carbonate	0.58	1.11	0.001	1.07
Sodium chloride	0.31	0.42	0.22	0.39
Vitamin/mineral premix[3]	0.50	0.50	0.50	0.50
Choline chloride	0.10	0.10	0.10	0.10
L-Lysine.HCL	0.50	0.40	0.59	0.47
DL-Methionine	0.20	0.20	0.21	0.21
L-Threonine	0.15	0.12	0.18	0.16
Glycine	0.29	0.42	0.18	0.41
Arginine	0.16	0.16	0.20	0.25
Isoleucine	0.15	0.10	0.18	0.20
Valine	0.10	0.11	0.12	0.09
AVIZYME® 1302[4]	0.05	0.05	0.05	0.05
Celite	1.33	2.36	0.80	1.00
Calculated ME (MJ/kg) and nutrients (%)				
ME	13.17	13.17	13.17	13.17
Total protein	20.50	19.00	22.50	21.00
AID protein	16.00	16.00	18.00	18.00
Neutral detergent fiber	10.6	10.5	11.4	11.1

Calcium	0.92	0.92	0.92	0.92
Available Phosphorus	0.40	0.40	0.40	0.40
Linoleic acid	3.50	3.58	1.74	2.34
Sodium	0.18	0.18	0.17	0.17
AA (%) - analyzed and (calculated digestible[5])				
Arginine	1.30 (1.08)	1.21 (1.08)	1.35 (1.10)	1.26 (1.10)
Isoleucine	0.91 (0.72)	0.81 (0.72)	0.93 (0.74)	0.85 (0.74)
Leucine	1.45 (1.12)	1.39 (1.12)	1.44 (1.12)	1.49 (1.12)
Lysine	1.33 (1.04)	1.14 (1.04)	1.39 (1.05)	1.29 (1.04)
Methionine	0.57 (0.47)	0.51 (0.47)	0.59 (0.47)	0.55 (0.47)
Methionine+Cystine	0.97 (0.77)	0.85 (0.77)	0.98 (0.78)	0.91 (0.77)
Threonine	0.85 (0.67)	0.76 (0.66)	0.86 (0.68)	0.80 (0.68)
Valine	1.05 (0.82)	0.95 (0.82)	1.09 (0.84)	1.10 (0.84)
Tryptophan	(0.17)	(0.17)	(0.18)	(0.18)
Protein digestibility (calculated%)	78.0	84.2	80.0	85.7

[1] *LoPro*: low digestible protein, *HiPro*: high digestible protein, *LoDig*: low protein digestibility, *HiDig*: high protein digestibility

[2]Wheat distiller's dried grains with solubles

[3]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.

[4]Xylanase 5000 U/g; Protease 1600 U/g (Halchemix Canada Inc.)

[5]Standardized ileal digestible: based on values determined in Exp. 1 for W-DDGS and MBM and values obtained for all other ingredients from AminoDat 3.0 Platinum version (Evonik-Degussa GmbH, Hanau, Germany)

and Jensen (1989a), the tissue surrounding the gizzard and intestines, extending within the ischium, and neighboring the cloaca, bursa of Fabricius and adjoining the abdominal muscle was considered as the abdominal fat pad, in the present study.

3.3.3. Chemical analysis

The feed ingredients, feed and freeze-dried digesta samples were ground through a 1 mm screen using a Retsch Mill (Model ZM 100, Haan, Germany) prior to chemical analyses. Chemical analyses were conducted in duplicate. The ingredient, feed and digesta samples were analyzed for dry matter (DM) content by drying at 135°C in an airflow type oven for 2 h (method 930.15; AOAC 1990). For the digestibility study, feed and digesta samples were analyzed for chromic oxide after ashing at 450°C (Fenton and Fenton 1979) and for the performance study, grower diets and digesta samples were analyzed for acid insoluble ash after ashing at 500°C (MaCarthy et al. 1974). Samples were analyzed for CP and AA content (method 982.30E; AOAC 1990) by the Evonik Corporation (Evonik-Degussa GmbH, Hanau, Germany).

3.3.4. Calculations and statistical Analysis

The AID coefficients of AA for the W-DDGS and MBM evaluated in the Exp. 1 were calculated using the method of Ten Doeschate et al. (1993) from the formulae:

$$DC_{diet} = 1 - [(M_{diet}/M_i) \times (C_i/C_{diet})]$$

$$DC_{ti} = (DC_{diet} \times C_{diet} - DC_{ref} \times C_{ref} \times 0.60)/(C_{diet} - C_{ref} \times 0.60)$$

Where DC_{diet} and DC_{ti}= digestibility coefficient of an AA in the diet and test ingredient

M_{diet} and M_i = chromic oxide content of the feed and the ileal digesta

C_i, C_{diet} and C_{ref} = AA content of ileal digesta, of feed, and reference diet

DC_{ref} is the digestibility coefficient for the reference diet and was calculated using the same equation as for DC_{diet}.

The AID coefficients of DM, CP and AA were determined for the four dietary treatments used in Exp. 2 with the same formula previously described for DC_{diet}, but using acid insoluble ash as the marker. For both experiments, SID of AA was calculated from AID coefficients using the method of Yin et al. (2002), based on the mean endogenous AA composition of basal endogenous protein (Kadim et al. 2002). The digestible AA content was calculated using total nutrient content multiplied by the digestibility coefficients. The PER was calculated as grams of weight gain per gram of protein intake, for each growth phase and the overall experimental period. Abdominal fat, breast fillets, breast tenders and total white muscle (breast fillet + breast tenders) yields were calculated relative to the final BW.

Pen was considered as the experimental unit for the digestibility and growth performance variables while individual birds were considered as the experimental unit for the carcass variables in the Exp 2. Data from Exp 2 were analyzed as a 2×2 factorial arrangement with two levels of dietary protein and two levels of protein digestibility. Digestibility and carcass variables were analyzed using the GLM procedure of SAS (SAS Institute, Inc. Version 9.2) at the 5% level of significance. Performance variables were analyzed by analysis of variance using the MIXED procedure of SAS. For the performance and carcass variables, the initial BW was used as a covariate.

3.4. Results

The AA composition and the AA digestibility values of W-DDGS and MBM samples used in Exp. 1 are shown in Table 3.5. Of particular note, the SID for lysine in the W-DDGS was only 56.5% indicating that the product may have been overheated during drying causing the formation of Maillard reaction products (Widyaratne and Zijlstra 2007).

The DM digestibility of grower diets of Exp.2 was affected by the digestible protein level ($P < 0.05$), but not by the protein digestibility (Table 3.6). The DM digestibility was higher for *HiPro* diets than for *LoPro* diets. The CP digestibility was significantly affected by both main effects ($P < 0.05$) and was higher for *HiPro* diets and *HiDig* diets. The interaction between protein level and digestibility had a *P*-value of 0.06 and was due to the birds fed the *HiPro:HiDig* diet having significantly higher ileal protein digestibility than birds fed the other three diets. This suggests that the effects of *HiPro* and *HiDig* are additive for CP digestibility. The interaction between protein level and digestibility was significant for the SID of most of the AA ($P < 0.05$). As with AID for CP, the SID coefficients of most of the AA were significantly higher for the *HiPro:HiDig* diet compared to the other three diets. Similarly, the main effects interacted for the SID AA content of all tested AA, except for glutamate ($P < 0.05$; Table 3.7), and digestible AA contents were lower for the *LoPro:HiDig* diet than for the other grower diets.

During the starter phase of Exp. 2, neither of the main effects significantly affected growth performance ($P > 0.05$), but the protein level and digestibility interacted for ADG ($P < 0.05$; Table 3.8). Compared to *LoDig* proteins, the *HiDig* proteins in the *LoPro* diet increased the ADG of starter birds by 5.2% ($P < 0.05$) while protein

Table 3.5. Moisture (%), crude protein, ammonia and amino acid composition (% DM) and, apparent (AID) and standardized (SID) ileal digestibility (%) of amino acids in the test ingredients measured in 28-d old broiler chickens in Exp. 1.

Item	Wheat DDGS[1]			Meat and bone meal		
	Composition	AID[2]	SID[2]	Composition	AID	SID
Moisture	9.77			4.73		
Crude protein	39.58			60.69		
Ammonia	1.37			0.77		
Amino acid						
Alanine	1.53	64.0 ± 3.89[3]	67.4 ± 3.89	4.20	72.4 ± 1.33	73.6 ± 1.33
Arginine	1.78	76.4 ± 2.68	79.1 ± 2.68	3.83	72.0 ± 1.27	73.2 ± 1.27
Aspartate	2.04	47.0 ± 5.38	49.9 ± 5.38	4.65	47.5 ± 1.70	48.7 ± 1.70
Glutamate	10.52	85.9 ± 1.71	86.6 ± 1.71	7.40	62.5 ± 1.57	63.5 ± 1.57
Glycine	1.62	63.2 ± 3.30	65.9 ± 3.30	6.95	64.4 ± 0.97	65.0 ± 0.97
Histidine	0.95	67.2 ± 2.55	69.1 ± 2.54	1.42	69.3 ± 1.14	70.6 ± 1.14
Isoleucine	1.36	68.2 ± 3.86	71.4 ± 3.85	1.87	64.7 ± 1.59	67.1 ± 1.60
Leucine	2.58	75.7 ± 3.24	77.8 ± 3.24	3.89	69.9 ± 1.34	71.2 ± 1.34
Lysine	1.05	52.5 ± 4.46	56.5 ± 4.46	3.34	70.7 ± 1.21	72.0 ± 1.21
Methionine	0.63	74.6 ± 2.88	77.6 ± 2.88	0.96	74.9 ± 1.09	76.9 ± 1.09
Phenylalanine	1.72	80.2 ± 2.90	82.6 ± 2.90	2.08	68.0 ± 1.32	70.0 ± 1.32
Proline	3.68	84.1 ± 1.56	85.4 ± 1.56	4.58	59.7 ± 1.53	60.8 ± 1.53
Serine	1.78	72.4 ± 2.88	76.4 ± 2.88	2.44	57.3 ± 1.50	60.3 ± 1.50
Threonine	1.26	62.9 ± 3.40	68.7 ± 3.40	2.14	63.1 ± 1.55	66.5 ± 1.55
Valine	1.66	68.9 ± 3.68	72.1 ± 3.68	2.65	67.8 ± 1.36	69.8 ± 1.36
Total	36.29			54.00		

[1]Wheat distillers dried grains with solubles

[2]Values were calculated from results of Exp. 1

[3]Values are means ± standard deviation

Table 3.6. Effect of the interaction between digestible protein level and protein digestibility on apparent (AID: DM and CP) and standardized (SID: AA) ileal digestibility (%) measured in 35-d old broiler chickens.

Item	*LoPro*[1]		*HiPro*[1]		SEM	*P* Value		
	LoDig[2]	*HiDig*[2]	*LoDig*	*HiDig*		Protein	Digestibility	Interaction
Dry matter	68.79	68.20	70.52	70.73	0.78	0.011	0.809	0.613
Crude protein	77.06*b*	77.24*b*	77.25*b*	80.89*a*	0.87	0.040	0.037	0.057
Amino acid								
Lysine	86.96*ab*	86.09*b*	86.53*b*	89.26*a*	0.90	0.138	0.307	0.054
Methionine	92.41*ab*	91.31*b*	91.44*b*	93.72*a*	0.71	0.321	0.416	0.025
Threonine	82.48	83.15	83.11	87.40	1.08	0.031	0.029	0.104
Isoleucine	87.11*b*	86.64*b*	87.47*b*	90.08*a*	0.87	0.038	0.230	0.087
Arginine	88.23*ab*	88.09*ab*	86.06*b*	88.98*a*	0.75	0.402	0.073	0.051
Phenylalanine	85.35	85.16	86.45	89.11	1.00	0.017	0.225	0.163
Histidine	83.10*b*	82.60*b*	81.95*b*	88.22*a*	0.90	0.019	0.003	0.001
Leucine	84.50*b*	84.21*b*	84.30*b*	88.60*a*	0.94	0.035	0.042	0.022
Valine	84.23*b*	84.30*b*	84.39*b*	88.34*a*	0.87	0.023	0.029	0.035
Alanine	82.50*b*	82.41*b*	80.62*b*	86.53*a*	1.09	0.311	0.012	0.010
Aspartate	74.13*b*	75.62*b*	68.72*c*	79.66*a*	1.19	0.569	<0.0001	0.001
Glutamate	89.09*b*	88.66*b*	90.07*b*	92.24*a*	0.63	0.001	0.175	0.049
Glycine	82.95*c*	85.19*b*	79.50*d*	87.52*a*	0.74	0.457	<0.0001	0.001
Proline	84.18	86.30	86.73	90.78	0.70	<0.0001	0.0001	0.182
Serine	82.37	84.53	82.86	87.71	0.99	0.075	0.002	0.186

[1] *LoPro*: low digestible protein, *HiPro*: high digestible protein [2] *LoDig*: low protein digestibility, *HiDig*: high protein digestibility

[a-d]Means within rows with different superscripts are significantly different ($P < 0.05$)

Table 3.7. Effect of the interaction between digestible protein level and protein digestibility on standardized ileal digestible amino acid contents (%) measured in 35-d old broiler chickens.

Amino acid	*LoPro*[1]		*HiPro*[1]		SEM	*P* Value		
	LoDig[2]	*HiDig*[2]	*LoDig*	*HiDig*		Protein	Digestibility	Interaction
Lysine	1.16*b*	0.99*c*	1.20*a*	1.16*b*	0.01	<0.0001	<0.0001	<0.0001
Methionine	0.53*b*	0.46*c*	0.54*a*	0.52*b*	0.00	<0.0001	<0.0001	<0.0001
Threonine	0.70*a*	0.63*b*	0.72*a*	0.70*a*	0.01	<0.0001	<0.0001	0.010
Isoleucine	0.80*a*	0.71*c*	0.81*a*	0.77*b*	0.01	<0.0001	<0.0001	0.005
Arginine	1.14*ab*	1.07*c*	1.16*a*	1.12*b*	0.01	0.0004	<0.0001	0.050
Phenylalanine	0.74*b*	0.72*b*	0.80*a*	0.82*a*	0.01	<0.0001	0.832	0.018
Histidine	0.40*b*	0.37*c*	0.40*b*	0.52*a*	0.00	<0.0001	<0.0001	<0.0001
Leucine	1.23*b*	1.17*c*	1.22*b*	1.33*a*	0.01	<0.0001	0.060	<0.0001
Valine	0.89*c*	0.80*d*	0.92*b*	0.97*a*	0.01	<0.0001	0.037	<0.0001
Alanine	0.75*a*	0.69*b*	0.77*a*	0.77*a*	0.01	<0.0001	0.002	0.002
Aspartate	0.98*b*	1.06*a*	0.88*c*	1.07*a*	0.02	0.009	<0.0001	0.002
Glutamate	3.79*c*	3.47*d*	4.46*a*	4.03*b*	0.03	<0.0001	<0.0001	0.062
Glycine	1.14*a*	1.05*b*	1.15*a*	1.15*a*	0.01	<0.0001	<0.0001	<0.0001
Proline	1.30*b*	1.14*c*	1.55*a*	1.33*b*	0.01	<0.0001	<0.0001	0.009
Serine	0.71*c*	0.72*c*	0.78*a*	0.75*b*	0.01	<0.0001	0.209	0.022

[1] *LoPro*: low digestible protein, *HiPro*: high digestible protein [2] *LoDig*: low protein digestibility, *HiDig*: high protein digestibility

[a-d]Means within rows with different superscripts are significantly different ($P < 0.05$)

Table 3.8. Effect of the interaction between digestible protein level and protein digestibility on growth performance during starter, grower and overall periods of broiler chickens.

Variable	*LoPro*[1]		*HiPro*[1]		SEM	*P* Value		
	LoDig[2]	*HiDig*[2]	*LoDig*	*HiDig*		Protein	Digestibility	Interaction
Starter								
ADG[3]	33.94[b]	35.72[a]	34.92[ab]	34.14[ab]	0.57	0.603	0.389	0.034
ADFI[4]	44.00	45.88	45.49	45.34	0.69	0.504	0.223	0.153
FCR[5]	1.30	1.29	1.31	1.33	0.02	0.240	0.803	0.425
Grower								
ADG	83.07	85.65	85.31	86.10	1.31	0.312	0.207	0.499
ADFI	134.35[b]	144.45[a]	139.83[ab]	141.22[a]	1.92	0.562	0.006	0.031
FCR	1.55*b*	1.60*a*	1.57*ab*	1.58*ab*	1.01	0.795	0.026	0.077
Overall								
ADG	58.51	60.69	60.11	60.12	0.85	0.545	0.209	0.212
ADFI	89.18[b]	95.17[a]	92.66[a]	93.28[a]	1.12	0.483	0.007	0.024
FCR	1.42	1.44	1.44	1.45	0.01	0.353	0.189	0.883
Final BW	2483[b]	2649[a]	2627[a]	2592[ab]	0.04	0.322	0.137	0.023

[1] *LoPro*: low digestible protein, *HiPro*: high digestible protein [2] *LoDig*: low protein digestibility, *HiDig*: high protein digestibility

[3]average daily gain (g/d); [4]average daily feed intake (g/d); [5]feed conversion ratio (g/g); [6]Final body weight at 35d of age (g)

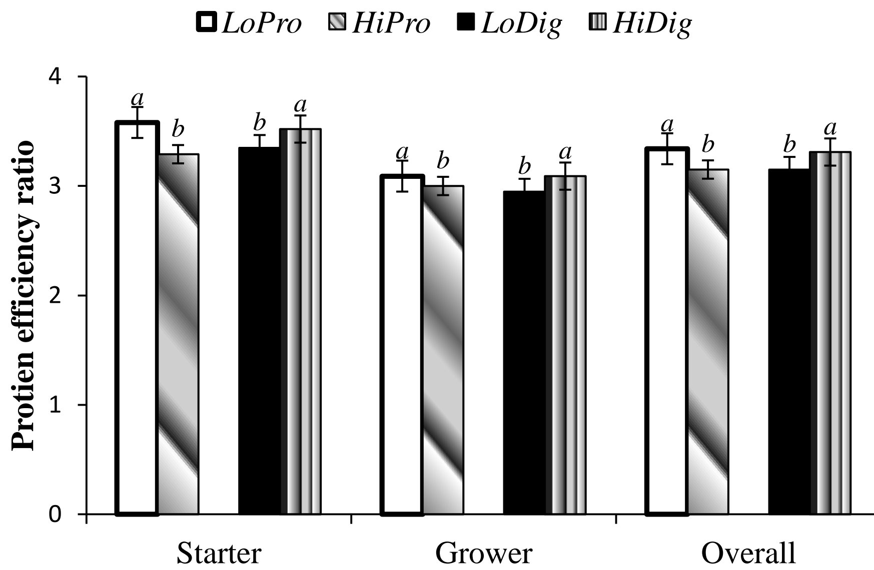
[a,b]Means within rows with different superscripts are significantly different ($P < 0.05$)

digestibility did not affect the growth of starter birds fed *HiPro* diets ($P > 0.05$). Neither dietary protein level nor protein digestibility affected the ADG of birds during the grower phase ($P > 0.05$), but an interaction between the two factors affected ADFI ($P < 0.05$). The *LoDig* proteins reduced ADFI of growers fed *LoPro* diets by 7.5% ($P < 0.05$), but not of birds fed *HiPro* diets ($P > 0.05$). The FCR was 1.9% higher for growers fed *HiDig* proteins ($P < 0.05$). The dietary protein level did not affect the FCR ($P > 0.05$), only tended to interact with digestibility ($P = 0.08$). The protein level, protein digestibility or their interaction did not affect the overall growth and the FCR of the birds ($P > 0.05$), but an interaction between the two main effects affected the overall ADFI of birds ($P < 0.05$). Similar to grower phase, *LoDig* proteins reduced the overall ADFI of birds fed *LoPro* diets by 6.3% ($P < 0.05$), but not of birds fed *HiPro* diets ($P > 0.05$). The interaction between protein level and digestibility was significant for the final BW of the birds ($P < 0.05$; Table 3.8). The overall growth of birds fed the *LoPro:HiDig* diet was significantly higher than those fed the *LoPro:LoDig* diet ($P < 0.05$) and similar to birds fed *HiPro* diets ($P > 0.05$).

Both main effects were significant for PER during the starter and grower periods as well as over the whole experiment ($P < 0.05$; Figure 3.1). Birds fed *LoPro* diets and *HiDig* proteins significantly increased the PER throughout the experiment than those fed *HiPro* diets and *LoDig* proteins, respectively. In addition, the PER was significantly higher for starter birds than for growers ($P < 0.05$; Figure 3.1).

Protein level but not digestibility affected the *Pectoralis major*, *P. minor* and total breast muscle yield ($P < 0.05$; Table 3.9); the birds fed *HiPro* diets yielded 6.7, 3.6 and 6.2% more *P. major*, *P. minor* and total breast meat, respectively than birds fed *LoPro* diets. In contrast, protein digestibility but not level significantly increased the

(A)



(B)

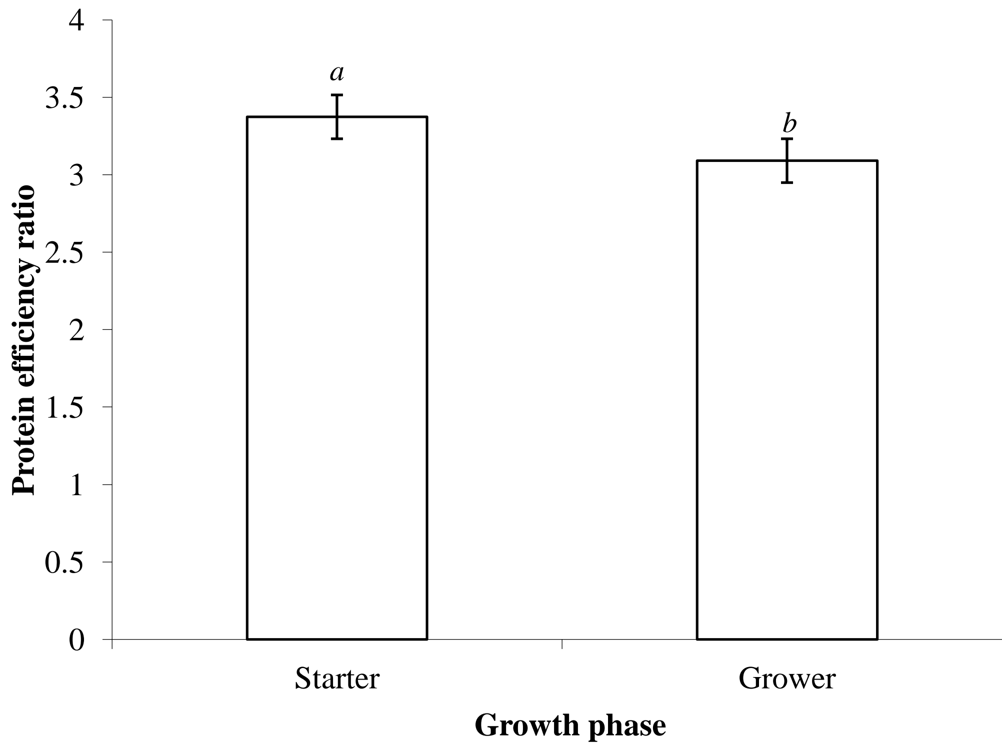


Figure3.1. Effect of dietary protein level and protein digestibility (A) and, growth phase (B) on the PER of broiler chickens used in Exp.2.
[a,b]Means for each factor within starter, grower and overall growth periods with different superscripts are significantly different **(A)** and means with different superscripts are significantly different **(B)** ($P < 0.05$).

