Necrotic Enteritis, disease induction, predisposing factors and novel biochemical markers in broiler chickens

BY

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A thesis is submitted for the degree of Doctor of Philosophy, at the College of Medical, Veterinary and Life Sciences, University of Glasgow

Research conducted at the Avian Science Research Centre
Scottish Agricultural College, Ayr

November 2012

ABSTRACT

Necrotic enteritis (NE) is an important enteric disease in poultry production that has re-emerged as a major problem following an EU wide ban on the use of in-feed antimicrobials. Although the primary aetiological agent of disease is *Clostridium perfringens* type A, a commensal in the gastrointestinal tract (GIT) of chickens, numerous additional influential factors have been reported that can predispose chickens to NE. These precipitating factors mainly include diet, co-infection with other pathogens particularly coccidia, as well as environmental and management factors. Despite being first described almost more than 50 years ago, a reliable, consistently reproducible experimental model for NE induction is still lacking. Here, a series of experiments were conducted to investigate the importance of the various potential predisposing factors, in isolation and in combination, that are believed to play a role in sub-clinical NE development: feed withdrawal, dietary protein sources, co- infection with coccidia, *C. perfringens* dose and contact with reused litter. In addition, chicken breed sensitivity to NE was assessed, and last, but not least, blood and gut tissue samples were used to identify novel biochemical markers for sub-clinical NE.

Chapter 3 reports an experiment showing that feed withdrawal up to 24 hrs in experimentally challenged birds did not result in NE specific lesions. Chapter 4 shows that replacing dietary soyabean meal with potato protein concentrate or canola meal or adding synthetic trypsin inhibitor to the soyabean meal control diet did not induce sub-clinical NE in birds housed on reused litter, a natural source of *C. perfringens* challenge. Chapter 5 describes that *in vitro* growth of *C. perfringens* on *in vitro* digested grower diets was prolonged following the addition of fishmeal, suggesting that the role of fish meal as a predisposing factor for *in vivo* sub-clinical NE cannot be excluded. All subsequent diets therefore contained high levels of fish meal. When this was used in combination with high dose of coccidial vaccine, a repeated in-feed challenge for three days at 10^2 colony forming units (cfu) *C. perfringens* per g feed did not result in sub-clinical NE, though at 10^9 cfu/g resulted in 10% of challenged birds (3 out of 30) showing NE-specific lesions (Chapter 6). Further study is needed to determine if the two Ross birds with gross NE lesions compared to the one Hubbard bird (out of 15 birds each) was due to a lower level of NE resistance.

The failure to significantly induce sub-clinical NE in the previous experiments suggests that challenging the birds with *C. perfringens* in the isolated presence of suspected predisposing factors may not provide a suitable experimental model. Indeed, when birds were dosed twice daily with 10^8 cfu *C. perfringens* for three days in the presence of high levels of fishmeal, canola meal as main protein source, coccidial and IBD vaccinations, and feed withdrawal prior to challenge, 40.6% of the challenged birds developed lesions of sub-clinical NE without inducing mortality (Chapter 7). This concurred with reduced growth performance relative to the sham-infected control birds, and thus is a successful model for induction of sub-clinical NE.

Finally this work has, for the first time provided novel information on potential biomarkers (Chapter 8). Whilst challenge did not impact on the expression of genes previously shown to be differentially expressed upon *C. perfringens* toxin exposure, the serum ceruloplasmin concentration increased, suggesting that monitoring this acute phase protein may indicate the presence of *C. perfringens* infection in poultry. However, as such markers generally lack specificity, further research confirming its role in response to sub-clinical NE is needed to provide a fully effective diagnostic and prognostic marker for flock health and welfare, as well as ultimately helping to gain better understanding of the pathophysiology of sub-clinical NE.

Improved knowledge of the effect of different dietary components on the growth of C. *perfringens* may help in the formulation of broiler diets to assist in further reducing the incidence of NE particularly in the absence of antimicrobial growth promoters. It is hoped that host responses in terms of acute phase proteins, and possibley gene expression, will also provide greater insight into the pathogenesis of NE. Provided that the developed experimental sub-clinical NE model is reproducible, this will benefit the understanding of this billion dollar disease and enable further investigation of various chemical and non-chemical interventions to reduce its severity and impact on poultry production.

DECLARATION

This thesis has been composed by myself and is a record of work carried out in an original line of research, except where due reference is made to the contribution of others. All such sources of information are again listed in the reference section. All help provided during my work is sincerely appreciated and appropriately acknowledged.

None of the work has been presented in any previous degree application.

Gulbeena Saleem

November, 2012

PUBLICATION

Part of the work reported in this thesis has been communicated in different conference meetings:

Saleem G., N. Sparks, J. Houdijk and V. Pirgozliev (2012) Interactive effects of diet composition and litter quality on growth performance and incidence of sub-clinical necrotic enteritis in broiler chickens British Poultry Abstracts pp 7, Vol 8, Number 1, August, 2012.