Table 3.9. Effect of dietary protein level and protein digestibility on breast muscle yield and accumulation of abdominal fat in broiler chickens in Exp. 2.

	Level		Digestibility			*P* Value		
Variable	Low	High	Low	High	SEM	Level	Digestibility	Interaction
Yield (% of live weight)								
Pectoralis major	15.3^b	16.3^a	15.9	15.7	0.11	<0.01	0.39	0.79
Pectoralis minor	3.29^b	3.41^a	3.38	3.32	0.04	0.01	0.19	0.79
Total breast meat	18.6^b	19.7^a	19.2	19.1	0.12	<0.01	0.26	0.87
Abdominal fat	1.33	1.27	1.20^b	1.40^a	0.03	0.17	<0.01	0.39

[a,b]Means within a main effect with different superscripts are significantly different ($P < 0.05$)

amount of abdominal fat ($P < 0.05$). Birds fed *HiDig* proteins yielded 16.7% more abdominal fat than birds fed *LoDig* diets.

3.5. Discussion

One of the main limitations associated with this type of study is the inability to meet the required nutrient specifications with the same set of ingredients. This results in the problem of distinguishing nutrient effects from ingredient effects. Balancing diets based on SID may reduce this problem (Lemme et al. 2004). The premise of formulating feeds based on SID is that diets with equal SID AA should give equal growth performance (Angkanaporn et al. 1996). This result is independent of feed intake and the ingredients used in the diets (Angkanaporn et al. 1996; Ravindran and Bryden 1999; Stein et al. 2001; Lemme et al. 2004). In the current study, the low digestible (*LoDig*) diets were prepared using W-DDGS and MBM as the primary sources of protein. Given that the digestible nutrient contents of these products are extremely variable, a digestibility trial was performed so that feed formulation would be accurate. In contrast, soybean meal is consistent in its digestible nutrient content to a great extent and the fishmeal used was of high quality and had previously been analyzed for total nutrient content and nutrient digestibility calculated from a reliable database.

The AID of DM was higher in birds fed *HiPro* diets than in birds fed *LoPro* diets at d 35. This can be attributed to the ingredient composition of the grower diets used in this study. Wheat was the main cereal grain in the *HiPro* diets while barley was included, albeit at low levels, in *LoPro* diets in addition to wheat. All diets contained xylanase which degrade arabinoxylan, the key anti-nutritional factor presents in wheat (Zijlstra et al. 1999). Nevertheless, no enzyme was included in diets to break down β-

glucan, the main anti-nutritional factor in barley (Bach Knudsen 1997). Accordingly, the intact β-glucan present in *LoPro* diets might have constrained the DM digestibility of respective diets. As expected, the AID of CP was higher in diets containing high digestible (*HiDig*) protein sources. In addition, *HiPro* diets demonstrated higher CP digestibility, entirely due to markedly higher CP digestibility in *HiPro:HiDig* diet.

Birds fed *LoPro:HiDig* diet demonstrated the lowest values for SID content for most of the AA, despite lower and similar SID, compared to birds fed *HiPro:HiDig* and *LoDig* diets, respectively. Since the diets were formulated on the SID AA basis, the standardized ileal digestible AA contents should hypothetically be similar for each AA among the four grower diets. Nevertheless, the standardized ileal digestible AA content in those birds fed *LoPro:HiDig* diet closely resembled the numbers used in feed formulation. Digestible nutrient content is a factor of digestibility of particular nutrient. Ileal AA digestibility is calculated considering the disappearance of AA at the terminal ileum as a percentage of dietary intake, assuming the portion that disappeared on its way to the terminal ileum is digested and absorbed by the host animal. However, an amount of this disappeared portion is subjected to intestinal microbial metabolism and utilization. Accordingly, that assumption makes ileal AA digestibility, hence the ileal digestible AA content overestimated. The amount utilized by microbes correlates to the degree of intestinal microbial activity. In the present study, however, the ADG of birds fed *LoPro:HiDig* diet was similar to that of birds fed the other three diets, during the grower and the overall experimental periods. The PER data demonstrated maximum protein utilization in birds fed *LoPro* diets and *HiDig* proteins. The birds fed *LoPro:HiDig* diet demonstrated the highest final BW. These observations suggest that the maximum utilization of disappeared portion of AA for protein accretion occurred in

birds fed *LoPro:HiDig* diet. In birds fed the other three diets, a considerable amount of AA may have been utilized for increased microbial activities. Accordingly, it can be predicted that feeding poorly digestible proteins or high dietary protein may increase the intestinal microbial activity (Apajalahti et al. 2004). However, intestinal bacterial numbers or microbial activity was not evaluated in this study, therefore this hypothesis needs to be tested.

Neither protein level nor digestibility affected the feed intake of the birds in the starter phase. This was probably as a result of physical restrictions on feed intake of birds due to limited gut capacity (Griffiths et al. 1977). However, birds fed *LoPro:HiDig* diet grew faster than the birds fed *LoPro:LoDig* diet. It is well understood that, to be of any value to the animal, dietary protein must be digested and the ensuing AA absorbed prior to reaching the hind gut. The nutrients available to intestinal bacteria are primarily derived from the indigestible portion of feeds. Changes in the composition of intestinal contents can lead to changes in intestinal microbial numbers, species and species diversity (Drew et al. 2002; Hill et al. 2005; Dahiya et al. 2006; Pieper et al. 2008). Intestinal microbes of the birds fed diets containing *LoDig* proteins would likely receive relatively higher amounts of AA as undigested proteins. Some metabolites derived as a result of bacterial fermentation of certain AA can affect the enterocyte turnover thus overall growth performance of the host (Ewing and Cole 1994; Macfarlane and Macfarlane 1995; Gaskins 2001; van der Klis and Jansman 2002). However, broiler starters fed *LoPro:HiDig* diet had a better growth compared to the starters fed *LoPro:LoDig* diet, likely due to higher availability of AA for hind gut bacterial activity, as described above.

The capacity of the GIT of bird increases with age, therefore the physical state of restriction on the quantity of feed intake should be decreased (Hidalgo et al. 2004; Kamran et al. 2008), However, the feed intake of growers fed *LoPro* diets was not increased in the present study. Instead, the feed intake of birds fed the *LoPro* diet containing *LoDig* proteins reduced by 7.5% compared to *LoPro:HiDig* diet fed counterparts. This trend was observed in birds fed *HiPro:LoDig* diet, as well. Despite the equal amounts of digestible protein within each of the *LoPro* and *HiPro* diets used in the present study, the diets with *HiDig* proteins contained a lesser amount of total protein (CP) than in diets with *LoDig* proteins. Aletor et al. (2000) reported an increase in feed intake in broiler growers fed *l*CP – AA supplemented diets. On the other hand, higher amounts of nitrogen received by the gut microbes of the birds fed diets containing *LoDig* proteins as undigested proteins may result in an increased in hind gut fermentation (Piva et al. 1995). The physical distention of the hind gut and the resultant low digesta passage rate caused by high microbial fermentation might reduce feed consumption (Cherbut et al. 1988). Despite reduced feed intake, birds fed *LoDig* proteins (*h*CP) utilized feeds more efficiently compared to the birds fed *HiDig* proteins (*l*CP), as reflected by the FCR. This observation was in agreement with Aletor et al. (2000), who reported a corresponding increase in FCR, associated with increased feed consumption in broiler growers fed *l*CP – AA supplemented diets.

Despite the similar SID AA levels in all experimental diets, utilization of dietary protein was more effective in birds fed *LoPro* (either total or digestible) diets and *HiDig* protein than in birds fed *HiPro* diets and *LoDig* proteins, respectively, as indicated by the PER. This observation was in agreement with the findings of Cheng et al. (1997) and Aletor et al. (2000), who reported a significant increase in PER with the reduction in

dietary CP content. In addition, non essential AA supplementation of *l*CP diets reduced the PER values compared to the un-supplemented counterparts (Aletor et al. 2000). Broilers used in this study utilized dietary protein more efficiently during the starter phase compared to the grower phase. Similarly, Johnson and Parsons (1997) and Kamran et al. (2008) measured the PER in broiler chickens at different growth stages and reported a reduction in PER with increasing age. These findings can probably be attributed to the increasing nutrient requirements for maintenance with the progression of the age. Overall, the PER results suggest that for the most efficient utilization of dietary protein, high quality proteins should be assimilated in broiler rations and essential AA should be included in amounts not greater than compelled for optimum performance. This was well supported by the final BW of the birds which was optimized in birds fed *LoPro* diets containing *HiDig* proteins.

Birds fed *HiPro* diets yielded more breast muscle (breast fillets, breast tenders and total white muscle) compared to birds fed *LoPro* diets. Since the experimental diets of the current study were formulated to be iso-energetic, the ME: CP ratio of *HiPro* diets was lower than that of *LoPro* diets. Some previous studies reported similar observations in broilers fed diets with low ME:CP ratio with an increase in breast meat yield with the age of the birds (Moran 1977; Peng et al. 1985; Swatland 1989; Bartov and Plavnik 1998; Corzo et al. 2005; Dozier et al. 2006). The reported findings suggest that breast muscle yield is responsive to dietary protein level above that required for growth. Accordingly, the propensity to reduce dietary protein level in diets fed during the latter phases of broiler production should be reassessed if maximal breast meat yield is of importance and the birds are schedule for marketing at a relatively advanced age.

The content of abdominal fat was higher in the birds fed *HiDig* proteins than in birds fed *LoDig* proteins. Many previous studies have reported that birds fed *l*CP had increased feed intake with a concurrent increase in the deposition of abdominal fat (Fancher and Jensen 1989b,c; Rosebrough and McMurtry 1993). Although the iso-energetic diets used in the present study contained equal amounts of digestible protein at each low and high level, the ME:CP ratio was higher in diets containing *HiDig* proteins, compared to respective diets with *LoDig* proteins. It is generally accepted that feeds containing a high energy: protein ratio promote the retention of excess energy as fat (Kamran et al. 2008) and as a result, the birds fed those diets containing *HiDig* proteins with a higher ME:CP ratio deposited the excess energy as body fat in the present study.

In conclusion, the overall findings of this study suggest that the use of highly digestible feed ingredients when formulating low-protein diets may significantly reduce the negative effects of low-protein diets on the growth performance of broilers. This interaction between protein level and protein digestibility may partially explain the lack of consensus in the literature on the effects of low-protein diets on the growth performance of broiler chickens. However, the relationship between lowered gut AA content and improved growth performance requires further investigation to elucidate the mode of action.

4. EVALUATION OF MICROBIOLOGICAL, PATHOLOGICAL AND HEMATOLOGICAL PARAMETERS IN AN EXPERIMENTAL DISEASE MODEL FOR NECROTIC ENTERITIS IN BROILER CHICKENS RAISED ON LITTER

4.1. Abstract

Two experiments were conducted to establish a potential experimental model for NE in broiler chickens raised on litter. In Exp. 1, 96 birds were placed in two rooms in eight floor pens/room. Birds in one room were challenged, either by gavaging or offering feed mixed with cooked meat medium containing *C. perfringens* type A. In Exp. 1, *C. perfringens* challenge reduced the ADG of birds during d 14 to 21, d 29 to 35 and d 14 to 35 (entire) periods, by 9.14, 16.06 and 8.65%, respectively ($P < 0.05$). The ADG was not affected by challenging mode during particular periods ($P > 0.05$). The two main effects interacted for the ADFI and FCR of birds for the period of d 14 to 21 ($P < 0.05$); *C. perfringens* challenge reduced the feed consumption and in-feed challenge reduced the ADFI by 19.8% compared to gavaging. The FCR in unchallenged birds was not affected by the mode of challenging, but the pathogen administration with feed reduced the FCR by 17.34% compared to gavaging. In the d 29 to 35 period, ADFI was reduced and the FCR was increased 9.88 and 8.33%, respectively, by the *C. perfringens* challenge ($P < 0.05$). *C. perfringens* challenge reduced the ADFI during the overall experimental period by 9.39% compared to the unchallenged birds ($P < 0.05$). Neither *C. perfringens* challenge nor challenge mode affected the growth performance during the d 22 to 28 period ($P > 0.05$) and the challenge mode did not affect the parameters either in d 29 to 35 or the overall experimental period ($P > 0.05$). Neither of the main effects affected the FCR during the overall experimental period ($P > 0.05$). Two challenge

modes did not affect the *C. perfringens* counts either in the ileum or cecum ($P > 0.05$). In Exp. 2, 48 broiler chickens raised on litter were orally inoculated with *C. perfringens* in cooked meat medium, using two modes (water or feed) at two dose rates (2 or 4 mL per bird). Neither the mode nor the dose of *C. perfringens* administration did not affected the growth performance, during d 14 to 21, d 21 to 28 and d 14 to 28 (entire) periods ($P > 0.05$). The two main effects did not affect the *C. perfringens* counts in the ileum and the cecum and histopathological and hematological variables ($P > 0.05$). The results suggest that each modes of *C. perfringens* administration described herein can be exploited with either dose rates to induce NE in broiler chickens raised on litter.

4.2. Introduction

Necrotic enteritis is a bacterial infection with a considerable economic impact on the poultry industry (McDevitt et al. 2006). This infection is caused by some *C. perfringens* type A strains carrying the genes for α or NetB toxins (Keyburn et al. 2008, Van Immerseel et al. 2008). The disease may emerge in clinical or subclinical forms (Van Immerseel et al. 2004). Broiler producers experience high flock mortality with the acute clinical version of the disease (Wages and Opengart 2003), while the subclinical form of the disease negatively affects the growth performance of broiler chickens (Elwinger et al. 1992; Porter 1998; Lovland and Kaldhusdal 2001). Traditionally, NE prevention strategies have taken the form of antibacterial feed additives, often referred to as growth promoters (Maxey and Page 1977; George et al. 1982; Stutz and Lawton 1984; Hofshagen and Kaldhusdal 1992; Ficken and Wages 1997; Watkins et al. 1997). The current debate regarding the non-therapeutic use of antibiotics in animal feed has suggested the requirement of a substantial understanding of the factors associated with

the pathogenesis of NE. Nevertheless, the major problem in the evaluation of the factors associated with the development of NE and potential controlling methods is the lack of a reproducible disease model. Several approaches to reproduce NE in broiler chickens have been documented in the literature, describing different disease models. The majority of approaches have used the factors which predispose birds to NE, such as coccidial co-infection, to induce the disease (Kageyama et al. 1987; Williams et al. 2003). However, none of the methods developed so far is recognized as a robust disease model for NE.

Several parameters are used in estimating the severity of NE; intestinal gross lesions and bacterial counts in digesta contents are the main determinants for the subclinical form of the disease while mortality is the key response for the acute form of NE (Kaldhusdal and Lovland 2000). Methods such as whole-blood chemiluminescence (WBCL) assay (Papp and Smits 2007; Papp et al. 2009) and microscopic lesion scoring systems (Gholamiandehkordi et al. 2007) are emerging as novel approaches to quantify the severity of the disease.

The current challenge model used in our laboratory involves the gavage of chickens on d15-19 post-hatch with 1 ml of an overnight culture of *C. perfringens* (Wilkie et al. 2005; Dahiya et al. 2005; 2007a,b). This method requires the handling of every bird daily for 5 consecutive days, and this limits the size of the experiment to a relatively small number of birds. However, studies examining the effect of *C. perfringens* infection on growth performance of broilers require a larger number of birds making the current method of infection impractical. The current study was thus conducted to evaluate the mode and the dose of *C. perfringens* challenge on the growth

performance, innate immune status, intestinal lesions and growth of *C. perfringens* in ileal and cecal contents in broiler chickens.

4.3. Materials and methods

Two experiments were conducted at the Animal Care Unit in the Western College of Veterinary Medicine, University of Saskatchewan. Experimental protocols for both experiments 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 2005).

4.3.1. Birds and management

One-day-old male broiler chicks (Ross 308; *Gallus gallus*), obtained from a local broiler hatchery (Lilydale Hatchery, Wynyard, Saskatchewan, Canada) were used in both experiments and standard management procedures were followed. Floor pens used in both experiments were equipped with a pan feeder, a manual drinker and new soft-wood shavings. Ambient temperatures gave optimal comfort for the birds with controlled temperature and humidity. The lighting schedule used was intended not to restrict early growth and consisted of 23 h light and 1 h dark from day 1 to 14 and, 20 h light and 4 h dark from d 15 onwards. Feed and water were available for *ad libitum* consumption, throughout both experiments. Upon arrival, a total of 96 birds were randomly placed in two rooms (48 birds in eight pens/room) in Exp. 1 and 48 birds were randomly distributed into eight pens in one room in Exp. 2.

4.3.2. Feed and feeding

The birds were fed the same corn/soybean meal-based starter crumble diet (3,200 kcal/kg of ME; 1.2% Lys) described in chapter 3 (Table 3.1: Federated Coop Feeds, Saskatoon, Saskatchewan, Canada) for the first 14 d of both experiments. On d 14, the birds were weighed and introduced to ideal protein-balanced, unmedicated grower rations. In Exp. 1, the crumbled grower diet contained 18% AID CP and 13.17 MJ/kg of ME (Table 4.1). In Exp. 2, the grower diet was in mash form and formulated to contain 23% AID CP and 12.75 MJ/kg of ME (Table 4.2). Both grower diets met or exceeded the NRC requirements for broiler chickens for all other nutrients [National Research Council (NRC) 1994] and were fed until the end of the experiments.

4.3.3. Preparation of innoculum

For this study a *C. perfringens* field strain isolated from a clinical case of NE in broiler chickens was used (Dr. M. Chirino, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK). The particular strain was characterized using Polymerase chain reaction (PCR) technique as a type A toxin producer (Dumonceaux et al. 2006). The organism was cultured anaerobically on BBL Blood Agar Base (Becton Dickinson Co., Sparks, MD) containing 5% sheep blood and 100 mg/L of Neomycin Sulphate (The Upjohn Co., Orangeville, ON, Canada) for 18 h at 37ºC, then aseptically inoculated into a sterilized preparation of Robertson's cooked meat broth (Difco Laboratories, Detroit, MI), and incubated anaerobically and statically for 8 h at 37ºC.

Table 4.1. Ingredient and nutrient composition of grower diet used for all birds from d 14 to 35 of age in Exp. 1 (as fed basis).

Component	Inclusion (%)
Ingredient content	
Wheat	74.50
Peas	0.64
Canola meal	3.28
Wheat distiller's dried grains with solubles	6.50
Meat and bone meal	8.50
Blood meal	0.23
Canola oil	2.70
Dicalcium phosphate	0.33
Calcium carbonate	0.001
Sodium chloride	0.22
Vitamin-mineral premix[1]	0.50
Choline chloride	0.10
L-Lysine.HCL	0.59
DL-Methionine	0.21
L-Threonine	0.18
Glycine	0.18
Arginine	0.20
Isoleucine	0.18
Valine	0.12
AVIZYME® 1302[2]	0.05
Celite	0.80
Calculated energy and nutrient content (%)	
ME (MJ/kg)	13.17
AID CP	18.00
AID Glycine	1.00
Calcium	0.92
Available phosphorus	0.40
Linoleic acid	1.74
Sodium	0.17

SID Lysine	1.05
SID Methionine	0.47
SID Met+Cys	0.78
SID Threonine	0.68
SID Tryptophan	0.18
SID Arginine	1.10
SID Valine	0.84
SID Isoleucine	0.74
SID Leucine	1.12

[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.

[2]Xylanase 5000 U/g; Protease 1600 U/g (Halchemix Canada Inc.)

Table 4.2. Ingredient and nutrient composition of the grower diet used for all birds from d 14 to 28 of age in Exp. 2 (as fed basis).

Component	Inclusion (%)
Ingredient content	
Corn	34.00
Wheat	30.00
Peas	0.08
Soybean meal	11.93
Canola meal	0.66
Corn gluten meal	5.72
Blood meal	1.83
Meat and bone meal	4.69
Fish meal	1.16
Gelatin	4.00
Canola oil	1.00
L-Lysine HCl	0.22
DL-Methionine	0.13
Dicalcium phosphate	1.81
Calcium carbonate	0.97
Sodium chloride	0.20
Choline chloride	0.10
Vitamin-mineral premix[1]	0.50
Celite	1.00
Calculated energy and nutrient content (%)	
ME (MJ/kg)	12.75
AID CP	23.00
Calcium (%)	1.20
Available phosphorus	0.60
AID Met + Cys	0.83
AID Lysine	1.11
AID Threonine	0.72
AID Trptophan	0.20
AID Arginine	1.36

AID Isoleucine	0.73
AID Leucine	2.08
AID Valine	1.03
AID Glycine	1.75

[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.

4.3.4. Experimental design and challenge procedure

Both experiments were conducted based on 2 x 2 factorial arrangements. The main effects for the Exp. 1 were *C. perfringens* infection (challenged or unchallenged) and challenge mode (oral gavage or in-feed) whereas for the Exp. 2, mode (feed or water) and dose (2 or 4 ml of broth culture/bird) of challenging the birds. Respective birds were challenged once daily with the actively growing broth culture of *C. perfringens* on d 15 to 19 (inclusive). Fresh cultures were prepared daily and culture-feed/water mixing was done immediately before offering to the birds. Bacterial counts were performed on the culture daily before inoculation and the counts ranged from 5.89×10^6 to 7.24×10^6 colony forming units per ml (cfu/ml).

In Exp.1, all birds in one room were challenged, either by gavaging or offering feed mixed with cooked meat medium broth containing *C. perfringens* type A. Each bird in four pens received 1/3 of ADFI (24 g of feed each at the particular age) mixed with 4 ml of the broth culture whereas the birds in the remaining four pens in the same room were orally gavaged with 1.0 ml of same broth culture. Birds in the other room were either orally gavaged with 1.0 ml sterile cooked meat medium broth or fed 1/3 of ADFI mixed with 4 ml of sterile cooked meat medium broth (Table 4.3).

In Exp. 2, birds in four pens received 1/3 of ADFI mixed with either 2 or 4 ml of broth culture (2 pens each) whereas the birds in the remaining four pens received 0.1% sterile peptone buffer with 5 g/L Cys hydrochloride (Sigma Chemical Co., St. Louis, MO), an amount equivalent to 1/4 of average daily water consumption (35 ml each at the particular age) mixed with either 2 or 4 ml of the same broth culture (2 pens each: Table 4.3). To ascertain a better survival of the organism in the environment, peptone water containing Cys hydrochloride (5g/L) was used, instead of tap water.

Table 4.3. Experimental designs to evaluate mode and dose of *C.perfringens* challenge in broiler chickens.

Experiment 1

Treatment	Number of birds	*C.perfringens* challenge	Mode
1	24	Challenged	Oral gavage[1]
2	24	Challenged	Feed[2]
3	24	Unchallenged	Oral gavage[3]
4	24	Unchallenged	Feed[4]

Experiment 2

Treatment	Number of birds	Mode	Dose[5] (ml)
1	12	Water[6]	2
2	12	Water	4
3	12	Feed	2
4	12	Feed	4

[1] 1.0 ml of an 8 h broth culture of *C. perfringens* in cooked meat medium

[2] 1/3 of ADFI mixed with 4 ml of the broth culture

[3] 1.0 ml of sterile cooked meat medium broth

[4] 1/3 of ADFI mixed with 4 ml of the sterile broth

[5] An 8 h broth culture of *C. perfringens* in cooked meat medium

[6] Sterilized peptone water with 5 g/L Cys hydrochloride

Prior to daily challenge, feed and water were restricted in respective pens for 4 h, overlapping the dark period of the lighting schedule and feed/water restriction time together. Accordingly, the culture mixed feed and peptone water were offered right after the dark period to make certain rapid consumption of large numbers of live bacterial cells by all birds. The respective feeders were top-dressed with culture/sterile broth mixed feed and a separate set of drinkers were used to offer culture mixed peptone water. The drinkers with normal drinking water were placed in the particular pens when the birds had consumed all peptone water with broth culture.

4.3.5. Sample and data collection

Birds were weighed on pen basis at the initiation of feeding the grower diet (d 14), and weekly thereafter. Feed disappearance was measured on each weigh day. The data were used to calculate ADG, ADFI and FCR for the periods of d 14 to 21 and d 21 to 28 in both experiments and for d 28 to 35 in Exp. 1. Randomly selected 50% of the birds in each pen in Exp. 1 and all birds in Exp. 2 were killed by cervical dislocation on d 28 and the contents of the ileum (from Meckel's diverticulum to 2 cm proximal to the ileocecal junction) and the contents of both ceca were aseptically collected from each bird. One aliquot (0.1 – 0.2 g) of each digesta sample was aseptically transferred into preweighed 15 ml sterilized conical plastic tubes containing 1 ml of 0.1% sterile peptone buffer with 5 g/L Cys hydrochloride (Sigma Chemical Co., St. Louis, MO) for plating. Immediately after collection, each tube was shaken well to facilitate sample mixing in the peptone water and kept on ice until plated within 3 h of collection. In Exp 1, a second aliquot was frozen at -20ºC for subsequent DNA extraction. Digesta samples from both

locations were collected from the remaining 50% of birds on d 35 of Exp. 1, as previously described, for DNA extraction.

Peripheral blood was collected from the brachial vein from all birds on d 28 of Exp. 2 for the WBCL assay as described by Papp and Smits (2007). Approximately 3 ml blood was collected into 4 ml commercial sodium heparin tubes (Vacutainer®, BD Oakville, Ontario, Canada). The blood samples were stored at 4ºC immediately after collection until tested within 10 h of collection.

4.3.6. Whole-blood chemiluminescence assay

Samples were run in duplicate using a Clear Polysorp 96-well microplate (Nunc, Roskilde, Denmark). Zymosan-stimulated, Lucigenin-enhanced WBCL assay was performed according to the method of Papp and Smits (2007) with slight modifications. Briefly, blood was diluted first to achieve 1:10 dilution. Then Zymosan A from *Saccharomyces cerevisiae* (Sigma Chemical Co., St. Louis, MO) was prepared as described by Marnila et al. (1995), and was used non-opsonized as a stimulator of cells at 1mg/ml. Lucigenin (N,N'-Dimethyl-9,9'-biacridium dinitrate; Sigma Chemical Co., St. Louis, MO) working solution was prepared to achieve 0.5 mM concentration. Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, MO) was used for the dilution of blood and for the preparation of working solutions of reagents. For the assay, diluted blood samples were added to the microplate first followed by the addition of zymosan and finally the working solution of lucigenin. Both the prepared reagents were added to the wells using a multi-channel pipettor directly prior to reading the plate in a Fluoroskan Ascent FL luminometer (Thermo Scientific Inc. Hudson NH). Diluted blood, zymosan and lucigenin were added 50 µl each into respective wells to achieve the final

reaction volume of 150 µl in each well. Light emission was recorded for 48 cycles, at 4 minute intervals and 1.5 seconds/reading at 39 ºC. Plates were shaken for 5 seconds before each interval. Light emission results were presented as relative light units (RLU). Background light emission was subtracted. The peak RLU is the peak CL response. Accordingly, peak RLU values were used in all statistical analyses.

4.3.7. Pathological examination

All birds were kept under constant observation for any sign of NE, which include depression, diarrhea, dehydration, somnolence, ruffled feathers and reduced feed intake or mortality (Helmboldt and Bryant 1971; Al-Sheikhly and Truscott 1977a,b; Al-Sheikhly and Al-Saieg 1980; Gazdzinski and Julian 1992). As described above, birds were euthanized by cervical dislocation on the respective sample collection days and their intestinal tracts were removed. Prior to digesta collection, intestinal lesions were scored blindly, according to the criteria described by Dahiya et al. (2005), using a scale from 0 to 4 (Table 4.4).

4.3.8. Genomic DNA extraction and purification

Digesta samples (350 mg per tube) were placed in a bead-beating tube (Mo-Bio Laboratories, Solano Beach, CA) and incubated first at 37°C for 30 min with RNase A (73 µg), lysozyme (750 µg), and proteinase K (400 µg) in 0.365 ml of buffer containing 50 mM Tris-Cl, 50 mM EDTA, 0.5% Tween and 0.5% Triton X-100, followed by another 30 min incubation at 50°C, upon addition of 0.135 ml of a buffer containing 3 mM guanidine-HCl and 20% Tween 20. The samples were subjected to one freeze–thaw

Table 4.4. Intestinal gross lesion scoring system for necrotic enteritis (after Dahiya et al. 2005).

Score	Observation/s
0	No gross lesions, normal intestinal appearance
0.5	Severely congested serosa and mesentery engorged with blood
1	Thin-walled, friable intestines with small red petechiae (> 5)
2	Focal necrotic intestinal lesions
3	Sizable patches of necrosis (1 to 2 cm long)
4	Diffused necrosis typical of field cases

cycle (-70°C/25°C), then placed in a bead beater (Bio101 ThermoSavant FP120) and shaken three times for 20 s (setting 5) with 1 vol. (700 μl) of 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma). The tubes were centrifuged at 13,000 x g for 15 min, the top aqueous phase was transferred to a new tube, mixed with an equal volume (~ 700 μl) of chloroform and centrifuged again at 13,000 x g for 10 min. The top aqueous phase was removed to a new tube and nucleic acids were precipitated with 1 vol isopropanol and 0.1 vol 3 M Na acetate, pelleted by centrifugation for 10 min. The pellet was washed once in 1 ml of 70% ethanol, aspirated, air dried, and re-suspended in 100 μl of sterile filtered UV'd water.

The extracted DNA samples were purified using FastDNA kit (Bio 101). Briefly, the DNA samples were mixed with 300 μl of binding matrix and incubated at room temperature for 10 min. Then the samples were centrifuged at 13,000 x g for 30 sec and the supernatants were discarded. The pellets were re-suspended in SEWS-M wash buffer and centrifuged at 13,000 x g for 1 min. The supernatants were discarded and the resulting pellets were air dried over night at room temperature. The next day, the pellets were re-suspended in 100 μl of sterile filtered UV'd water and incubated at 55°C for 20 min. Upon a 5 min centrifugation at 13,000 x g, the supernatants were transferred to new tubes and DNA was precipitated by adding 100 μl isopropanol and 10 μl 3 M Na acetate. The pellet was washed once in 1 ml of 70% ethanol, aspirated, air dried, and re-suspended in 50 μl of sterile filtered UV'd water. The purified DNA samples were stored at -20°C.

4.3.9. Bacterial enumeration

The ileal and cecal digesta samples collected for plating in both experiments were weighed and further diluted to an initial 10^{-1} dilution using peptone water. Ten-fold dilutions were spread in duplicate with an automated spiral plater (Autoplate, Spiral Biotech Inc., Bethesda, MD) on BBL blood agar base (Becton, Dickinson and Co.) containing 5% sheep blood and 100 mg/L of Neomycin Sulphate (The Upjohn Co., Orangeville, ON, Canada). The plates were incubated anaerobically for 24 h at 37ºC. The α- and β-hemolytic colonies on the plates were counted as *C. perfringens*, with presumptive colonies being randomly picked, Gram stained, plated on mannitol yolk polymixin agar (Oxoid Inc., Nepean, Ontario, Canada), and examined under the microscope to confirm as *C. perfringens*. The *C. perfringens* strain obtained from the infected birds was characterized by PCR technique as a type A toxin producer. Counts were expressed as $\log_{10}$ cfu per gram of intestinal contents.

The purified DNA was used for molecular enumeration of *C. perfringens* in digesta samples. PCR primers (50-CTGGGGTATCAACTAAAGTCTACGC-30 and 50-CCTTTGCTGCATAATCCCAATC- 30) were designed to amplify a 693 base pair (bp) fragment (PLC-A) of the *C. perfringens* α - toxin gene (GenBank accession number X17300). These primers (500 nM each) were used in a PCR containing *C. perfringens* genomic DNA (40 ng), 1.5 mM MgCl2, 0.2 mM each dNTP, and 1 U Taq DNA polymerase (Invitrogen) under the following conditions: 94°C, 4 min followed immediately by 40 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min. The PCR product was purified on an agarose gel and incubated with 1 U Taq polymerase (Invitrogen) and 0.2 mM each dNTP at 72°C for 15 min, then purified using a QiaQuick PCR purification column (Qiagen). Purified PCR products were ligated into pGEM-T Easy (Promega)

overnight at 4°C and the ligation mixture was used to transform E. coli JM109 (Promega). A single insert-containing clone was retrieved and used as a PCR standard as described below.

A second set of α - toxin-specific PCR primers was designed for molecular enumeration of *C. perfringens* using real-time quantitative PCR (qPCR). These primers (50-GAAGCTATGCACTATTTTGGAGAT-30 and 50-ATACTGTTCTTTCCTTTCTT- -CTGC- 30) amplified a 120 bp fragment (PLC-B) of the *C. perfringens* α - toxin gene that was nested within the larger cloned PLC-A fragment. To enumerate *C. perfringens* in digesta samples, we first optimized the dilution of the DNA extracts to mitigate the effects of PCR inhibition (Dumonceaux et al. 2005). DNA extracts were diluted 1:16 in TE buffer (10 mM Tris–Cl pH 8.0, 1 mM EDTA) and used in a qPCR assay along with standards consisting of known amounts of PLC-A plasmid DNA. Plasmid standards were expressed as copy number per qPCR assay, which was calculated as the weight of plasmid DNA in each standard divided by the plasmid molecular weight (2.4 - 106 g/mol based on a plasmid size of 3697 bp). qPCR assays used Platinum1 SYBR Green Quantitative PCR SuperMix-UDG (Invitrogen) and included 3 mM MgCl2 and 500 nM each primer. Amplifications were proceeded by the following steps: 50°C, 2 min (UDG activation), then 95°C, 3 min (well factor collection); this was followed immediately by 40 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s. Data collection was set at the extension step. Amplifications were performed using an iCycler (BioRad). qPCR counts were converted to genomes detected/g digesta based on the weight of digesta extracted and the dilution factors.

3.3.10. Statistical analysis

Pen was considered the experimental unit for the growth performance variables and individual birds for the other variables. Performance variables were analyzed by analysis of variance using the MIXED procedure of SAS (SAS Institute, Inc. Version 9.2) using a statistical model with the following factors: mode and challenge in Exp.1 and mode, dose and mode x dose in Exp. 2. Initial BW was used as a covariate. All other variables were analyzed using the GLM procedure of SAS. Means were reported as least-square means [± pooled standard error of the mean (SEM)] and were separated using the probability of difference. Differences were considered significant when $P < 0.05$.

4.5. Results

Subsequent to challenge with *C. perfringens*, most of the birds initially became dull and depressed and, had abnormally wet droppings. Those symptoms subsided after few days and the birds exhibited no obvious signs of morbidity 7 to 9 d post-challenge. No mortality was observed during the course of the study.

During d14 - 21 in Exp. 1, the *C. perfringens* challenge affected the ADG of birds ($P < 0.05$; Table 4.5), reducing the ADG by 9.14%. The challenge mode did not affect the ADG of birds during this period ($P > 0.05$), but interacted with *C. perfringens* challenge for ADFI and FCR ($P < 0.05$): feed consumption was reduced in challenged birds and introduction of the pathogen with feed reduced the ADFI by 19.8% compared to gavaging. The FCR in unchallenged birds was not affected by the mode of challenge, but the pathogen administration with feed reduced the FCR by 17.3% compared to gavaging. Neither *C. perfringens* challenge nor challenging mode affected the growth performance parameters in the d 22 to 28 growth period ($P > 0.05$). During d29 – 35, the

Table 4.5. Effect of *Clostridium perfringens* challenge and mode of inoculation (oral gavage vs feed) on growth performance of broiler chickens in Exp. 1.

Variable	*C. perfringens* infection		Mode		SEM	*P* Value		
	Unchallenged	Challenged[1]	Gavage	Feed		Infection	Mode	Interaction
d14 – d21								
Average daily gain (g)	64.43	58.54	60.78	62.20	1.37	0.01	0.48	0.16
Average daily feed intake (g)	117.74	92.21	109.08	100.87	1.98	<.001	0.01	0.01
Feed conversion ratio (g/g)	1.83	1.58	1.80	1.61	0.03	<.001	0.01	0.03
d22 – d28								
Average daily gain (g)	84.51	85.02	84.11	85.42	2.67	0.89	0.73	0.64
Average daily feed intake (g)	126.78	130.39	124.43	132.74	4.24	0.56	0.19	0.55
Feed conversion ratio (g/g)	1.50	1.56	1.48	1.58	0.08	0.62	0.39	0.42
d29 – d35								
Average daily gain (g)	101.40	85.12	95.21	91.31	3.00	0.01	0.38	0.72
Average daily feed intake (g)	170.68	153.81	160.18	164.32	5.07	0.04	0.57	0.53
Feed conversion ratio (g/g)	1.68	1.82	1.69	1.82	0.05	0.08	0.11	0.21
d14 – d35								
Average daily gain (g)	83.45	76.23	80.03	79.64	1.89	0.02	0.89	0.45
Average daily feed intake (g)	138.40	125.47	131.23	132.65	2.66	0.01	0.71	0.73
Feed conversion ratio (g/g)	1.66	1.65	1.64	1.67	0.03	0.87	0.48	0.48

[1] An 8 h broth culture of *C. perfringens* in cooked meat medium

ADG, ADFI and FCR were affected by the *C. perfringens* challenge ($P < 0.05$). The ADG and ADFI were reduced by 16.06 and 9.88%, respectively and the FCR was increased 8.33%, by the *C. perfringens* challenge. None of the growth performance parameters were affected either by challenge mode or challenge x mode interaction ($P > 0.05$), in this period. For the entire experimental period (d14 – 35), the ADG and ADFI were affected by the *C. perfringens* challenge ($P < 0.05$), reducing by 8.65 and 9.39%, respectively. The challenging mode or challenge x mode interaction did not affect these parameters and the two main effects or the interaction did not affect the FCR during the overall experimental period ($P > 0.05$).

Neither the mode nor the dose of *C. perfringens* administration affected the ADG, ADFI and FCR of birds, for the periods of d 14 to 21 and d 21 to 28 of age and for the entire experimental period (d 14 to 28) in Exp.2 ($P > 0.05$; Table 4.6). Furthermore, there was no significant interaction between the mode and dose of challenging for various performance parameters ($P > 0.05$).

The mean intestinal gross lesion score of chickens under different treatments and killed on 28 d of age in both experiments and 35 d of age in experiment 1 is shown in Figure 4.1. The mode of challenging in Exp. 1 and the mode or the dose of oral administration of *C. perfringens* in Exp. 2 had no significant effect on NE-related intestinal gross lesion score of birds ($P > 0.05$). No significant interaction was noted between the two factors of Exp. 2 for the intestinal gross lesion score ($P > 0.05$). Thin-walled and friable small intestines with congested serosa and blood-engorged mesenteric vessels were detected in most of the birds in both experiments, irrespective of the treatment. Some birds had focal necrotic lesions while some displayed sizable (1 to 2 cm long) patches of necrosis in various segments of the small intestine, more frequently in

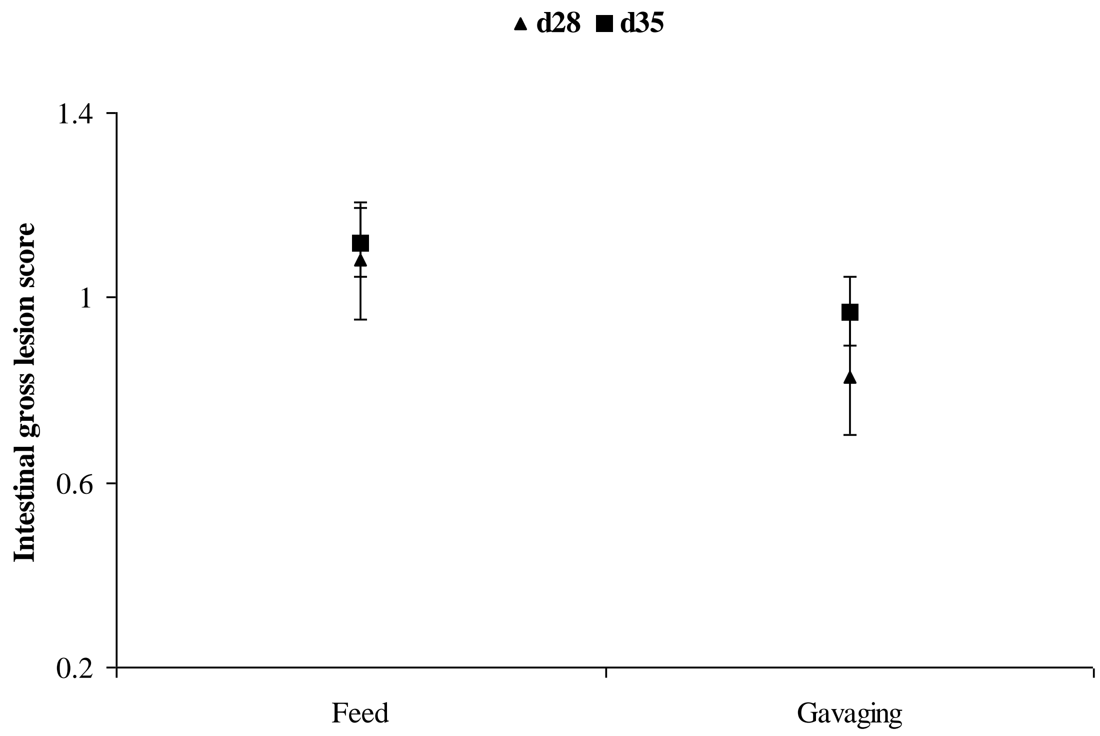
Table 4.6. Effect of mode (water and feed) and dose of administration of *Clostridium perfringens* on growth performance of broiler chickens in Exp. 2.

Variable	Mode		Dose[1] (ml)		SEM	*P* Value		
	Water[2]	Feed	2	4		Mode	Dose	Interaction
d14 – d21								
Average daily gain (g)	47.06	46.01	46.40	46.67	1.25	0.697	0.918	0.764
Average daily feed intake (g)	79.53	76.97	75.24	81.25	1.38	0.405	0.094	0.951
Feed conversion ratio (g/g)	1.69	1.68	1.63	1.74	0.03	0.779	0.114	0.662
d22 – d28								
Average daily gain (g)	70.15	69.52	70.15	69.52	1.32	0.824	0.824	0.259
Average daily feed intake (g)	126.91	134.35	122.26	138.99	7.16	0.631	0.307	0.818
Feed conversion ratio (g/g)	1.82	1.93	1.75	2.00	0.10	0.591	0.261	0.516
D14 – d28								
Average daily gain (g)	58.60	57.77	58.27	58.10	1.22	0.749	0.945	0.428
Average daily feed intake (g)	103.21	105.65	98.75	110.12	4.07	0.779	0.235	0.832
Feed conversion ratio (g/g)	1.77	1.83	1.70	1.90	0.07	0.654	0.216	0.540

[1] An 8 h broth culture of *C. perfringens* in cooked meat medium

[2] Sterilized peptone water with 5 g/L Cys hydrochloride

(A)



(B)

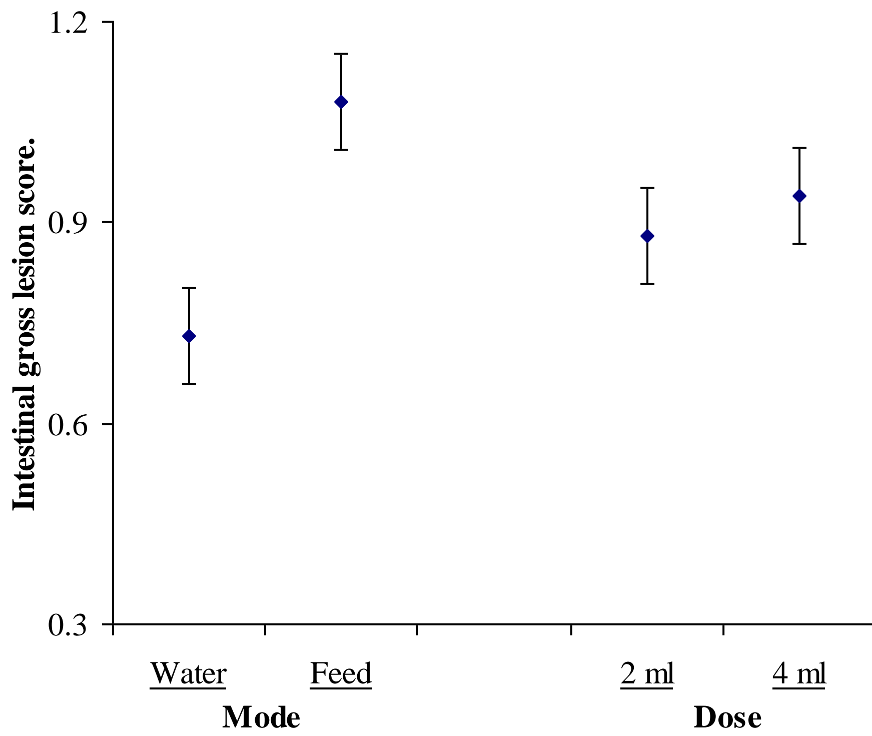


Figure 4.1. Mean intestinal gross lesion scores of NE in broiler chickens challenged with *C. perfringens* in an 8 h cooked meat broth, (A) using two modes (oral gavage or feed) at d28 and d35 of age in Exp. 1, and (B) using two modes (water or feed) at two dose rates (2 and 4 mL) at d28 of age in Exp. 2. Lesions were scored on a 0 to 4 scale where 0 indicates no intestinal gross lesions (apparently normal), 0.5 = severely congested serosa and mesentery engorged with blood, 1 = thin walled and friable intestines with small red petechiae (>5), 2 = focal necrotic intestinal lesions, 3 = sizable patches of necrosis (1 to 2 cm long), and 4 = severe extensive necrosis typical of field cases.

the distal and proximal parts of the jejunum and ileum, respectively. Intestines with large amounts of gas and severe extensive necrosis with marked hemorrhage were not observed in any of the birds, as seen in typical field cases.