Saleem G., N. Sparks, J. Houdijk and V. Pirgozliev (2012) Effect of canola meal and litter condition on gut health. British Poultry Abstracts pp 18, Vol 8, Number 1, August, 2012.

Saleem G., N. Sparks, J. Houdijk and V. Pirgozliev (2011) Experimental induction of sub-clinical necrotic enteritis in broiler chickens. British Poultry Abstracts pp 58, Vol 7, Number 1, August, 2012.

Saleem G., N. Sparks, J. Houdijk and V. Pirgozliev (2011) Proliferation of *Clostridium perfringens* on *in vitro* digested poultry feed with and without fish meal Proceedings of 18th European Symposium on Poultry Nutrition, pp. 778-780, Turkey.

Saleem G., N. Sparks., J. Houdijk., T. Acamovic and V. Pirgozliev (2011) Does feed withdrawal assist experimental induction of sub-clinical necrotic enteritis in broiler chickens? British Poultry Abstracts pp 4, Vol 7, Number 1, August, 2011.

Publication that does not form an integral part of the thesis *per se*, but is related to the main subject of the thesis is mentioned below as evidence of additional work:

Saleem G., N. Sparks, V. Pirgozliev, T. Acamovic, J. Houdijk, J. Wiseman, A. Peron, J. Snape (2010) “Effect of wheat cultivar, type and xylanase supplementation on growth performance and gut microflora of broilers” Book of Abstracts, XIIIth European Poultry Conference, pp 375.

AWARDS

During my PhD studies, I have received the following awards and travel grants:

- Travel grant to present an oral paper at 18th European Poultry Conference, 2011 Izmir Turkey.

- Highly commended in British Council Shine International Student Awards.

- Represented SAC at Science for Life Inter-institute Competition (2011).

- Internal SAC award for best presentation at SAC post- graduate conference, Edinburgh (2011).

- Invitation, by the organizing committee, to attend EPC 2010 in Tours, France as well as to participate in the Youth Programme of conference.

- Overseas research student Award (ORSAS), University of Glasgow, Scotland, UK.

ACKNOWLEDGEMENT

All praises and thanks to Almighty Allah and beloved prophet Hazrat Muhammad (PBUH).

I wish to acknowledge many people who have made this thesis an achievable reality. Dr Jos. Houdijk for his precious supervision, endless support, kind understanding and moral encouragement. I am grateful for the opportunity both to learn how to improve my scientific writing and presentation skills. His contributions and advice to my thesis have been invaluable.

Professor Dr. Nicholas Sparks for his endless patience and understanding with the numerous problems encountered throughout my research. I am truly grateful for his support, guidance and insight during my tenure at SAC's Avian Science Research Centre (ASRC). Professor Dr. David Eckersall for all the support provided during acute phase protein analysis and for assistance with Glasgow University matters.

The late Dr. Tom Acamovic was a naturally joyful human being whose contact with others extended his joy to their lives. He is surely missed as a joyful, supportive and ultimate professional human being.

Dr. Spiridoula Athanasiadou for all the help and kind guidance with gene work both practical and theoretical, that enabled me to do gene expression analysis. Dr. Tombermont from University of Ghent for kindly providing the strain 56 of *C. perfringens* without which this research would not have been possible. Dr. Tom Pennycott for the knowledge and expertise he shared with me on post-mortem and histopathological examination.

I am greatly acknowledged to Higher Education Commission Pakistan for the PhD Scholarship.

I am truly grateful to Dr. Oluyinka Olukosi for all the help, kindness and technical support throughout these years. I would like to thank all the members of ASRC, Dr. Victoria Sandilands, Dr. Visil Pirgozliev and Dr. Sarah Brocklehurst for their friendly

advice and invaluable assistance. Dr. Farina Malik Khattak for being an excellent friend and colleague, who encouraged and enabled my survival during the initial days of PhD and settling in UK. To technical support staff at the Microbiology Laboratory and Veterinary Services; Libby Deans, Fraser White and Emily O'Reilly for the support provided during sample analysis. To fellow graduate students of ASRC, Waseem Mirza, Laura Beeson, Rita, Adekunle Adebiyi, Nanung Dono, Naser Asheen, and all in post-graduate office, who helped me in many different ways throughout the years.

I would like to express my sincere gratitude to my parents, Muhammad Saleem and Majeeda Saleem: for their unconditional love. I cannot express the depth of my love and gratitude for their nurturing and belief in me and for teaching me the importance of learning and for making me who I am.

My deepest and cardinal thanks goes to my sister, Samina Aslam for her love, care and being with me through the thick and thin of my life. None of these would have been possible had it not been for the guidance and inspiration of my sister Samina Aslam. Heartfelt thanks to my brothers, Muhammad Tariq Saleem, Muhammad Babar Saleem and my sister Rizwana who all pray for me everyday, and to my mother-in-law and sisters-in-law for extending their support and praying for me and my family. A lovely thank to Suaad Walker for her peaceful company.