The ileal and cecal *C. perfringens* counts enumerated using plate count and q-PCR technique on d 28 and 35 of Exp. 1 are shown in the Table 4.7. The pathogen count by plate count on d 28 of Exp. 2 is shown in the Table 4.8. Both ileal and cecal pathogen numbers revealed no significant differences among challenged groups in both experiments ($P > 0.05$). There was no significant interaction between the mode and dose of challenge for *C. perfringens* counts either in ileal or cecal contents in Exp. 2 ($P > 0.05$).

The WBCL response was not significantly affected by the mode or dose of *C. perfringens* administration or the interaction ($P > 0.05$: Figure 4.2).

4.6. Discussion

NE is considered difficult to reproduce experimentally in broiler chickens, therefore, no widely recognized infection model exists for the experimental induction of the disease (Kaldhusdal et al. 1999). Several methods have been described in the literature but little information about their repeatability is available (Williams et al. 2003). Such methods include the rearing of birds on *C. perfringens* infected poultry-house litter (Maxey and Page 1977; Wicker et al. 1977; Hamdy et al. 1983; Cowen et al. 1987; Kaldhusdal et al. 2001), oral administration of *C. perfringens* through feed (Long and Truscott 1976; Prescott 1979), drinking water (Jansson et al. 1990; Takeda et al. 1995) or by gavage (Bernier et al 1977; Baba et al. 1997; Répérant and Humbert 2002; Wilkie et al. 2005; Dahiya et al. 2005, 2007a,b), intravenous administration of *C. perfringens* (Bernier and

Table 4.7. Effect of *Closrtidium perfringens* challenge and mode of pathogen administration on organism proliferation in the ileum and the cecum of broiler chickens at 28 and 35 d of age in Exp. 1.

Closrtidium perfringens counts	Challenge		Mode		SEM	*P* Value		
	Unchallenged	Challenged	Gavaging[1]	In feed[2]		Challenge	Mode	Interaction
Plate counts (d 28)								
Ileal digesta *(log_{10} cfu/g)*	0.00	3.13	1.61	1.58	0.180	<0.0001	0.708	0.708
Caecal digesta *(log_{10} cfu/g)*	0.00	2.99	1.68	1.37	0.207	<0.0001	0.225	0.225
q-PCR counts (d 28)								
Ileal digesta *(log_{10} cfu/g)*	0.00	3.55	1.83	1.72	0.095	<0.0001	0.422	0.422
Caecal digesta *(log_{10} cfu/g)*	0.00	3.26	1.79	1.48	0.127	<0.0001	0.093	0.093
q-PCR counts (d 35)								
Ileal digesta *(log_{10} cfu/g)*	0.00	3.59	1.87	1.72	0.121	<0.0001	0.408	0.408
Caecal digesta *(log_{10} cfu/g)*	0.00	3.07	1.65	1.42	0.151	<0.0001	0.289	0.289

[1] 1 ml/bird of an 8 h broth culture of *C. perfringens* in cooked meat medium
[2] 4 ml in 24g of feed/bird of an 8 h broth culture of *C. perfringens* in cooked meat medium

Table 4.8. Effect of mode (water and feed) and dose of administration of *Closrtidium perfringens* on organism proliferation in the intestinal contents of broiler chickens at 28 d of age in Exp. 2.

Closrtidium perfringens counts	Mode		Dose[1] (mL)		SEM	*P* Value		
	Water[2]	Feed	2	4		Mode	Dose	Interaction
Plate counts								
Ileal digesta *(log_{10} cfu/g)*	3.96	3.67	3.95	3.68	0.19	0.445	0.477	0.567
Caecal digesta *(log_{10} cfu/g)*	4.86	3.98	4.53	4.30	0.35	0.214	0.743	0.994

[1] An 8 h broth culture of *C. perfringens* in cooked meat medium

[2] Sterilized peptone water with 5 g/L Cys hydrochloride

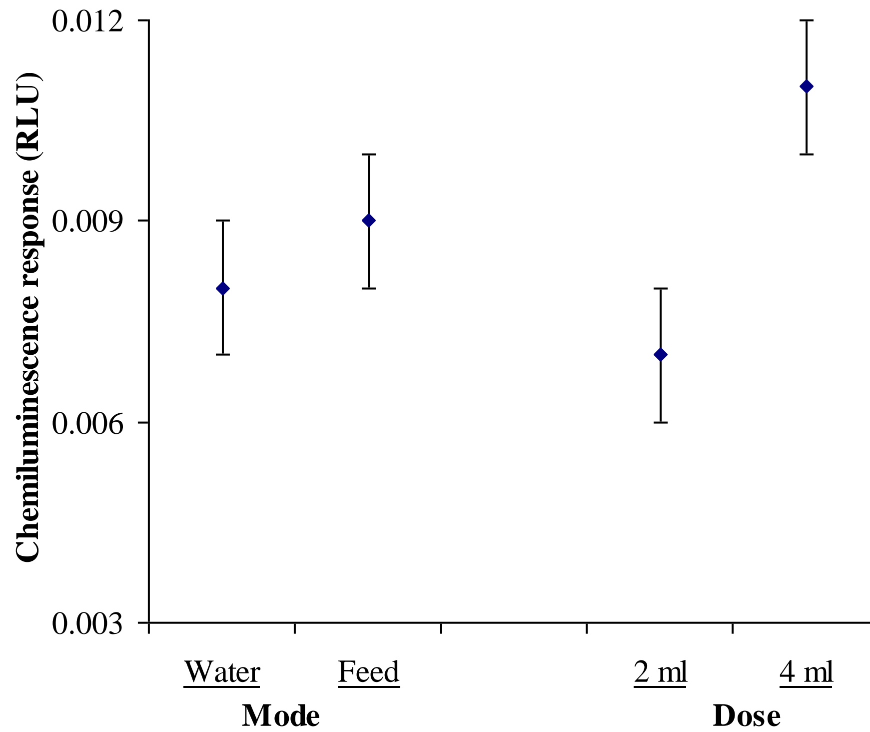


Figure 4.2. Mean peak whole blood chemiluminesence detected in 28-d-old broiler chickens challenged with *C. perfringens* in an 8 h cooked meat broth, using two modes (water or feed) at two dose rates (2 and 4 ml) in Exp. 2. Lucigenin-enhanced, non-opsonized zymosan-stimulated whole blood chemiluminesence response was measured 9 d post-challenged.

Filion 1971; Bernier et al. 1977), inoculation of the organism into the cannulated duodenum (Truscott and Al-Sheikhly 1977) or into the cloaca (Williams et al. 2003) and infusion of crude toxins of *C. perfringens* directly into the duodenum (Truscott and Al-Sheikhly 1997). The seeder-contact method practiced in the spreading diseases like salmonellosis in poultry flocks is another approach that could have been considered in developing a model for NE, but the anaerobic characteristics of *C. perfringens* might have limited the employment of such methods.

Coccidiosis is considered as a factor associated with the occurrence of the disease NE in broiler chickens, under practical field (Long 1973) and controlled laboratory (Al-Sheikley and Al-Saige 1980) conditions. This is probably due to the frequent sharing of the site in the intestinal tract by the respective causative organisms plus the intestinal mucosal damage initiated by Eimeria species (Bradley and Radhakrishnan 1973; Long 1973; Kimura et al. 1976) and greater affinity of *C. perfringens* for adhesion to the previously damaged mucosa (Kageyama et al. 1987; Baba et al. 1992b). Many studies have taken this phenomenon into account and consequently, several coccidial species have been used in the process of developing a disease model for NE, such as *E. acervulina* (Al-Sheikley and Al-Saige 1980; Shane et al. 1985), *E. brunetti* (Baba et al. 1992b), *E. maxima* (Williams et al. 2003), *E. necatrix* (Al-Sheikley and Al-Saige 1980; Baba et al. 1997) or *E. tenella* (Kageyama et al. 1987). In general, these studies demonstrated that simultaneous infection with coccidia and *C. perfringens* produces more extensive pathogenic effects than each individual infection. Supporting these observations, Williams et al. (2003) further demonstrated a reduction in the severity of a virulent *C. perfringens* challenge in broiler chickens vaccinated

against coccidiosis, probably by preventing severe coccidial lesions that might predispose birds to NE.

In the present investigation, one strain-pure culture of *C. perfringens* was used without a concurrent coccidial infection. Reproduction of NE without coccidial co-infection permits the study of treatment effects on *C. perfringens* -induced enteritis without the possible confounding influence of the second pathogen. In addition, the separation of effects due to coccidiosis from those associated with NE can be complicated in immunological and molecular studies.

Despite the lack of overt clinical signs of NE in both experiments, the relatively poor growth performance of birds was probably due to subclinical disease caused by *C. perfringens* challenge. In Exp. 1, the ADG of the challenged birds was reduced during the first week of challenge compared to unchallenged birds. The disease NE in poultry can arise either in acute clinical form or in subclinical version (Shane et al. 1985; Kaldhusdal and Hofshagen 1992). The clinical disease is associated with high mortality in broiler barns, as a result of severe necrotic damage to the small intestinal mucosa (Wages and Opengart 2003), whereas reduced growth performance in broilers is commonly accompanied by the subclinical form (Kaldhusdal and Hofshagen 1992; Porter 1998; Lovland and Kaldhusdal 2001; Hofacre et al. 2003). This inferior growth performance can be attributed to the necrosis of the intestinal epithelium caused by *C. perfringens* and subsequent impairment in nutrient digestion and absorption, substandard growth rate and increased FCR (Elswinger et al. 1992; Kaldhusdal and Hofshagen 1992; Kaldhusdal et al. 2001; Hofacre et al. 2003). In addition, evidence exists to link *C. perfringens* infections to hepatitis and cholangio-hepatitis in broiler chickens, which leads to substandard growth performance as well (Lovland and Kaldhusdal 1999, 2001).

Impaired overall performance has previously been reported in broiler chickens highly infected with *C. perfringens* (Stutz et al. 1983; Stutz and Lawton 1984; Kaldhusdal and Hofshagen 1992; Dahiya et al. 2005, 2007a, b). The C. *perfringens* challenge reduced the ADFI of birds and it was further affected by the pathogen administration with feed. This might be due to the undesirable effects of toxins and other metabolites produced by *C. perfringens* on the palatability of the feed. Accordingly, the FCR of that treatment group was reduced as well.

However, during the second week of Exp. 1 (d22 – 28), neither C. *perfringens* challenge nor mode of challenge affected the growth performance of the birds, likely due to diminishing intensity of the infection. But the growth performance of C. *perfringens* challenged birds was reduced again during the third week, probably due to re-infection caused by the ingestion of the pathogen or their spores on the litter, shed by the infected birds. None of the growth performance parameters evaluated in Exp. 2 showed a significant difference among treatment groups.

Although gross intestinal lesions are not always observed in birds infected with *C. perfringens* (Olkowski et al. 2006), intestinal lesion scoring is widely and effectively being used to quantify the severity of NE in broiler chickens (Riddell and Kong 1992; Jackson et al. 2003; Williams et al. 2003; Dahiya et al. 2005, 2007a,b; Gholamiandehkordi et al. 2007; Kulkarni et al. 2007). Kaldhusdal and Hofshagen (1992) and Kaldhusdal et al. (1999) reported grossly visible intestinal lesions as an estimable indicator of an occurrence of NE. A perfect infection model to examine the pathogenesis of acute and subclinical form of NE is a setting with a high percentage of the challenged birds exhibiting grossly visible intestinal lesions without mortality (Gholamiandehkordi

et al. 2007). Accordingly, the demonstration of the development of NE-specific lesions is an important feature of the current study.

The *C. perfringens* numbers observed in the gut contents of challenged birds of the present study were much higher than that found in healthy birds ($<10^4$ cfu/ g of digesta) and high populations of the organism in the intestinal (as high as 6.18 and 8.10 $\log_{10}$/ g in ileal and cecal, respectively) contents were detected even in clinically healthy looking birds, confirming the subclinical version of NE, as reported by Dahiya et al. (2005). The plate count is based on the cfu formed only by live bacterial cells whereas in q-PCR, DNA from all live and dead bacterial cells contributes in enumeration, thereby overestimating the numbers (Dumonceaux et al. 2006). Therefore, the pathogen count determined using q-PCR technique on d28 of Exp. 1 was comparatively higher than that of plate counts.

In challenged birds, the *C. perfringens* counts in both ileal and cecal digesta were considerably higher in Exp. 2 than those found in Exp. 1. This may have been caused by the higher contents of AID CP (23 vs 18%), animal protein (11.7 vs 8.7%) and AID glycine (1.75 vs 1.00%) in the grower diet fed in Exp.2. Previous studies with broiler chickens have reported the stimulatory effect of high dietary protein levels, higher amounts of animal protein supplements in the diet in particular, and high dietary glycine level on the proliferation of *C. perfringens* and predisposing of birds to NE (Ispolatovskaya 1971; Nakamura et al. 1978; Kaldhusdal 2000; Kocher 2003; Drew et al. 2004; Dahiya et al. 2005; Wilkie et al. 2005). In addition, the grower diet fed in Exp. 2 contained no crystalline glycine, whereas a considerable amount of the AID glycine (18%) in the grower diet fed in Exp. 1 was provided by crystalline form, which is considered as 100% true digestible (Izquierdo et al. 1988; Chung and Baker 1992) and

rapidly absorbed in the upper part of the GIT (Lingens and Molnar 1996; Maenz and Engele-Schaan 1996a; Drew et al. 2003), making it unavailable to the microbes residing in the distal part. The grower diet fed in Exp. 1 contained supplementary xylanase while the diet used in Exp. 2 did not. Previous studies reported a significant decrease in intestinal *C. perfringens* numbers in broilers fed wheat-based diets supplemented with xylanase-based enzyme preparations (Jia et al. 2009b; Choct et al. 2006). This can be due to the reduction in NSP-associated digesta viscosity by the enzyme and thus increased nutrient digestion and absorption by the host, leaving less nutrients for the growth of gut microbes. Further, the birds in Exp. 1 received a pelleted grower diet whereas the grower diet fed in Exp. 2 was in mash form, which is considered as a triggering factor for *C. perfringens* proliferation in the GIT of broilers (Engberg et al. 2002). Feeding fine particle (mash) feeds reduces the normal gastric reflux and gizzard activity which in turn reduces the nutrient digestion and absorption by the host. Consequently, more nutrients would be available to fuel microbial overgrowth.

The intestinal lesion score and *C. perfringens* counts in the ileal and cecal digesta observed in both experiments of the present study bare the hallmarks of subclinical version of NE in most of the birds, as described by Lovland and Kaldhusdal (2001). While NE-induced mortality was not experienced during the course of the study, previous studies have suggested that *C. perfringens* counts and intestinal lesion score as more reliable and sensitive indicators of the disease than NE-related mortality (Kaldhusdal and Hofshagen 1992; Kaldhusdal et al. 1999).

The WBCL assay is a simple but relatively quick and inexpensive analytical technique used in the assessment of reactive oxygen species (ROS) produced by circulating phagocytes (De Chatelet and Shirley 1981; Tono-Oka et al. 1983; Nagahata

et al. 1991). Thus, a positive correlation exists between the chemiluminescence response of a phagocyte and its ability to produce ROS (Ginsburg et al. 1993; Merrill et al. 2001), which are supposed to fight against invading pathogens, providing a first-line immunologic defense. The WBCL assay has widely and successfully been utilized in evaluating differences in innate immune responses measured using polymorphonuclear (PMN) status of peripheral blood among experimental groups or populations of some animal species such as fish (Nikoskelainen et al. 2004, 2006), frogs (Marnila et al. 1995; Gilbertson et al. 2003), badgers (McLaren et al. 2003), mallards (Laudert et al. 1993), bears, raptors, and domestic chickens (Papp and Smits 2007; Papp et al. 2009). The WBCL peak reflects the functional capacity of circulating PMNs and a positive correlation exists between the WBCL peak and the numbers of both PMNs and total white blood cells in peripheral blood (Tono-Oka et al. 1983; Nagahata et al. 1991). However, in chickens, the peak is correlated with the absolute heterophil numbers and heterophil: mononuclear cell ratio (Papp and Smits 2007). Papp et al. (2009) demonstrated a positive correlation between WBCL response and NE-related clinical findings such as elevated intestinal lesion score and increased proliferation of *C. perfringens,* in *C. perfringens*-challenged broiler chickens fed different experimental diets. In Exp. 2, no difference was observed among values of WBCL response of birds in different treatment groups. Here, the absence of an uninfected control group of birds hindered the chances of comparing the findings with that of uninfected birds. However, the WBCL values of the current study together with the other findings suggested the similarity among the treatments in terms of developing a disease model for NE.

The overall findings of the present study suggest that any of the evaluated treatments (either dose of culture with either mode of challenge) can be exploited in the process of establishing a repeatable challenge model for NE in broiler chickens raised on litter, without a concurrent coccidial infection. This model stimulates a subclinical form of NE and is suitable for investigating the effect of diet on the pathogen proliferation and the disease progression.

5. EFFECTS OF DIETARY PROTEIN LEVEL AND PROTEIN DIGESTIBILITY ON THE GROWTH PERFORMANCE AND INTESTINAL ECOSYSTEM OF BROILER CHICKENS INFECTED WITH *Clostridium perfringens*

5.1. Abstract

To evaluate the effects of dietary protein level and digestibility on the growth performance and intestinal ecosystem of *C. perfringens* challenged and unchallenged broilers, 640 birds were fed four different ideal protein-balanced, isocaloric diets in a 2 x 2 x 2 factorial design with two levels of AID protein (*HiPro*: 20 and 18% and *LoPro* 18 and 16% on days 1-14 and 15-35 respectively) and two levels of Ileal protein digestibility (*HiDig* and *LoDig*; approximately 85% and 80% CP digestibility respectively) under challenged and unchallenged situations. During the starter period, neither protein level nor digestibility affected the ADG ($P > 0.05$), but interacted for ADFI ($P < 0.05$): in *LoPro* diets, *HiDig* proteins increased ADFI of birds compared to *LoDig* proteins, but protein digestibility did not affect the ADFI of birds fed *HiPro* diets. Not protein level ($P > 0.05$), but protein digestibility significantly reduced the FCR of starters fed *LoDig* proteins ($P < 0.05$). None of the main effects affected the growth performance parameters during the grower period or the final BW ($P > 0.05$). The C. *perfringens* challenge significantly affected the ADG of grower birds ($P < 0.05$), but not the ADFI or FCR ($P > 0.05$). Birds fed *LoPro* diets significantly improved PER throughout the experiment and *HiDig* proteins increased PER during the grower and overall periods ($P < 0.05$). The prospective challenge group demonstrated superior growth performance during the starter period however challenge reduced their PER ($P < 0.05$). The overall PER of the birds was not affected by the challenge ($P > 0.05$). PER was significantly higher during starter than grower period ($P < 0.05$). Ileal *C.*

perfringens counts and intestinal gross lesion score were higher in birds fed *HiPro* and *LoDig* proteins compared to *LoPro* and *HiDig* proteins fed counterparts, respectively ($P < 0.05$). Protein level, digestibility or C. *perfringens* challenge did not affect the cecal acetate level ($P > 0.05$), but challenged birds had more propionate than unchallenged counterparts ($P > 0.05$). The three main effects interacted for cecal butyrate and total VFA levels ($P < 0.05$). Unchallenged birds fed *LoPro-HiDig* diet had more cecal butyrate and total VFA than *LoPro-LoDig* diet fed birds. In birds fed *LoPro-LoDig* diet, C. *perfringens* challenge significantly increased cecal butyrate and total VFA levels than in unchallenged counterparts. None of the main effects affected cecal branched chain VFA levels ($P > 0.05$). The three main effects did not affect the cecal ammonia production ($P > 0.05$). Overall findings from this study revealed the potential use of *LoPro* diets containing highly digestible proteins to minimize the risk of *C. perfringens* in broilers without AGP.

5.2. Introduction

Necrotic enteritis is a common bacterial infection in current broiler flocks and has a great impact on poultry welfare and the economics of broiler production (Van der Sluis 2000a). Some *C. perfringens* type A strains carrying the genes for α or NetB toxins are responsible for this economically important disease (Keyburn et al. 2008, Van Immerseel et al. 2008). Dietary incorporation of antibiotics in broiler rations has been the most common strategy employed to control the proliferation of this pathogen (Dahiya et al. 2006). However, with the ban on prophylactic antibiotic usage in animal feed industry in some countries, a higher incidence of NE is re-emerging in broiler chickens (Grave et al. 2004; Van Immerseel et al. 2004; Williams 2005). In this context,

an intense interest exists in the establishment of management and/or dietary approaches which substitute in-feed antibiotics to combat this enteric pathogen. Further these alternatives must maintain growth performance and the health and safety of broiler meat for human consumption (Ewing and Cole 1994).

Clostridium perfringens is normal member of the intestinal microflora of chickens (Gazdzinski and Julian 1992; Branton et al. 1997) and can be isolated in high numbers from the intestinal tract-from the crop, through the gizzard, the small intestine and in the ceca of apparently healthy birds (Ficken and Wages 1997; Engberg et al. 2002; Pedersen et al. 2003). Despite this, the mere presence of this pathogen in the GIT is not the only factor that required to elicit the disease NE in broiler chickens (Shane et al. 1984; Cowen et al. 1987; Kaldhusdal et al. 1999; Craven et al. 2001; LaRagione and Woodward 2003) and other nutritional and management cofactors are normally required to induce the infection (Dahiya et al. 2006). Nevertheless, with the withdrawal of antibiotics from broiler ration, diet related risk factors have attracted much attention due to their substantial role in the pathogenesis of NE.

Two major diet-related factors are associated with poultry NE. The first factor is cereal grains that used in poultry ration formulation. (Riddell and Kong 1992; Kaldhusdal and Skjerve 1996). The other factor that predisposes birds to NE is dietary protein (Kaldhusdal and Skjerve 1996). Previous studies have reported the marked effects that dietary protein source and level have on intestinal proliferation of *C. perfringens* in broiler chickens (Kaldhusdal 2000; Drew et al. 2004). Diets containing high concentrations of protein and high concentrations of animal-derived proteins in particular, increase intestinal *C. perfringens* numbers (Kaldhusdal 2000; Kocher 2003; Drew et al. 2004; Wilkie et al. 2005). However, these have used much higher inclusion

rates of the respective feed ingredients and protein levels than would be used in practical broiler rations.

The study described in Chapter 3 demonstrated significantly inferior growth performance of birds fed *LoPro* diets formulated to contain *LoDig* protein supplements compared to those fed *LoPro* diets with same nutrient specifications but formulated with highly digestible protein sources. The nutrients available to intestinal bacteria are primarily derived from the indigestible portion of feeds. Thus, alterations in indigestible nutrients can cause changes in intestinal microbial numbers, species and species diversity (Drew et al. 2002; Hill et al. 2005; Dahiya et al. 2006; Pieper et al. 2008). This has the potential to negatively affect the health and growth performance of broilers (Gaskins 2001; van der Klis and Jansman 2002). Bacterial metabolism of undigested AA may result in the production of toxic metabolites including ammonia (Visek 1978a; Pond and Yen 1987), biogenic amines (Tisljar et al. 2002), phenols and indoles (Yokoyama et al. 1982) that can affect the turnover of enterocytes and thus the overall growth performance of the host (Macfarlane and Macfarlane 1995; Gaskins 2001; van der Klis and Jansman 2002). Based on these observations, we hypothesize that diet digestibility significantly affects gut *C. perfringens* populations and toxic metabolites, resulting in reduced growth performance. The purpose of this study was to examine the effects of ileal digestible protein level and protein digestibility in commercial type diets - formulated based on SID AA content - on growth performance and the intestinal microbial fermentation profile in broiler chickens experimentally infected *with C. perfringens.*

5.3. Materials and methods

A total of 640 broiler chickens were reared in two successive batches of 320 birds under identical conditions at the Animal Care Unit in the Western College of Veterinary Medicine, University of Saskatchewan. Experimental protocols for the study 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 (1993).

5.3.1. Birds and management

One-day-old conventional male broiler chicks (Ross 308), obtained from a local broiler hatchery (Lilydale Hatchery, Wynyard, Saskatchewan, Canada) were used in both trials and standard management procedures were followed. The temperature was maintained at 34 °C at placement and reduced as the birds progressed in age with a final temperature of 23 °C at d 35. The lighting schedule used consisted of 23 h light and 1 h dark from d 1 to 14 and, 20 h light and 4 h dark from d15 to the end (d 35) of each trial. Feed and water were available to the birds on an *ad libitum* basis in both trials. Upon arrival to the research facility, 320 birds in each trial (43.89±1.06 g) were randomly allocated into 32 floor pens in a room having thermostatically controlled heating and ventilation (10 birds/pen). Each pen (1 m^2) was equipped with one pan feeder, one manual drinker and new soft-wood shavings. A feeder lid was placed in each pen from d 1 to 5 to ensure chicks had adequate access to feed. The room was thoroughly cleaned, disinfected and rested for more than two weeks between the trials.

5.3.2. Experimental design

The birds were subjected to a 2-phase feeding regimen consisting of starter (1 to 14d) and grower (15 to 35d) phases and fed the same diets used in the experiment described in Chapter 3 in a 2 x 2 x 2 factorial design. Pens were randomly assigned to one of four dietary treatment groups in each feeding regimen and each treatment group was replicated eight times.

5.3.3. *C. perfringens* challenge procedure

This study used an avian *C. perfringens* field strain isolated from a clinical case of NE in broiler chickens (Dr. M. Chirino, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK). The strain was characterized using PCR technique as a type A toxin producer (Dumonceaux et al. 2006). The organism was cultured anaerobically on BBL Blood Agar Base (Becton Dickinson Co., Sparks, MD) containing 5% sheep blood and 100 mg/L of Neomycin Sulphate (The Upjohn Co., Orangeville, ON, Canada) for 18 h at 37ºC, then aseptically inoculated into a sterilized preparation of Robertson's cooked meat broth (Difco Laboratories, Detroit, MI), and incubated anaerobically overnight at 37ºC.

All 320 birds in trial 1 were challenged daily right after the 4 h dark period on d 15 to 19 (inclusive), offering the inoculum in feed, as described in Chapter 4. Briefly, each bird in all pens received 1/3 of ADFI mixed with 2 ml of broth culture. All birds in trial 2 were kept unchallenged and received 1/3 of ADFI mixed with 2 ml of sterile cooked meat medium broth per bird. The respective feeders were top- dressed with culture/sterile broth mixed feed and fresh cultures were prepared daily. Culture-feed mixing was done immediately before offering to the birds. Bacterial counts were

performed on the culture daily before inoculation and the count ranged from 3.7 to 6.4 x 10^6 cfu/ml.

5.3.4. Sample and data collection

Birds and feeders were weighed on a pen basis at the initiation of the experiment (d 1), and weekly thereafter, (d 7, 14, 21, 28, and 35). Feed consumption was measured on each weigh day. The data were used to calculate ADG, ADFI and FCR for starter, grower and overall experimental periods. Birds were monitored twice a day (8:30 and 15:30) and in the case of mortality, the weight of the dead bird/s and remaining feed in the respective feeder/s were recorded. At 35 d of age, all birds were euthanized by cervical dislocation and the contents of the ileum (from Meckel's diverticulum to 2 cm proximal to the ileocecal junction) and the contents of both ceca were aseptically collected from each bird. One aliquot each from both locations and one additional aliquot only from cecum were collected and frozen at -20ºC for subsequent DNA extraction and, ammonia and volatile fatty acid (VFA) analysis, respectively.

5.3.5. Pathological examination

All birds were observed for any sign of clinical NE during the trial, including depression, diarrhea, dehydration, somnolence, ruffled feathers and reduced feed intake or mortality (Helmboldt and Bryant 1971; Al-Sheikhly and Truscott 1977a,b; Al-Sheikhly and Al-Saieg 1980; Gazdzinski and Julian 1992). On d 35 prior to collecting digesta, intestinal lesions were scored blindly, according to the criteria described by Dahiya et al. (2005), using a scale from 0 to 4 (Table 4.4).

5.3.6. Genomic DNA extraction and purification

The DNA extraction and purification on the collected ileal digesta samples were peformed based on the method described in Chapter 4.

5.3.7. Bacterial enumeration

The purified DNA was used for molecular enumeration of *C. perfringens* in ileal digesta samples. The enumeration of *C. perfringens* was performed using qPCR technique as described in Chapter 4. The qPCR counts were converted to genomes detected/g digesta based on the weight of digesta extracted and the dilution factors.

5.3.8. Volatile fatty acid and ammonia analyses

Upon thawing of cecal digesta samples collected for VFA analysis, 100 mg was transferred in duplicate into 1.8 ml Eppendorf™ centrifuge tubes. Then 20 µL of 25% mata – phosphoric acid was added into each Eppendorf™ tube followed by 500 µL of 1 mM trimethyl acetic acid in acetonitrile to act as the internal standard. Finally, 830 µL of acetonitrile was added into each tube. Samples were vortexed for 3 seconds before centrifuging at 14,000 x g for 5 minutes in a Microfuge® 18 Microcentrifuge (Beckman Coulter™, Palo Alto, CA). The sample supernatant was pipetted (1 ml) into an Agilent™ GC glass vial and capped. An Agilent 6890 Series Gas Chromatography System (Wilmington, DE) including an Agilent 7683 Series injector (5 µl) fitted with a Zebron ZB-FFAP High Performance GC Capillary Column (30.0 m × 320 µm × 0.25 µm, Phenomenex, Torrance, CA) was used to identify and quantify VFA in every sample. A standard curve was made from purchased standards (Nu-Chek Prep, Inc. Elysian, MN) and used to quantify the concentration (mM) of the individual VFA.

Samples were prepared daily and placed on the autosampler twice daily. Samples that were prepared daily, but not initially placed on the autosampler were stored in the fridge to minimize the loss by volatization. The concentrations of VFA were measured as μmol/g and the molar ratio (MR) of each VFA was calculated as a proportion to the total VFA concentration.

For ammonia analysis, cecal digesta samples were thawed overnight at 4°C and 500 mg was transferred in duplicate into 15 ml screw cap culture tubes. Upon addition of 10 ml of deionized water into each tube, the tubes were shaken well and stored overnight at 4°C. After overnight soaking, the tubes were vortexed and centrifuged at 500 x g for 5 minutes at 4°C in an ICE MicroCL 17R Centrifuge (Thermo Electron Corporation, Waltham, MA). Upon sample preparation, the phenol-hypochlorite procedure described by Broderick and Kang (1980) was applied to quantify the ammonia concentration.

5.3.9. Statistical analysis

Pen was considered as the experimental unit for the growth performance variables and, each individual bird was considered as the experimental unit for the other variables. The PER was calculated as grams of weight gain per gram of protein intake, for each growth phase and the overall experimental period. The variables were analyzed by analysis of variance using the MIXED procedure of SAS (SAS Institute, Inc. Version 9.2). Means were reported as least-square means [± pooled standard error of the mean (SEM)] and were separated using the probability of difference; $P < 0.05$ was considered significant to test the hypotheses. For the performance variables, the initial BW was used as a covariate and the statistical model contained the following main factors and interactions

among them: dietary digestible protein level, protein digestibility and *C. perfringens* challenge.

5.4. Results

Neither protein level nor digestibility affected the ADG of the birds during the starter period ($P > 0.05$), and only tended to interact for the ADG ($P = 0.06$; Table 5.1). The ADG of the group to be challenged was significantly higher than that of the control group during the starter phase ($P < 0.05$). During this growth phase the interaction between protein level and digestibility was significant for ADFI ($P < 0.05$). When incorporated into *LoPro* starter diets, *HiDig* proteins increased ADFI of birds by 4.2% compared to *LoDig* proteins, but protein digestibility did not affect the ADFI of birds fed *HiPro* diets (data not shown). The ADFI of the prospective challenge group was significantly higher than that of the control group during this phase ($P< 0.05$). The protein level did not significantly affect the FCR of starters ($P > 0.\ 05$). The protein digestibility significantly affected the FCR during the starter phase, increacing it by 3.1% in birds fed diets containing *LoDig* proteins ($P < 0.05$). The FCR of birds to be challenged was 3.1% lower than the control group during the starter phase ($P < 0.05$). During the grower phase, the main effects protein level and digestibility tended to interact for the ADG of birds ($P = 0.06$), but did not affect either ADFI or FCR ($P >$ 0.05).

Subsequent to challenge with *C. perfringens*, most birds became dull and depressed and, had abnormally wet droppings. Those symptoms subsided after a few days and the birds exhibited no obvious signs of morbidity by 7 to 9 d after challenge. The C. *perfringens* challenge significantly affected the ADG of birds ($P < 0.05$), but not

Table 5.1. Effect of dietary protein level, protein digestibility and *Clostridium perfringens* challenge on growth performance of broiler chickens during a 35 day growth trial.

Main effects	d0 body weight[5]	Starter (d0 – 14)				d14 body weight[5]	Grower (d15 -35)				Final body weight[5]	Overall PER
		ADG[1]	ADFI[2]	FCR[3]	PER[4]		ADG	ADFI	FCR	PER		
Protein level												
Low	43.7	33.4	43.7	1.26	3.52*a*	513.8	89.3	143.4	1.62	3.16*a*	2395	3.33*a*
High	44.1	33.0	44.2	1.28	3.22*b*	506.8	90.6	146.1	1.59	3.05*b*	2432	3.13*b*
Pooled SEM	0.170	0.394	0.510	0.015	0.031	4.249	0.641	1.139	0.025	0.019	20.59	0.021
Protein digestibility												
Low	44.1	33.1	43.1*b*	1.25*b*	3.34	508.3	89.6	143.1	1.60	3.02*b*	2398	3.18*b*
High	43.7	33.2	44.8*a*	1.29*a*	3.41	512.3	90.3	146.4	1.61	3.19*a*	2429	3.29*a*
Pooled SEM	0.170	0.393	0.506	0.014	0.031	4.249	0.637	1.313	0.029	0.019	20.37	0.021
C. perfringens challenge												
Unchallenged	43.5	31.9*b*	43.2*b*	1.29*a*	3.31*b*	493.7	92.1*a*	145.4	1.56	3.15*a*	2455	3.21
Challenged[z]	44.3	34.4*a*	44.7*a*	1.25*b*	3.44*a*	526.9	87.9*b*	144.0	1.65	3.06*b*	2373	3.25
Pooled SEM	0.170	0.339	0.494	0.014	0.031	4.249	0.902	2.085	0.047	0.019	20.50	0.019
Source of variation (P – value)												
Protein level	0.159	0.488	0.450	0.208	<.0001	0.246	0.154	0.254	0.567	0.001	0.414	<.0001
Protein digestibility	0.159	0.765	0.011	0.029	0.142	0.504	0.468	0.255	0.805	<.0001	0.252	0.001
C. perfringens challenge												

	0.001	<.0001	0.001	0.010	0.001	<.0001	0.008	0.754	0.434	0.012	0.467	0.151
Level x Digestibility	0.519	0.058	0.045	0.696	0.297	0.052	0.064	0.107	0.784	0.398	0.678	0.215
Level x Challenge	ND	ND	ND	ND	ND	ND	0.888	0.787	0.520	0.672	0.480	0.866
Digestibility x Challenge	ND	ND	ND	ND	ND	ND	0.614	0.464	0.542	0.177	0.851	0.270
Level x Digestibility x Challenge	ND	ND	ND	ND	ND	ND	0.367	0.216	0.604	0.740	0.648	0.141

[1]average daily gain (g/d); [2]average daily feed intake (g/d); [3]feed conversion ratio (g/g); [4]PER (g/g); [5] d0, d14 and final body weight at d35d (g)

[z]birds were challenged on d14, therefore growth performance of birds to be challenged during the starter phase and challenged birds during the grower and the overall experimental periods

a,b: means with different letters within a column for each main effect differ ($P < 0.05$)

ND: not determined since birds were not challenged in the starter phase

the ADFI or FCR ($P > 0.05$). The C. *perfringens* challenge reduced the ADG of birds by 4.6%. On a weekly basis, the C. *perfringens* challenge significantly affected the ADG of broilers during wk 3 and 5 ($P < 0.05$), but not in wk 4 ($P > 0.05$; Figure 5.1). None of the main effects or interactions among them affected the final BW of birds ($P > 0.05$; Table 5.1).

Dietary protein level during starter, grower and overall experimental periods and, protein digestibility during the grower and overall experimental periods, affected the PER of birds ($P < 0.05$; Table 5.1). Birds fed *LoPro* levels significantly increased the PER throughout the experiment and *HiDig* proteins optimized the PER during the grower and overall experimental periods. The PER of birds to be challenged was higher than that of the control group during the starter period ($P < 0.05$). However, the C. *perfringens* challenge significantly affected the PER ($P < 0.05$), which was 2.9% lower in challenged birds than in unchallenged birds. The overall PER of the birds was not affected by the challenge ($P > 0.05$). In addition, the PER in both unchallenged and challenged birds was significantly higher for starter birds than for grower birds ($P < 0.05$).

Protein level and digestibility significantly affected both ileal *C. perfringens* counts and NE-related intestinal gross lesion score of birds ($P < 0.05$: Table 5.2): both parameters were higher in birds fed *HiPro* levels and *LoDig* proteins compared to *LoPro* levels and *HiDig* proteins fed counterparts, respectively. No interaction between the two factors was observed either for the pathogen counts or for the intestinal lesion score of birds ($P > 0.05$).

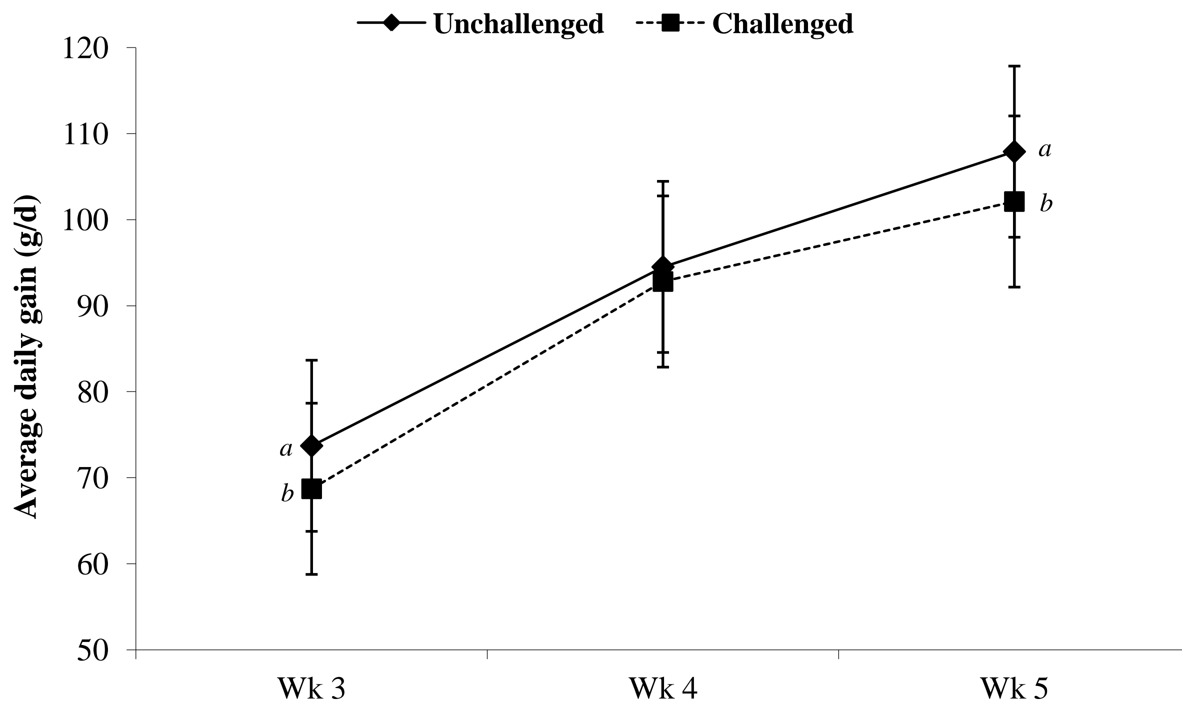


Figure 5.1. Average daily gain (g/d) of broiler chickens during the grower period (Wk 3: d15 – 21, Wk 4: d22 – 28, Wk 5: d29 – 35; SEM = 1.641).

^{a,b}Means within each week with different superscripts are significantly different.

Table 5.2. Effect of dietary protein level and protein digestibility on ileal *Clostridium perfringens* population and intestinal gross lesion score of broiler chickens on d 35 of age.

Main effects	Ileal *C.perfringens* counts *(log_{10} cfu/g)*	Intestinal gross lesion score[1]
Protein level		
Low	2.94*b*	1.06*b*
High	3.61*a*	1.59*a*
Pooled SEM	0.220	0.151
Protein digestibility		
Low	3.60*b*	1.56*b*
High	2.95*a*	1.09*a*
Pooled SEM	0.220	0.151
Source of variation (*P* – value)		
Protein level	0.039	0.019
Protein digestibility	0.046	0.037
Level x Digestibility	0.286	0.664

[1]According to the criteria described by Dahiya et al. (2005): 0-No gross lesions, normal intestinal appearance; 0.5-Severely congested serosa and mesentery engorged with blood; 1- Thin-walled, friable intestines with small red petechiae (> 5); 2-Focal necrotic intestinal lesions; 3- Sizable patches of necrosis (1 to 2 cm long); 4- Diffused necrosis typical of field cases.

The main effects of protein level, protein digestibility or C. *perfringens* challenge did not affect the microbial acetate production in the cecum ($P > 0.05$), but an interaction among the three factors tended to affect the level of acetate ($P = 0.09$; Table 5.3). Only the main effect C. *perfringens* challenge affected the cecal propionate level ($P < 0.05$), challenged birds having 25% more propionate than unchallenged counterparts. The three main effects interacted for the levels of butyrate and total VFA in the cecum ($P < 0.05$). Protein digestibility affected the cecal butyrate and total VFA concentrations of unchallenged birds fed *LoPro* diets ($P < 0.05$), *LoPro-HiDig* diet fed birds having 9.64 and 29.05 µmol more butyrate and total VFA, respectively, per g of cecal digesta compared to birds fed *LoPro-LoDig* diet. However, in birds fed *LoPro-LoDig* diet, *C. perfringens* challenge significantly increased the amounts of cecal butyrate and total VFA by 10.02 and 29.30 µmol, respectively, per g of digesta, compared to unchallenged counterparts. Except for the *LoPro-LoDig* treatment, the amounts of cecal butyrate and total VFA were similar between unchallenged and challenged birds fed the other dietary treatments ($P > 0.05$; Figure 5.2). Dietary protein level did not affect ($P > 0.05$), but protein digestibility and *C. perfringens* challenge interacted for the amount of valerate produced in the cecum ($P < 0.05$). When challenged, birds fed *LoDig* proteins had 71.4% more valerate, compared to birds fed *HiDig* proteins, but protein digestibility did not affect the cecal valerate level in unchallenged birds (data not shown). Only the main effect protein level affected the amount of *iso*caproate in the cecum ($P < 0.05$). Dietary *HiPro* level reduced cecal *iso*caproate concentration by 46.7% compared to *LoPro* level. The main effects did not affect the amount of cecal caproate in the birds ($P > 0.05$), but an interaction between protein level and C. *perfringens* challenge tended to affect the level of caproate ($P = 0.09$). None of the main effects and their interactions affected

Table 5.3. Effect of dietary protein level, protein digestibility and *Clostridium perfringens* challenge on volatile fatty acid profile, total volatile fatty acid concentration (μmol/g) and ammonia concentration (mmol/g) in the ceca of broiler chickens at d 35.

Main effects	Acetic	Propionic	Butyric	*iso* Butyric	*iso* Valaric	Valaric	*iso* Caproic	Caproic	Heptonic	Total	Ammonia
Protein level											
Low	70.04	3.94	19.37	0.25	0.41	1.16	0.15*a*	0.16	0.17	95.65	435.24
High	74.56	3.71	16.71	0.19	0.36	1.04	0.08*b*	0.19	0.19	97.50	485.88
Pooled SEM	3.51	0.27	1.01	0.02	0.03	0.08	0.03	0.03	0.02	4.58	29.26
Protein digestibility											
Low	69.78	3.77	16.60	0.21	0.36	1.31	0.13	0.15	0.20	92.98	480.60
High	74.82	3.88	19.47	0.23	0.41	0.90	0.10	0.19	0.16	100.17	440.52
Pooled SEM	3.51	0.27	1.01	0.02	0.03	0.08	0.03	0.03	0.02	4.58	29.26
C. perfringens challenge											
Unchallenged	74.14	3.40*b*	17.59	0.20	0.40	0.97	0.10	0.16	0.17	97.14	455.03
Challenged	70.46	4.25*a*	18.49	0.24	0.37	1.23	0.13	0.19	0.18	96.02	466.10
Pooled SEM	3.51	0.27	1.01	0.02	0.03	0.08	0.03	0.03	0.02	4.58	29.26
Source of variation (P – value)											
Protein level											
	0.366	0.547	0.068	0.129	0.360	0.296	0.039	0.540	0.491	0.775	0.226
Protein digestibility											
	0.314	0.766	0.049	0.658	0.388	0.001	0.448	0.422	0.103	0.272	0.337
C. perfringens challenge											
	0.462	0.028	0.533	0.321	0.544	0.026	0.362	0.617	0.585	0.863	0.790

Level x Digestibility	0.586	0.105	0.953	0.846	0.227	0.214	0.562	0.933	0.937	0.840	0.317
Level x Challenge	0.277	0.536	0.093	0.813	0.734	0.171	0.990	0.098	0.455	0.268	0.630
Digestibility x Challenge	0.223	0.067	0.056	0.667	0.969	0.034	0.928	0.577	0.725	0.116	0.667
Level x Digestibility x Challenge	0.090	0.178	0.009	0.758	0.668	0.056	0.389	0.141	0.661	0.052	0.749

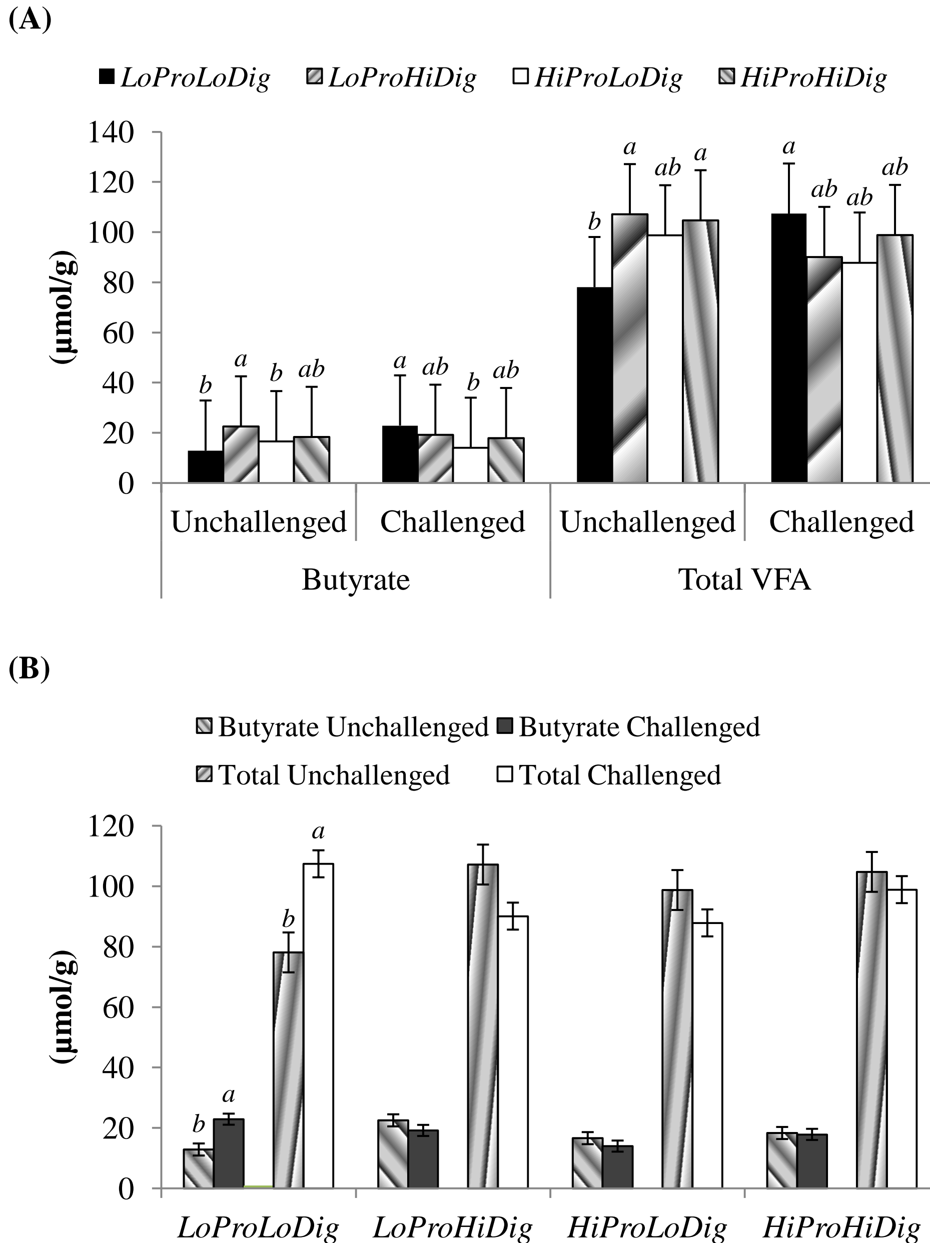


Figure 5.2. Effect of *C. perfringens* challenge on the microbial butyrate and total VFA production in caca of broilers fed different dietary treatments.

LoPro: low digestible protein level, *HiPro*: high digestible protein level, *LoDig*: low protein digestibility, *HiDig*: high protein digestibility.

[a,b]Means for each dietary treatment within butyrate and total VFA with different superscripts are significantly different (A), and means for butyrate and total VFA within each dietary treatment with different superscripts are significantly different (B).

*iso*butyrate, *iso*valerate and heptonate concentrations in the cecum (*P* > 0.05).

The three main effects and their interactions did not affect the ammonia production in the cecum of birds (*P* > 0. 0.05; Table 5.3). However, *HiPro* level, *LoDig* proteins and C. *perfringens* challenge resulted numerical increases in cecal ammonia production.

The three main effects did not affect the acetate MR (Table 5.4) in the cecum of birds (*P* > 0.05), but interactions between protein level and digestibility and, protein digestibility and C. *perfringens* challenge affected that MR (*P* < 0.05). Compared to *HiDig* proteins, *LoDig* proteins in *LoPro* diets increased acetate MR by 3.4%. Protein digestibility did not affect the acetate MR in the cecum of birds fed *HiPro* diets. In birds fed *LoDig* proteins, C. *perfringens* challenge reduced the acetate MR by 5.7% compared to unchallenged birds, but C. *perfringens* challenge did not affect the acetate MR in birds fed *HiDig* proteins (data not shown). The main effect C. *perfringens* challenge and the interaction between protein level and digestibility affected the propionate MR in birds (*P* < 0.05). The MR was 25.1% higher in challenged birds than in unchallenged counterparts. In birds fed *HiPro* diets, *LoDig* proteins increased propionate MR by 35.6% compared to *Hidig* proteins, but protein digestibility did not affect the propionate MR in birds fed *LoPro* diets (data not shown). An interaction among the main effects affected the butyrate MR (*P* < 0.05). When challenged, the MR was higher in birds fed *LoPro* diets than in birds fed *HiPro* diets, irrespective of protein digestibility. Among unchallenged treatment groups, butyrate MR was higher in birds fed *LoPro-HiDig* diet (data not shown). The main effect C. *perfringens* challenge affected the valerate MR (*P* < 0.05) and the interaction between protein level and digestibility tended to affect the MR in the cecum (*P* = 0.06). The C. *perfringens* challenge increased the valerate MR by

Table 5.4. Effect of dietary protein level, protein digestibility and *Clostridium perfringens* challenge on molar ratio (% of total) of volatile fatty acid in the ceca of broiler chickens at d 35.

Main effects	Acetic	Propionic	Butyric	*iso* Butyric	*iso* Valaric	Valaric	*iso* Caproic	Caproic	Heptonic
Protein level									
Low	73.44*b*	4.16	19.93*a*	0.28	0.45	1.20	0.18	0.17	0.19
High	76.43*a*	4.10	16.88*b*	0.21	0.40	1.10	0.10	0.22	0.20
Pooled SEM	0.515	0.301	0.525	0.031	0.045	0.077	0.032	0.041	0.021
Protein digestibility									
Low	75.16	4.30	17.51*b*	0.25	0.44	1.40*a*	0.17	0.19	0.24
High	74.71	3.96	19.30*a*	0.23	0.42	0.90*b*	0.11	0.21	0.16
Pooled SEM	0.515	0.301	0.525	0.031	0.045	0.077	0.032	0.041	0.021
C. perfringens challenge									
Unchallenged	76.33*a*	3.67	17.77	0.23	0.45	1.04*b*	0.13	0.18	0.19
Challenged	73.54*b*	4.59	19.05	0.25	0.40	1.26*a*	0.15	0.21	0.20
Pooled SEM	0.515	0.301	0.525	0.031	0.045	0.077	0.032	0.041	0.021
Source of variation (P – value)									
Protein level	0.0001	0.889	0.0001	0.124	0.430	0.383	0.088	0.394	0.662
Protein digestibility	0.541	0.421	0.019	0.662	0.764	<.0001	0.223	0.729	0.014
C. perfringens challenge	0.0003	0.035	0.090	0.631	0.419	0.048	0.740	0.581	0.662
Level x Digestibility	0.009	0.039	0.541	0.949	0.370	0.063	0.761	0.820	0.868
Level x Challenge	0.503	0.225	0.043	0.335	0.620	0.367	0.621	0.055	0.060

Digestibility x Challenge	0.033	0.626	0.147	0.234	0.355	0.234	0.421	0.957	0.200
Level x Digestibility x Challenge	0.096	0.962	0.038	0.251	0.566	0.489	0.833	0.311	0.351

a, b: means with different letters within a row for each main effect differ ($P < 0.05$)

21.2%. The protein level tended to affect the *iso*caproate MR (P = 0.09), but the other two main effects did not affect (P > 0.05). None of the main effects affected the caproate MR (P > 0.05), but the interaction between protein level and C. *perfringens* challenge tended to affect (P = 0.06). The main effect protein digestibility affected the heptonate MR in the cecum (P < 0.05) and the interaction between protein level and C. *perfringens* challenge tended to affect (P = 0.06). The *LoDig* proteins in the diet increased the MR by 50% compared to *HiDig* proteins.

5.5. Discussion

The previous study described in Chapter 3 reported a significant interaction between protein level and digestibility for ADG during the starter period of the birds fed the same experimental diets. This interaction was due primarily to decreased performance in birds fed the *LoPro-LoDig* diet compared to birds fed the other three diets. The similar trend observed in the current experiment supports the previous findings. When proteins with low digestibility values are fed, comparatively higher amounts of AA may leave the upper GIT and thus be freely available to the microbes residing in the lower GIT. Some enteric bacteria acquire the ability to metabolize AA, producing toxins and other metabolites which may affect enterocyte turnover and growth performance of birds (Ewing and Cole 1994; Macfarlane and Macfarlane 1995; Gaskins 2001; van der Klis and Jansman 2002). This combined with reduced dietary protein would put the *LoPro-LoDig* fed birds under greater stress and resulted in the decreased growth seen in both experiments.

Birds fed *LoPro-HiDig* diet consumed more feed than birds fed *LoPro-LoDig* diet during the starter period of the present study. The study described in Chapter 3 reported the same observation only during the grower period of birds, but not in the starter phase. Diets with *HiDig* proteins contained a lesser amount of CP compared to diets with *LoDig* proteins, notwithstanding the inclusion of equal amounts of digestible protein in feed formulation in each of the *LoPro* and *HiPro* situations. An increase in feed consumption was reported by Aletor et al. (2000), feeding broilers *l*CP – AA supplemented diets during the grower phase. In addition, higher amounts of nitrogen pass as undigested proteins to the gut microbes of birds fed diets containing *LoDig* proteins, which may result in increased hindgut fermentation (Piva et al. 1995). The physical distention of the hindgut as a consequence of the increased hindgut fermentation might result in a reduction in feed consumption of the birds (Cherbut et al. 1988).

The growth performance of the birds that were challenged was superior to the other group during the starter phase, before the challenge occurred. However, subsequent to *C. perfringens* inoculation, the growth performance of these birds became inferior to the unchallenged group during the grower phase. Significantly higher initial BW of the group to be challenged might have led to the better growth performance during the starter phase, compared to the control group. The ADG of the birds was reduced in the first week of challenge (third week of experiment). Nevertheless, as reported in Chapter 4, the C. *perfringens* challenge did not affect the ADG of growers during the fourth week, likely due to diminishing intensity of the infection. However, the ADG of challenged birds was reduced again during the fifth week, due to possible re-infection caused by the ingestion of the pathogen or their spores on the litter, shed by the

infected birds. Reduced weight gain and increased FCR associated with impaired nutrient digestion and absorption due to the damage caused by *C. perfringens* to the intestinal mucosa can be linked to the substandard growth performance of challenged birds during the grower phase (Elswinger et al. 1992; Kaldhusdal and Hofshagen 1992; Kaldhusdal et al. 2001; Hofacre et al. 2003). In addition, gaining access to portal circulation and biliary system, *C. perfringens* gives rise to hepatitis and cholangio-hepatitis in broiler chickens (Lovland and Kaldhusdal 1999, 2001; Sasaki et al. 2000). The subsequent damage to the liver, bile ducts and gall bladder may result in reduced growth performance as well. Previous studies have reported impaired overall performance in broiler chickens, highly infected with *C. perfringens* (Stutz et al. 1983; Stutz and Lawton 1984; Kaldhusdal and Hofshagen 1992; Dahiya et al. 2005, 2007a, b).

One of the factors that determines the subsequent growth rate in broilers is the chick quality that includes the chick weight (Wilson 1991; Tona et al. 2003, 2004). Accordingly, significantly higher chick weight and the resultant better growth performance during the starter phase of birds to be challenged might have led to the final BW being equal to that of their unchallenged counterparts. Even under this situation, although not significant, the *C. perfringens* challenge demonstrated a considerable effect on the final BW of birds under different dietary treatments (Figure 5.3), which might have been significant and could have created a great economic impact, if the chicks with similar weight would have been used in challenged and unchallenged conditions. In this study, the *C. perfringens* challenge resulted only a 1.2 g reduction in the final BW of birds fed *LoPro-HiDig* diet, compared to unchallenged counterparts, but this weight difference for *LoPro-LoDig, HiPro-LoDig* and *HiPro-HiDig* diets fed birds were 134.5, 91.3 and 100.3 g, respectively. The findings of the current study clearly indicate that the

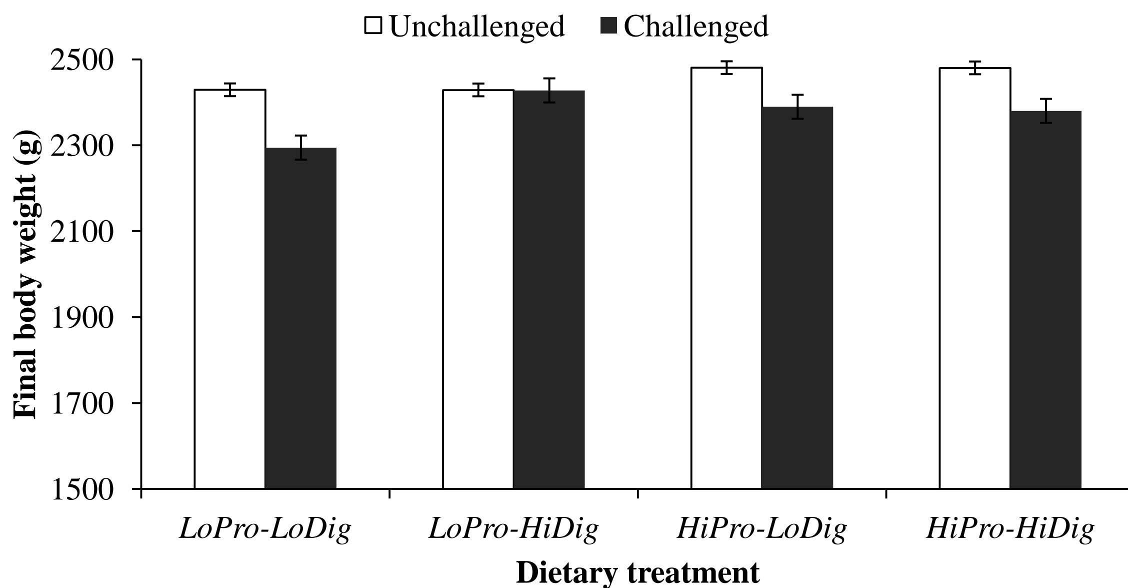


Figure 5.3. Effect of *C. perfringens* challenge on the final body weight at d35 of broilers fed different dietary treatments (*P* = 0.648; SEM = 39.51)

LoPro: low digestible protein level, *HiPro*: high digestible protein level, *LoDig*: low protein digestibility, *HiDig*: high protein digestibility.

impact of *C. perfringens* on broiler growth performance can be reduced to a great extent by using *LoPro* diets containing high quality protein supplements.

The efficiency of dietary protein utilization is generally reflected by the PER (Kamran et al. 2008). In the present study, the PER was higher in birds fed *LoPro* diets compared to birds fed *HiPro* diets during starter, grower and overall experimental periods. This finding was supported by previous studies (Cheng et al. 1997; Aletor et al. 2000) as well as the study described in Chapter 3, reporting a significant increase in PER with the reduction of dietary protein content. Increasing dietary protein level by adding non essential AA to *l*CP diets, Aletor et al. (2000) reported a reduction in PER compared to AA un-supplementation. However, lowering the CP content but maintaining a constant ME:CP ratio, Kamran et al. (2008) demonstrated a linear reduction in PER in broiler chickens during the grower, finisher and entire experimental periods. This observation can be attributed to the linear increase in feed consumption with the lowering ME:CP ratio, which in turn increased protein intake of birds during those growth phases.

Compared to *LoDig* protein supplements, *HiDig* protein supplements in broiler rations optimized the protein utilization, as reflected by the PER, in the present study. The previous study described in Chapter 3 with the same dietary treatments reported the same observations only during the grower and finisher periods, but not in the starter phase. As previously discussed, undigested proteins originated from *LoDig* protein sources are subjected to microbial metabolism in the hind gut and the consequential metabolites may negatively affect the growth of birds (Ewing and Cole 1994; Macfarlane and Macfarlane 1995; Gaskins 2001; van der Klis and Jansman 2002). As a result, the PER in birds fed diets containing *LoDig* proteins was decreased, despite the

similar protein intake. In addition, in unchallenged birds the PER was higher during the starter period compared to grower period. This observation was supported by Johnson and Parsons (1997), Kamran et al. (2008) and data from the study described in Chapter 3. Increasing demand for nutrients for maintenance with increasing age is a plausible explanation for this observation.

Similar to the other growth performance parameters, the PER as well, was superior in birds to be challenged during the starter period compared to the control group. Nevertheless, C. *perfringens* challenge at the beginning of the grower period reduced the PER of the challenged group. Damage caused by *C. perfringens* to the GIT and associated organs adversely affects the growth performance of birds (Elswinger et al. 1992; Kaldhusdal and Hofshagen 1992; Kaldhusdal et al. 2001; Lovland and Kaldhusdal 1999, 2001; Hofacre et al. 2003). The ADFI, hence the CP intake, was similar between the challenged and unchallenged birds during the grower period, so the weight gain suppressed by the pathogen reduced the PER of challenged birds. However, the PER was similar for challenged and unchallenged birds at the end of the experiment on account of the superior PER of challenged birds before challenging.

Overall, as previously suggested in Chapter 3, high quality protein supplements should be incorporated into broiler diets and essential AA should be supplemented in quantities not more than required for optimum performance, to optimize the utilization of dietary protein.

Based on the findings of previous studies, the distinct impact of the source and level of dietary protein have on *C. perfringens* proliferation in the GIT of broiler chickens is evident (Kaldhusdal 2000; Drew et al. 2004). Studies have demonstrated an over-proliferation *C. perfringens* in the small intestine of broiler chickens, subsequent to

an increased consumption of protein or a specific AA and, predisposing of birds fed higher amounts of proteins to NE (Titball et al. 1999; Drew et al. 2004; Kaldhusdal 2000; Kocher 2003; Wilkie et al. 2005). Although sufficient evidence exists to support the mechanisms behind the effects of other dietary factors on the proliferation of *C. perfringens* and development of NE, there is a scarcity of information to describe the mechanisms associated with the high prevalence of NE in broilers fed *HiPro* levels. Genetic makeup of broiler chickens determines the capacity of nutrient digestion and absorption and amounts beyond that level escapes the absorptive area of the GIT without being utilized. Accordingly, as previously described, feeding broilers with diets containing higher levels of proteins may increase the flow of unused protein to the latter part of the GIT and acting as a good source of nutrients for enteric pathogens, this portion of dietary protein may increase the proliferation of *C. perfringens.* The findings of the present study support this hypothesis with significantly higher *C. perfringens* numbers in the ileal digesta of broilers fed *HiPro* diets. In addition, when broilers are fed poorly digestible proteins, relatively higher amounts of undigested AA are present in the GIT (Lan et al. 2004; McDevitt et al. 2006) where they are available to the pathogenic microbes residing in the lower GIT. This hypothesis too, in accordance with the results of the current study, in which birds fed diets with *LoDig* protein supplements had higher ileal *C. perfringens* counts compared to *HiDig* proteins fed counterparts.

In view of the cost, complications and requirement of specialized training and equipments associated with the procedures for precise identification of bacterial genera and species, measurement of bacterial metabolites and end products of fermentation can be used to evaluate the overall shifts in bacterial activity in the GIT. The VFA are the major end products of microbial fermentation in the GIT (Ricke at al. 2004), thus

measuring VFA levels in digesta is used as a tool of evaluating changes in the bacterial activity in the GIT of broilers (Corrier et al. 1990; Choct et al. 1996, 1999; Vahjen et al. 1998; Yasar and Forbes 1999; Kocher et al. 2000). Ceca are the main site of microbial fermentation in the avian GIT because these chambers provide a relatively stable environment and harbor the largest as well as the most complex bacterial community in the GIT of poultry (Mead 1997). In addition, shorter digesta transit time in the upper GIT limits the microbial fermentation hence VFA concentration is normally low in the small intestine compared to the cecum of poultry (Rehman et al. 2007). Despite the highly efficient digestion and nutrient absorption process of the upper GIT, a constant supply of nutrients to the lower GIT still exists in the form of undigested dietary components, desquamated gut mucosal cells and host enzymes. Hence, the main energy sources available for cecal microbes are complex carbohydrates and undigested proteins (Piva et al. 1995; Józefiak et al. 2004). In the absence of simple sugars, inducible enzymes produced by the microbes facilitate utilization of dietary components not digested by the host (Bugaut 1987). In general, complex carbohydrates are fermented by the resident microflora in the cecum to VFA (Józefiak et al. 2004).

In the present study, the amount of microbial acetate produced in the caca of birds challenged with *C. perfringens* was similar among all treatment groups, but in unchallenged group, birds fed *HiPro-HiDig* diet had more acetate than *LoPro-LoDig* diet fed birds. However, the lowest acetate MR was reported by unchallenged birds fed *LoPro-HiDig* diet and challenged birds fed *LoPro* diets. The *C. perfringens* challenge increased the amount and the MR of cecal propionate. In addition, *HiDig* protein supplements reduced the propionate MR in birds fed *HiPro* diets. The low cecal MR of acetate and propionate in birds fed *LoPro* diets or *HiDig* proteins can be attributed to a

number of factors; proportionately lower production of these VFA, higher uptake by the host or utilization as energy sources by cecal microbes. Moreover, a possibility of utilizing some of these VFA by certain microbes as growth substrates cannot be excluded, because metabolic end products produced by one organism or species can serve as growth substrates for another. In fact, previous reports indicated the utilization of acetate by butyrate – producing microbes (Barcenilla et al. 2000) and lactobacilli (Meimandipour et al. 2009) for their metabolic activities.

In unchallenged birds fed *LoPro* diets, *LoDig* proteins reduced the cecal butyrate concentration compared to *HiDig* proteins. Nevertheless, birds fed *LoPro* diet containing *LoDig* proteins reported the highest amount of cecal butyrate when challenged with *C. perfringens*. This observation was same for the total VFA content in the cecum as well. The butyrate MR was higher in unchallenged birds fed *LoPro-LoDig* diet and challenged birds fed *LoPro* diets. Currently, butyrate attracts more attention than other VFA due to its beneficial effects on the GI epithelial cells (Cavaglieri et al. 2003). Lactate is a major precursor of butyrate synthesis (Bjerrum et al. 2006; Meimandipour et al. 2009) therefore an increase in the number of lactic acid bacteria is correlated with the increase in the butyrate synthesis (Duncan et al. 2004). Nevertheless, a negative correlation has been reported between presence of Lactobacillus and pathogenecity and/or proliferation of *C. perfringens* in the GIT of broiler chickens. For instance, Fukata et al. (1991) observed a lower intensity in pathogenecity of *C. perfringens* in chickens fed a monoflora of *Lactobacillus acidophilus*. In addition, a significant decrease in *C. perfringens* numbers was reported with increasing numbers of Lactobacilli in the cecum of broilers (Dahiya et al. 2007a). However, challenged birds fed *LoPro-LoDig* diet demonstrated the numerically worst growth performance in this study and that might

partly be due to the substantial damage caused by the pathogen to the intestinal mucosa. This damage may have limited the uptake and utilization of VFA by the GIT and that can be attributed to the higher butyrate and total VFA levels in challenged birds fed that particular diet, compared to unchallenged birds.

However, when carbohydrate sources are limited, carbohydrate: nitrogen ratio of the cecum decreases resulting in more proteolytic fermentation (Piva et al. 1995). Accordingly, a portion of the intestinal VFA, mainly branched – chain VFA, is protein derived and produced as a result of fermentation of branched – chain AA (valine, leucine and isoleucine: Macfarlane et al. 1992). However, a minimal proteolytic fermentation had taken place in the ceca of birds in the current study, as reflected by very low concentrations and MR of branched – chain VFA such as *iso*butyrate, *iso*valerate and *iso*caproate. It appears that cecal microbes utilized preferably carbohydrate substrates for fermentation resulting in a lower concentration of branch-chain VFA with higher proportion of straight chain VFA. This is beneficial to the host in terms of gut health and overall performance, because a proteolytic fermentation produces such potentially toxic metabolites as ammonia, biogenic amines, volatile phenols and indoles in addition to VFA (Yokoyama et al. 1982; Russell et al. 1983; Macfarlane et al. 1992). In this context, carbohydrate fermentation is preferred over proteolytic activity of microbes in the GIT.

In general, ammonia concentration is higher in the cecum compared to other segments of avian GIT (Isshiki 1980). Amino acids from both dietary and endogenous proteins are the main source of ammonia in the lower GIT (Wrong and Vince 1984). Accordingly, the concentration of ammonia in the cecum reflects the amount of AA reaching the lower GIT. In the present study, in the cecum of birds fed *LoPro* and *HiDig*

protein supplements the ammonia concentration was numerically lower by 10 and 8%, compared to ammonia in birds fed *HiPro* and *LoDig* proteins, respectively. This observation suggests that lower amounts of proteins escaped the digestion and absorption process of the upper GIT of broilers fed low dietary digestible proteins and highly digestible protein sources. In addition, the *C. perfringens* challenge of the birds resulted a numerical increase in cecal ammonia by 2.4%. In fact, *Clostridium* is among the few candidates who are able to metabolize proteins in the cecum, producing ammonia as an end product (Macfarlane et al. 1992). Therefore these findings have shown that feeding broilers with *LoPro* diets containing highly digestible proteins can improve the gut health by reducing effects of enteric pathogens.

In conclusion, data from this investigation revealed the capacity of *LoPro* diets with highly digestible protein supplements to minimize the *C. perfringens* challenge related growth suppression of broiler chickens. The key beneficial effects of that particular dietary approach were likely due to limited nutrient availability to the pathogen as a consequence of enhanced nutrient utilization by the host and a possibility for formation of host friendly intestinal ecosystem. The overall results of this study may support our efforts in the development of nutritional strategies to minimize the impact of *C. perfringens* on broiler production in the absence of AGP.

6. ORAL *Clostridium perfringens* CHALLENGE AFFECTS THE METHIONINE REQUIREMENTS OF BROILER CHICKENS

6.1 Abstract

Bacterial overgrowth of opportunistic pathogens often does not elicit clinical disease. However, it may result in changes in nutrient utilization altering the requirement for nutrients including AA. The effect of *C. perfringens* challenge of broiler chickens on the requirement for methionine was determined in a 21d growth trial. Broiler chickens (288) were randomly assigned to two rooms and fed a medicated commercial starter diet from d0-6. From d7-21, the birds were fed seven different diets: a methionine deficient basal diet (230 g/kg CP and 2.7 g/kg standardized ileal digestible methionine), of diets fortified with equi-sulfurous amounts of DLM (1.6, 3.2 or 4.8 g/kg) or HMB (1.8, 3.6 or 5.5 g/kg). On d7-20, the birds in one room were challenged using an overnight broth culture of *C. perfringens,* offered 2 ml per bird in 1/3 of ADFI, while the birds in the other room received feed mixed with sterile broth. Quadratic regressions were fitted to growth, feed intake and feed conversions, and methionine requirement was calculated as the inflection point of the regression line. The methionine requirement of the birds fed DLM was higher in infected birds compared to uninfected birds for ADG (6.03 and 5.07 g/kg; $P < 0.05$) while the infected and uninfected HMB fed birds had requirements that were not significantly different than the other two groups (5.30 and 5.39 g/kg). The methionine requirement for optimum FCR was significantly higher in infected birds than uninfected birds for both the DLM-fed birds (6.27 vs 5.41 g/kg) and the HMB-fed birds (6.50 and 5.94 g/kg). Further, the DLM fed birds had significantly lower methionine requirements for maximum FCR than the HMB fed bird in both infected and uninfected groups. The methionine requirements for maximum ADFI were not significantly

different between groups. Cecal *C. perfringens* populations and intestinal lesion scores were not significantly affected by either methionine source or level. *C. perfringens* infection has a significant effect on methionine requirement and may be responsible for the reduced growth performance of birds with subclinical NE.

6.2. Introduction

Necrotic enteritis is a potentially fatal bacterial infection of poultry and all poultry producing regions of the world have reported it as an economically important disease (Van Immerseel et al. 2004). The disease is associated with *C. perfringens* type A strains, carrying the genes for α or NetB toxins (Keyburn et al. 2008; Van Immerseel et al. 2008). The approximate annual global loss due to this disease has been estimated as US $2 billion (Van der Sluis 2000b). With the legal restrictions on the utilization of AGP in animal feed industry in some poultry producing countries, this disease is re-emerging in broiler chickens (Grave et al. 2004; Van Immerseel et al. 2004; Williams 2005). In this scenario, there is an earnest interest in finding alternative management as well as nutritional strategies to antibiotics to combat the occurrence and intensity of NE in poultry.

Dietary protein plays a crucial role in the development of NE in broiler chickens. The marked effect of the source and level of dietary protein on counts of *C. perfringens* in intestinal contents of broiler chickens is well documented (Kaldhusdal 2000; Drew et al. 2004). Studies have revealed that *HiPro* diets, in particular diets containing high level of animal-derived proteins, predispose broilers to NE (Kaldhusdal 2000; Kocher 2003; Drew et al. 2004; Wilkie et al. 2005).

In addition to dietary proteins, evidence exists to suggest a correlation between certain AA and *C. perfringens* growth and/or α toxin production. The presence of certain AA in the GIT triggers *C. perfringens* growth and α toxin production. Accordingly, increased levels of protein and/or a particular AA in the small intestine of broiler chickens can induce the over-proliferation of this organism (Titball et al. 1999). Several studies demonstrated the stimulatory effect of dietary glycine on the proliferation of this pathogen in the GIT of broiler chickens and thereby increasing the possibility of NE (Ispolatovskaya 1971; Nakamura et al. 1978; Dahiya et al. 2005; Wilkie et al. 2005). *C. perfringens* has demonstrated the requirement of peptides with glycine for the synthesis of α toxin (Nakamura et al. 1978; Stevens and Rood 2000). In contrast to the role of glycine in *C. perfringens* proliferation, methionine is required for sporulation and is highly stimulatory for growth, despite not being an essential nutrient for *C. perfringens* (Muhammed et al. 1975). However, *in vitro* experiments conducted in our laboratory observed a significant suppression of *C. perfringens* proliferation upon incubation of a mixed bacterial culture for a period of 24-h in minimal salt media supplemented with DLM at a higher concentration (10 mg/ml), compared to the unsupplemented media (Wilkie et al. 2005). Moreover, performing an *in vivo* study using the two commercially available methionine forms, DLM and HMB, Dahiya et al. (2007b) observed a significant decrease in *C. perfringens* numbers in the GIT of broilers fed relatively high concentrations of methionine.

Methionine is a limiting AA in commercial poultry feeds and is normally supplemented using dry DLM with approximately 99% methionine activity or dry or liquid HMB, commonly available as a product containing 88% methionine activity. For over four decades there has been debate in the scientific literature regarding the

nutritional effectiveness (Thomas et al. 1991; Van Weerden et al. 1992; Esteve-Garcia and Austic 1993; Huyghebaert 1993; Rostagno and Barbosa 1995; Schutte and de Jong 1996; Esteve-Garcia and Llaurado 1997; Wallis, 1999; Lemme et al. 2002) and the appropriate statistical methodology to evaluate the precise bioefficacy estimates (Littell et al. 1997; Kratzer and Littell 2006; Sauer et al. 2008) of DLM and HMB. The objective of current study was to evaluate the methionine requirement for optimum growth in broiler chickens fed diets supplemented with DLM or MHA under *C. perfringens* infected and uninfected situations.

6.3. Materials and methods

Experimental protocols for the study was approved by the Animal Care Committee of the University of Saskatchewan and was 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. Birds and management

One-day-old male Ross 308 broiler chicks (288), obtained from a local broiler hatchery (Lilydale Hatchery, Wynyard, Saskatchewan, Canada) were used in this study. Upon arrival to the research facility, birds were randomly allocated into 24 battery cages (12 birds/cage). On d 1-7, birds were fed the same corn/soybean meal-based starter crumble diet (3,200 kcal/kg of ME; 1.2% Lys) described in chapter 3 (Table 3.1: Federated Coop Feeds, Saskatoon, Saskatchewan, Canada). At the end of the pre-experimental period (d 7), the birds were weighed (142.85 ± 14.35 g) and randomly distributed in 48 battery cages in two rooms (24 cages/room; 6 birds/cage). The battery cages were arranged in

six tiers with wire floors plus fecal collection trays and were equipped with external feed and water troughs. Temperature was maintained at 34 °C at placement and reduced as the birds progressed in age with a final temperature of 27 °C at d 21. The lighting schedule used was intended not to restrict early growth and consisted of 23 h light and 1 h dark from d 1 to 7 and, 20 h light and 4 h dark from d 8 to the end (d 21) of experiment. Feed and water were available to the birds for *ad libitum* consumption, throughout the experiment.

6.3.2. Dietary treatments

On d 7, three cages of six birds each were assigned in a completely randomized design to one of the seven different experimental diets in mash form. The ideal protein-balanced, isocaloric diets were formulated based on SID AA content, calculated using QuickChick 1.0 program (Evonik-Degussa GmbH, Hanau, Germany). The birds were fed diets supplemented with either DLM (at 0, 0.16, 0.32, and 0.48%) or HMB (at 0, 0.18, 0.36, and 0.55%) thus providing four corresponding equimolar levels of each methionine source (Table 6.1). The control diet with no added methionine was formulated to contain 3100 kcal/ kg of ME, 23% CP, and 1.2, 0.27 and 0.35% SID lysine, methionine and cystine, respectively. Accordingly, the control diet contained only 50% of SID methionine required in d 8 – 21 period. The control diet was then supplemented with either DLM or HMB to achieve four equimolar levels of each methionine source (0, 0.16, 0.32, and 0.48%). The supplementation of methionine precursors was made at the expense of Celite (Celite 545, Celite Corporation, World Minerals Co., Lompoc, CA, USA). Except for methionine, the diets met or exceeded the NRC (1994) energy and nutrient requirements for broiler chickens for all other nutrients.

Table 6.1. Ingredient and nutrient composition of experimental diets to be fed from d8 to 21 of age (% as-is basis).

Ingredient (%)	**Control**	**DL-Methionine**			**DL-Hydroxy Methionine**		
		0.16	**0.32**	**0.48**	**0.18**	**0.36**	**0.55**
Wheat	58.00	58.00	58.00	58.00	58.00	58.00	58.00
Field Pea	14.50	14.50	14.50	14.50	14.50	14.50	14.50
Soybean meal	12.00	12.00	12.00	12.00	12.00	12.00	12.00
Canola meal	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Wheat DDGS[1]	0.39	0.39	0.39	0.39	0.39	0.39	0.39
Meat and bone meal	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Fish meal	0.42	0.42	0.42	0.42	0.42	0.42	0.42
Blood meal	0.14	0.14	0.14	0.14	0.14	0.14	0.14
Dried casein	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Canola oil	3.49	3.49	3.49	3.49	3.49	3.49	3.49
Dicalcium phosphate	0.81	0.81	0.81	0.81	0.81	0.81	0.81
Calcium carbonate	0.28	0.28	0.28	0.28	0.28	0.28	0.28
NaCl	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Vit/Min premix[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10	0.10
L-Lysine.HCL	0.32	0.32	0.32	0.32	0.32	0.32	0.32
L-Threonine	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Isoleucine	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Valine	0.09	0.09	0.09	0.09	0.09	0.09	0.09
Glycine	0.02	0.02	0.02	0.02	0.02	0.02	0.02
DL-Methionine	0.00	0.16	0.32	0.48	0.00	0.00	0.00

DL-Hydroxy Methionine	0.00	0.00	0.00	0.00	0.18	0.36	0.55
Xylanase[3]	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Celite	2.00	1.84	1.68	1.52	1.82	1.64	1.45
Energy and nutrient (%)							
ME (kcal/kg)	3100	3100	3100	3100	3100	3100	3100
Total protein	23.00	23.00	23.00	23.00	23.00	23.00	23.00
AID protein	19.00	19.00	19.00	19.00	19.00	19.00	19.00
AID Glycine	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Calcium	0.94	0.94	0.94	0.94	0.94	0.94	0.94
Available Phosphorus	0.42	0.42	0.42	0.42	0.42	0.42	0.42
Linoleic acid	2.09	2.09	2.09	2.09	2.09	2.09	2.09
Sodium	0.20	0.20	0.20	0.20	0.20	0.20	0.20
SID Lysine	1.20	1.20	1.20	1.20	1.20	1.20	1.20
SID Methionine	0.27	0.43	0.59	0.75	0.43	0.59	0.75
SID Cys	0.35	0.35	0.35	0.35	0.35	0.35	0.35
SID Met+Cys	0.62	0.78	0.94	1.10	0.78	0.94	1.10
SID Threonine	0.76	0.76	0.76	0.76	0.76	0.76	0.76
SID Tryptophan	0.19	0.19	0.19	0.19	0.19	0.19	0.19
SID Arginine	1.26	1.26	1.26	1.26	1.26	1.26	1.26
SID Valine	0.95	0.95	0.95	0.95	0.95	0.95	0.95
SID Isoleucine	0.82	0.82	0.82	0.82	0.82	0.82	0.82
SID Leucine	1.29	1.29	1.29	1.29	1.29	1.29	1.29

[1]Wheat distiller's dried grains with solubles

[2]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.

[3]Xylanase 5000 U/g; Protease 1600 U/g (Halchemix Canada Inc.)

6.3.3. *C. perfringens* challenge procedure

For this study an avian *C. perfringens* field strain isolated from a clinical case of NE in broiler chickens was used (Dr. M. Chirino, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK). The particular strain was characterized using PCR technique as a type A toxin producer (Dumonceaux et al. 2006). The organism was cultured anaerobically on BBL Blood Agar Base (Becton Dickinson Co., Sparks, MD) containing 5% sheep blood and 100 mg/L of Neomycin Sulphate (The Upjohn Co., Orangeville, ON, Canada) for 18 h at 37ºC, then aseptically inoculated into a sterilized preparation of Robertson's cooked meat broth (Difco Laboratories, Detroit, MI), and incubated anaerobically and statically for 8 h at 37ºC.

All 144 birds in one room were challenged right after the 4 h dark period on d 7 to 20 (inclusive), offering the inoculum in feed, as per the experimental model described in Chapter 4 with slight modifications. Briefly, each bird in that particular room received feed equivelent to 1/3 of ADFI mixed with 2 ml of broth culture. Similarly, each bird in the other room (unchellenged) received feed equivelent to 1/3 of ADFI mixed with 2 ml of sterile cooked meat medium broth. The respective feeders were top-dressed with culture/sterile broth mixed feed and fresh cultures were prepared daily. Culture-feed mixing was done immediately before offering to the birds. Bacterial counts were performed on the culture daily before inoculation and the count ranged from 4.46×10^4 to 6.87×10^6 cfu/ml.

6.3.4. Experimental design

Birds in six randomly selected cages in each room received the control diet whereas each experimental diet was offered to birds in three randomly selected cages in each room. Accordingly, each methionine source had three cages each fed the control diet and the three different methionine levels, to be considered in the statistical analysis.

6.3.5. Sample and data collection

Birds and feeders were weighed on a cage basis at the initiation (d 7) and termination (d 21) of the experiment. The data were used to calculate ADG, ADFI and FCR for the overall experimental period. Birds were monitored twice a day (8:30 and 15:30) and in the case of mortality, the weight of the dead bird/s and remaining feed in the respective feeder/s were recorded immediately, in order to consider in calculations. At 21 d of age, all birds were euthanized by cervical dislocation and the contents of both ceca were aseptically collected from each bird. One aliquot from both ceca was frozen at -20ºC for subsequent DNA extraction.

6.3.6. Pathological examination

All birds were kept under constant observation for any sign of NE, which include depression, diarrhea, dehydration, somnolence, ruffled feathers and reduced feed intake or mortality (Helmboldt and Bryant 1971; Al-Sheikhly and Truscott 1977a,b; Al-Sheikhly and Al-Saieg 1980; Gazdzinski and Julian 1992). As described above, birds were killed by cervical dislocation on d 21 of each trial and their intestinal tracts were removed immediately. Prior to digesta collection, intestinal lesions were scored blindly, according to the criteria described by Dahiya et al. (2005), using a scale from 0 to 4.

6.3.7. Genomic DNA extraction and purification

The DNA extraction and purification on the collected cecal digesta samples were performed based on the method described in Chapter 4.

6.3.8. Bacterial enumeration

The purified DNA was used for molecular enumeration of *C. perfringens* in cecal digesta samples. The enumeration of *C. perfringens* was performed using qPCR technique as described in Chapter 4. The qPCR counts were converted to genomes detected/g digesta based on the weight of digesta extracted and the dilution factors.

6.3.9. Statistical analysis

The 5% level of significance was used for all statistical analyses. The experiment was analyzed by fitting quadratic regression models to the data using the regression procedure of SPSS (version 18.0, SPSS INC., Chicago IL). All the effects were considered as fixed, and the interactions between the main effects were used in the model, with pen as the experimental unit. Requirements for methionine were calculated as the inflection point of regression equations. Differences between requirements were calculated using Tukey's honestly significant difference.

6.4. Results

The majority of challenged birds became dull and depressed, and excreted abnormally wet droppings upon initiation of challenging with *C. perfringens*. Cecal *C. perfringens* populations and intestinal lesion scores were not significantly affected by either methionine source or level ($P > 0.05$: Table 6.2). The methionine requirement of the

Table 6.2. Effect of different methionine sources and levels on intestinal lesion score and *Clostridium perfringens* populations in the ceca of broiler chickens on d 21 of age.

Main effects	Intestinal lesion score[1]	*C. perfringens* counts *(log_{10} cfu/g)*
Methionine source		
DL-methionine	1.04	2.76
HMB[2]	1.19	3.09
SEM	0.174	0.386
Methionine level (%)[3]		
0.27	1.08	2.85
0.43	0.83	2.41
0.59	1.38	3.28
0.75	1.17	3.16
SEM	0.246	0.545
Source of variation (*P* – value)		
Methionine source	0.557	0.553
Methionine level	0.485	0.677
Source x Level	0.437	0.353

[1]According to the criteria described by Dahiya et al. (2005): 0-No gross lesions, normal intestinal appearance; 0.5-Severely congested serosa and mesentery engorged with blood; 1- Thin-walled, friable intestines with small red petechiae (> 5); 2-Focal necrotic intestinal lesions; 3- Sizable patches of necrosis (1 to 2 cm long); 4- Diffused necrosis typical of field cases.

[2]HMB = 2-hydroxy-4-methylthiobutanoic acid.

[3]Standardized ileal digestible methionine

birds fed DLM was higher in infected birds compared to uninfected birds for maximum ADG (6.03 and 5.07 g/kg; $P < 0.05$) while the infected and uninfected HMB fed birds had requirements that were not significantly different than the other two groups (5.30 and 5.39 g/kg; $P > 0.05$: Table 6.3, Figure 6.1). The methionine requirement for optimum FCR was significantly higher in infected birds than in uninfected birds for both the DLM (6.27 vs 5.41 g/kg; $P < 0.05$) and the HMB (6.50 vs 5.94 g/kg; $P < 0.05$) fed birds. Further, the DLM fed birds had significantly lower methionine requirements for optimum FCR than the HMB fed bird in both infected and uninfected situations ($P < 0.05$). The methionine requirements for maximum ADFI were not significantly different between treatment groups ($P > 0.05$).

6.5. Discussion

The methionine requirement for the maximum broiler performance is variable and ranges from 1.8-5.7 g/kg (NRC 1994; Klain et al. 1960; Waldroup et al. 1979). This variability is due to environmental conditions and interaction between methionine and other nutrients. The requirements in the present study were within this range for BW gain, feed intake and feed conversion. The methionine requirement for maximum feed conversion is generally higher that for gain (Schutte and de Jong 1996; Tillman and Pesti 1985) and this agrees with the results of the present study.

Over the last several decades a large body of research has been performed to assess the relative bioefficacy of DLM and HMB. A recent review of this research by Jansman et al. (2003) reported that bioefficacy of HMB relative to DLM ranged from 53-98% in broiler chickens with a mean value of 77% for BW gain and 76% for FCR. In

Table 6.3. The effect of methionine source and *C. perfringens* infection on the quadratic regression coefficients and calculated methionine requirements for ADG, ADFI and FCR in broiler chickens.

		Regression Coefficients[1]					Calculated
Paramet	Treatment	b_0	b_1	b_2	r^2	*P*-value	Requirement
ADG	DLM[z] Uninfected	-36.78	19.94	-1.163	0.61	0.03	5.07*a*
	DLM Infected	-20.61	15.28	-0.802	0.74	0.03	6.03*b*
	HMB[y] Uninfected	-32.71	18.78	-1.067	0.61	0.07	5.30*ab*
	HMB Infected	-42.73	21.59	-1.214	0.65	0.04	5.39*ab*
ADFI[2]	DLM Uninfected	47.71	7.55	-0.464	0.33	0.90	4.64
	DLM Infected	52.78	6.93	-0.376	0.11	0.59	5.72
	HMB Uninfected	-19.78	23.26	-1.327	0.49	0.33	5.26
	HMB Infected	-27.51	26.00	-1.503	0.69	0.06	5.15
FCR	DLM Uninfected	2.50	-0.34	0.032	0.63	0.02	5.41*a*
	DLM Infected	2.46	-0.27	0.022	0.40	0.08	6.27*c*
	HMB Uninfected	1.92	-0.13	0.009	0.61	0.13	5.94*b*
	HMB Infected	1.99	-0.20	0.010	0.37	0.08	6.50*d*

[1]where regression equation is in the form: $y = b_0 + b_1x + b_2x^2$

[z]DLM- DL methionine, [y]HMB - 2-hydroxy-4-methylthiobutanoic acid

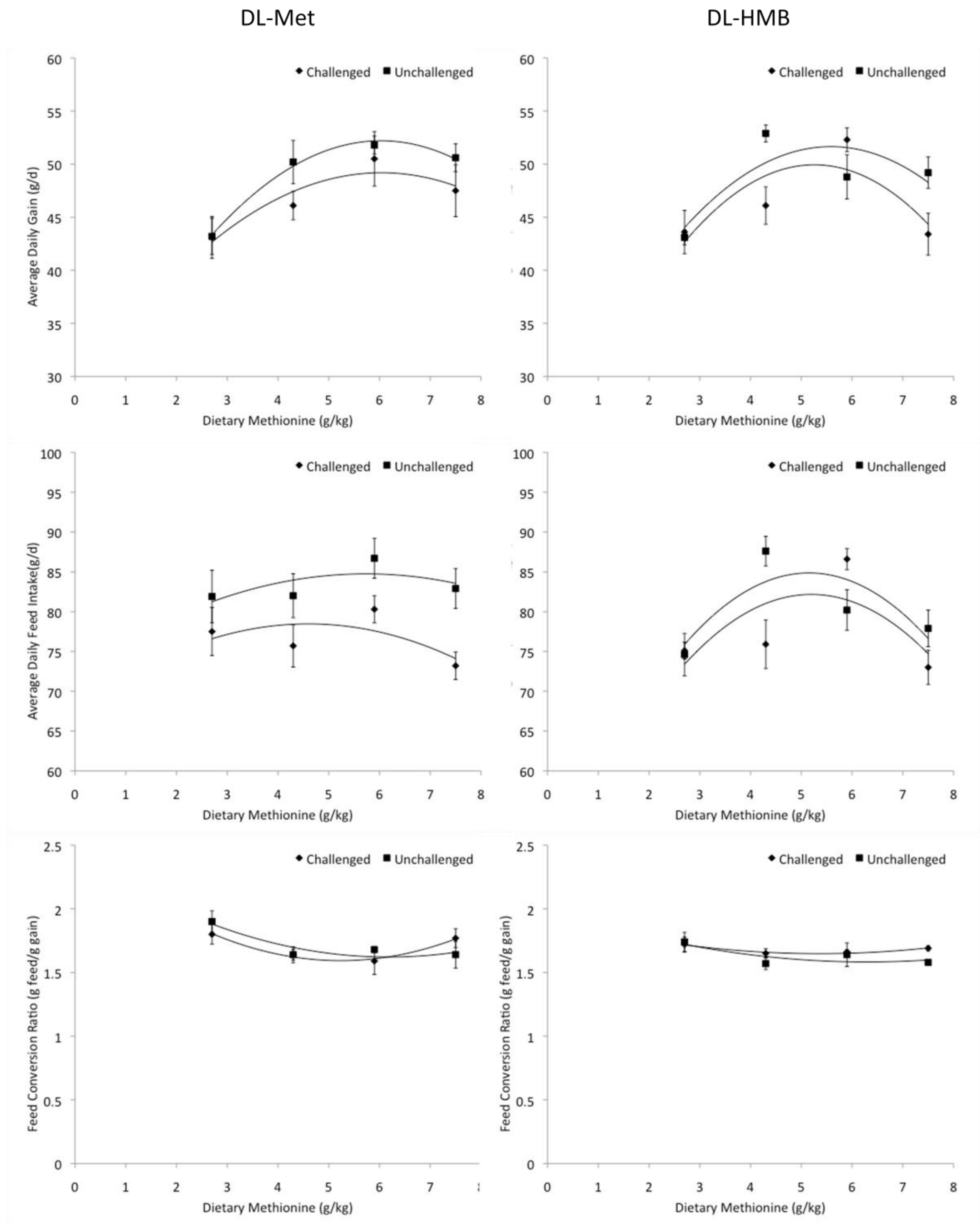


Figure 6.1. The effect of methionine source and *C. perfringens* inoculation on the requirement of broiler chickens for methionine.

the present study, the bioefficacy of HMB relative to DLM was 96% for ADG, 88% for ADFI and 74% for FCR. The high bioefficacy of HMB for ADG and ADFI may be a result of the small number of experimental units.

As previously observed with male (Bunchasak and Keawarun 2006) and female (Matsushita et al. 2007) broiler chickens, the methionine source did not affect the growth rate of uninfected birds in the current study. However, when infected, birds fed DLM needed 11% more methionine for maximum growth than uninfected counterparts. This indicates that *C. perfringens* infection elevates the methionine demand required for maximal growth in broiler chickens fed DLM supplemented diets. The growth performance, pathological observations and *C. perfringens* numbers of challenged birds indicate a subclinical form of NE, as indicated by Lovland and Kaldhusdal (2001). In the subclinical form of NE, nutrient digestion and absorption (including methionine) get impaired due to the damage caused by *C. perfringens* to the intestinal mucosa (Elwinger et al. 1992; Hofshagen and Kaldhusdal 1992; Lovland and Kaldhusdal 1999; Kaldhusdal et al. 2001; Hofacre et al. 2003). In addition, the intestinal epithelium processes absorbed AA prior to entry into the portal circulation, thereby determining the systemic availability of those AA (Brosnan 2003). Accordingly, the damaged intestinal epithelium of *C. perfringens* challenged birds may have affected the absorption and availability of methionine, limiting the absorptive and metabolic capacity, whereas no such limitation on methionine absorption and availability might have taken place at levels used in the present study in unchallenged birds with an intact intestinal epithelium. On the other hand, enteric infections in general increase the thickness of the intestinal wall which in turn reduces nutrient absorption (Thomke and Elwinger 1998). Methionine is usually considered as the first limiting AA in commercial broiler diets.

Therefore, the limited absorption of methionine might have limited the growth of the challenged birds while the growth of the unchallenged birds ascended with the increasing methionine level in the diet. In addition, the epithelial damage caused by the pathogen and associated metabolites elevates the methionine requirement since the healing process involves the use of nutrients to repair the damaged cells (Apajalahti et al. 2004). Moreover, intestinal tissue damage will lead to inflammatory and immune responses to promote the healing of damaged cells and to avoid the entry of enteric pathogen into the host tissues. AA deal with a number of metabolic or cellular responses as obligatory precursors with immune activation, acute phase protein production and the activation of peroxidative defenses (Reeds and Jahoor 2001). Accordingly, reduced absorption as well as enhanced utilization in processes other than production might have increased dietary methionine level required for growth.

However, dietary inclusion of HMB made no significant difference in the methionine requirement for optimum ADG in infected and uninfected birds. Reports exist on the competition between the host and intestinal bacteria for nutrients. Both sources provide L-methionine activity to monogastric species in the same way, but the compounds are chemically different in that the asymmetric alpha carbon has an amino group in DLM and hydroxyl group in HMB (Leeson and Summers 2008). Hence, the chemically different nature of the two methionine sources might have affected bacterial utilization of this nutrient. Evaluating the ability of three bacterial species (*Lactobacillus plantarum, Leuconostoc mesenteroides* and *Lactobacillus casei*) to utilize HMB for growth, Hegedus et al. (1993) reported that none of these bacteria could utilize HMB although they all could use methionine. This suggests a group of limited number of bacterial species may be responsible for the uptake and metabolism of HMB and *C.*

perfringens may not be one of them. In addition, feeding the two methionine sources at levels to meet the TSAA requirement of broilers, Matsushita et al. (2007) reported significantly lower plasma levels of acute phase proteins in birds fed HMB than the levels in birds fed DLM. The authors attributed this observation to the greater anti-inflammatory and anti-stress properties of HMB compared to DLM. This suggests lower utilization of dietary methionine in inflammatory responses of broilers fed HMB saving more for growth. Similarly, Zhang and Guo (2008) reported an increased innate and humoral immunocompetence in broiler chickens fed diets supplemented with HMB. Accordingly, as observed in the current study, *C. perfringens* infection *per se* does not elevate the methionine requirement for maximum growth when supplemented as HMB.

When infected, methionine required for optimal FCR was higher than in uninfected birds. As previously discussed, infections in general increase the nutrient demand by spending nutrients on the pathogen combat process, which otherwise available for the production purposes. In addition, the capacity of nutrient absorption plunges with infections like NE due to the damage caused by *C. perfringens* to the intestinal epithelium (Elwinger et al. 1992; Hofshagen and Kaldhusdal 1992; Lovland and Kaldhusdal 1999; Kaldhusdal et al. 2001; Hofacre et al. 2003). Moreover, previous reports explain how synergisms, antagonisms and vital interactions between nutrition and immunity can influence the growth and production performance of broiler chickens. Some studies observed higher requirements of dietary essential AA than the NRC recommended levels to achieve optimal immunocompetence in broilers (Kidd et al. 2001; Quentin et al. 2005). In fact, the NRC (1994) specifications have generally been created on the requirements of normal healthy birds raised in optimum management conditions, however birds in real industrial settings are usually under constant immune

challenge. Tsiagbe et al. (1987) reported higher requirement of methionine for maximum antibody titers than for growth of broilers. Methionine is considered to be a vital AA in both humoral and cell-mediated immunity (Swain and Johri 2000; Shini et al. 2005) owing to its ability to modulate the synthesis of glutathione, an essential intracellular antioxidant which provide protection against oxidative stress and subsequent inflammatory processes (Le Floc'h et al. 2004; Wu et al. 2004). Orally challenging broilers - fed graded level of methionine - with infectious bursal disease virus, Maroufyan et al. (2010) observed a positive correlation between the methionine level and antibody titer for that specific virus on 7d post challenge. These findings suggest a higher requirement of dietary methionine in broilers under immune challenge.

When the diets were supplemented with HMB, birds needed 6 and 2% more methionine for the optimum FCR than birds fed DLM supplemented diets in uninfected and infected situations, respectively. The chemical difference in the two compounds results in considerable variations *vis-à-vis* mechanism and site of absorption (Knight and Dibner 1984), transport within the body (Lobley et al. 2006; Wester et al. 2006) and metabolism and conversion by the body tissues (Dibner 2003). No explanation is available for the variability in the bioefficacy estimates of HMB relative to DLM, even after extensive research from different laboratories. According to some reports HMB absorption in the avian GIT is lower than DLM absorption (Lingens and Molnar 1996; Maenz and Engele-Schaan 1996a,b; Drew et al. 2003). Moreover, some researchers reported less efficient absorption of HMB oligomers in poultry (Saunderson 1991; Van Weerden et al. 1992). Therefore, the less efficient absorption of HMB may have increased the dietary methionine requirement for optimum protein accretion.

In the present study, methionine supplementation did not suppress the growth of *C. perfringens*, as observed in *in-vitro* (Wilkie 2006) and *in-vivo* (Dahiya et al. 2007b) studies. Conversely, supplementary methionine did not promote the pathogen growth as reported by Muhammed et al. (1975), who demonstrated a stimulatory effect of methionine on the proliferation and sporulation of *C. perfringens.* Instead, the pathogen proliferation was similar in the current study, in the ceca of birds fed unsupplemented control diet and diets with increasing levels of methionine. Nevertheless, compared to the high methionine levels examined in those studies, marginally deficient or excess levels were included in the experimental diets of the current study which may be not enough to exert an effect on the *C. perfringens* growth in the GIT of birds. However, both methionine sources equally supported the pathogen proliferation as observed by Dahiya et al. (2007b). Similar to Dahiya et al. (2007b), the NE-specific intestinal lesion score was not affected by either methionine source or level, but demonstrated a correlation with the pathogen counts.

The results of the present investigation indicate that *C. perfringens* infection increases the methionine requirement for maximal growth in broiler chickens when fed diets supplemented with DLM but not with HMB. The methionine requirement for optimal FCR is higher in infected than in uninfected birds and when the diets were supplemented with HMB, the methionine requirement for optimum FCR is higher than DLM supplemented diets in both uninfected and infected situations. Different beneficial effects of the two methionine sources suggest more favorable responses when the products are being used in combination, so more research in this area is warranted.

7. GENERAL DISCUSSION

Necrotic enteritis is a bacterial infection of poultry caused by some strains of *C. perfringens* and often left untreated in broiler chickens due to complexity in its detection in farm flocks. It can lead to significant economic losses due to high mortality or substandard growth performance in chickens with sub-clinical NE. Currently, the only reliable and successful preventative measure against this disease is the incorporation of antibiotics into broiler feeds. However, with the removal of AGP and coccidiostats from animal feed formulations, outbreaks of NE have drastically gone up, particularly in poultry producing countries in the European Union. This suggests the need for alternative methods of prophylaxis, hence a considerable amount of money and time are being spent throughout the world at present to develop cost-effective alternatives to AGP to combat this disease. Nevertheless, the antibiotic-free approaches developed so far to control *C. perfringens* in poultry are less than satisfactory. Much attention has paid on dietary and management practices in developing non-antibiotic approaches to control NE in broiler chickens.

The necessity of a well controlled and repeatable disease model for NE has ever been there, because no generally accepted robust and reproducible disease model has been established so far to induce the disease experimentally to investigate potential non-antibiotic approaches. Numerous efforts made to reproduce NE experimentally in different labs has resulted in markedly inconsistent results. Almost all current models depend on a multiorganism challenge using different *Eimeria* species in combination with *C. perfringens*, for the experimental induction of NE. The model used in this project was developed to be used in birds raised on the floor (litter) and tested with experimental as well as commercial type of diets comparing different challenging modes

without a concurrent coccidial infection. A more realistic approach was employed to mimic the normal way of infection, but to what degree this model would be successful in inducing the infection in a commercial setup is still uncertain. The implementation of this model in subsequent experiments did not elicit the clinical disease as seen in field outbreaks, but was able to produce sub-clinical version of NE in birds, ensuring very effective intestinal colonization of the pathogen. In addition, this model does not devastate the intestine of birds with coccidial pathogens interfering macroscopic and microscopic pathological evaluation and other findings. The absence of coccidial co-infection makes it easier in employing this method and in result interpretation. However, a microscopic lesion evaluation in the intestinal tissues was not performed but could have been done to enhance the validity of the challenge model.

The primary source of nutrients available to intestinal bacteria is that derived from the indigestible portion of feed. Thus, an alteration in intestinal microbial ecosystem can be expected with a modification in indigestible nutrients in the diet. *C. perfringens* is incapable of synthesizing not less than 13 out of 20 indispensable AA, hence heavily depends on proteins derived from the diet. The overwhelming impact of dietary protein level, protein digestibility and AA composition has on the occurrence and intensity of NE by triggering the *C. perfringens* proliferation and/or α toxin production and secretion is well documented in the literature. This indicates that one of the most efficient ways to control this pathogen without in-feed antibiotics is through appropriate feed formulation, particularly manipulating the protein level, quality (in terms of digestibility) and AA profile. In this context, use of *LoPro* diets can be speculated as a promising nutritional approach to control the proliferation of *C. perfringens* in broiler chickens, in the absence of AGP. However, based on available information the growth

and carcass performance seems to be impaired when broilers are fed *LoPro* diets even if formulated to meet all known nutrient requirements. Accordingly, one of the experiments (Chapter 3) conducted under this project was designed to evaluate the growth and production performance of broilers fed diets containing low and high ileal digestible protein levels, formulated with highly and poorly digestible protein supplements but equal amounts of nutrients. Based on the results of that experiment, the birds fed *LoPro* diets demonstrated inferior performance only when poorly digestible protein supplements were included in the diet. Birds fed *LoPro* diets with highly digestible protein supplements demonstrated equal performance as in birds fed *HiPro* diets. Hence, the findings of this experiment revealed that the negative effects of *LoPro* diets could be overcome with the inclusion of highly digestible protein supplements in broiler ration formulation. Nevertheless, this feeding strategy still has some limitations from the production point of view. Feeding *LoPro* diets is not an appropriate practice if maximal breast meat yield is the key intent of broiler production. In addition, compared to poorly digestible protein supplements, incorporation of highly digestible protein supplements into isoenergetic diets elevates the energy: protein ratio of the diet which increases the carcass fat accumulation. The precise energy: protein ratio for the optimum carcass quality could have been evaluated formulating diets with a series of ME levels, but higher numbers of dietary treatments, animals and experimental units in the same trial would have made the experiment more complicated and practically more difficult to deal with. Hence, more research in this area is warranted.

The study conducted to investigate the effect of dietary protein level and digestibility on *C. perfringens* proliferation and subsequent growth performance of broiler chickens (Chapter 5) demonstrated that the pathogen proliferation and associated

growth suppression of birds fed *LoPro* diets could be minimized with the inclusion of highly digestible protein supplements in such diets. In addition, this investigation revealed the ability of that particular dietary approach to create a host friendly intestinal ecosystem, with the formation of beneficial VFA and less ammonia. Nevertheless, one of the crucial constraints in this type of experiment is the inability to use the same set of ingredients to formulate the diets with different protein and digestibility levels, maintaining the same nutrient specifications. This makes it difficult to distinguish the nutrient effects from ingredient effects. In addition, each diet was made as one batch to be used in the respective experiments and stored in a cool room facility until being used in each experiment. However, the loss of nutritional characteristics of some ingredients, vitamins for instance, with time is inevitable, even if stored properly. Accordingly, the diets fed in the last experiment might nutritionally be somewhat different from those fed in the first experiment. In the study described in Chapter 3, the execution of *C. perfringens* challenged and unchallenged experiments at separate times in the same room ruled out the environment effect, but brought some confounding factors in. One such factor was the birds in the two groups might not receive exactly the same dietary treatments, as described previously. In addition, birds used in the two experiments were received at two different times from two different progenies with significantly different chick weights. Therefore, chick quality in the two groups might not be the same, which is considered as one of the critical factors that determines the subsequent growth performance of broiler chickens. In this scenario, the significantly higher chick weight of birds to be challenged apparently resulted in superior growth performance in that group up to the point of challenging, which in turn reduced the impact of the challenge, giving only numerically different final BW compared to the unchallenged counterparts.

The study conducted to evaluate methionine requirement for optimum growth performance in broiler chickens fed diets supplemented with DLM or HMB under *C. perfringens* infected and uninfected situations (Chapter 6) revealed that *C. perfringens* infection increases the dietary methionine requirement for maximal growth only when supplemented with DLM but not with HMB. However, different chemical nature and associated differences in absorption, metabolism and transport suggest different beneficial effects of the two compounds. Accordingly, the potential additive effect of the two methionine sources in combination would be more advantageous, so further research in this area would definitely add some interesting information to the current study.

The use of antibiotics in livestock industry is still in debate. However, these studies suggest that diet formulation, and ingredient quality can be manipulated to reduce the impact of *C. perfringens* on broiler production. Nevertheless, complete elimination of dietary predisposing factors remains a substantial challenge in broiler production. Further research investigating the effects of not only dietary protein, but also of other dietary components such as starch, fibre, lipid and minerals on *C. perfringens* are required.

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