Last and most importantly, I owe much to my husband, Jawwad Shabbir Yousafi, for his patience, support and encouragement in the long journey of achieving this degree. To my special angels: my son, Abdullah Jawwad Yousafi and my lovely daughter Nabiha Fatima Yousafi for their innocent understanding and love. It is their love that makes my life enjoyable and worth living. I owe you big time! To my whole family again, thank you for being such magnificent people. I love you all!!

Alhamdulillah for everything in these thesis years!

LIST OF ABBREVIATION

AFLP	Amplified fragment length polymorphism
AGPs	Antimicrobial growth promoter
APP	Acute phase proteins
APR	Acute phase reaction
BHI	Brain heart infusion agar
BW	Body Weight
Cp	Ceruloplasmin
CFU	Colony forming unit
CM	Canola meal
C. perfringens	*Clostridium perfringens*
CFU	Colony forming unit
CM	Canola meal
CNS	Central nervous system
CPH	*C. perfringens* associated hepatitis
DGGE	Denaturing gradient gel electrolysis
E. Coli	*Eschericia coli*
ELISA	Enzyme Linked Immunosorbent Assay
FCR	Feed Conversion Ratio
FI	Feed intake
GIT	Gastro-intestinal tract
GOI	Gene of interest
Hrs	Hours
IBD	Infectious bursal disease
MHC	Major Histocompatibility complex
MRD	Maximum Recovery Diluent
NE	Necrotic entertitis
OD	Optical density
OVT	Ovotransferrin
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFGE	Pulsed- field gel electrophoresis
PPC	Potato Protein concentrate
PPD	p-phenylenediamine
qPCR	Quantitative Polymerase chain reaction
RCA	Reinforced Clostridial agar
SBM	Soybean meal
SFPA	Shahidi Ferguson *Perfringens* agar
TG	Thioglycollate
TI	Trypsin inhibitor
TIA	Trypsin inhibitor activity
TSC	Tryptose Sulphite Cycloserine agar
WG	Weight gain

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1 GENERAL INTRODUCTION

Necrotic enteritis (NE) remains a major problem in the modern poultry industry, although in the past, it has been controlled by in-feed microbials, and ionophore anti-coccidials (Collier *et al.*, 2003). However it has re-emerged as a significant problem, causing reduced growth performance together with increased feed costs, following an EU wide ban on in-feed growth promoters. The sub-clinical form of NE is more disastrous since it can be more pervasive within the flock and mostly goes un-noticed and therefore undetected because of absence of evident clinical signs and/or symptoms (Kaldhusdal *et al.*, 2001; Hofacre *et al.*, 2003; Skinner *et al.*, 2010; Timbermont *et al.*, 2009a). This usually results in condemnation of carcasses at time of slaughter (Kaldhusdal & Hofshagen, 1992). Although it is becoming progressively more apparent, the economic impact of sub-clinical NE has not been formally investigated (Skinner *et al.*, 2010). Overall the main feature of the disease is the occurrence of necrotic lesions in the small intestine mostly in the jejunum and ileum but occasionally in the duodenum (Alsheikhly & Truscott, 1977b; Ficken & Wages, 1997). Decreased digestion and absorption, reduced weight gain and an increased feed conversion ratio are a direct result of the damage to intestinal mucosa caused by the sub-clinical form of the disease (Kaldhusdal *et al.*, 2001). Poultry farms cannot afford to ignore the economic losses caused by this disease, despite its sporadic nature in developing countries as it is still causing large scale outbreaks in chicken production units. However, the total impact of fully developed NE on chicken production is difficult to determine accurately due to the nature of sub-clinical NE.

The primary causative organism is a gram positive, anaerobic, spore forming bacterium, *Clostridium perfringens*, a commensal in the gastrointestinal tract (GIT) of poultry (Ficken & Wages, 1997) that has been isolated from feed, litter, dust, and faeces (Wages & Opengart, 2003). Despite identification of numerous factors that promote the development of sub-clinical NE, the exact field conditions that precipitate the out breaks of NE are still ambiguous. Together with a degree of co-infection with *Eimeria*, the predisposing factors are mainly dietary in nature. Currently, the only way to assess the degree of host response is by scoring gross pathological lesions within the intestine.

Under experimental conditions it has proved difficult to induce sub-clinical NE, although *C. perfringens* has been identified as the disease's primary etiological agent.

There is currently some understanding of the disease's progression, but is still unclear as to the predisposing factor(s) that lead to the induction of NE following an overgrowth of *C. perfringens* in the GIT. Contradictory results from experiments have left the numerous predisposing factors still ill defined (Kaldhusdal *et al.*, 1999). It is therefore essential to develop a reproducible experimental model for induction of sub-clinical NE in order to achieve more effective control of this costly disease as financial cost of NE has been estimated to be £1.6 billion per year to the world's poultry industry (Van der Sluis, 2000). This will enable control of strategies and exploration of new methods to allow testing of a variety of factors, such as feed additives, vaccines and a range of new approaches. The focus of this thesis is:

1) To clarify the role of the different, conflicting predisposing factors, in order to develop a model for induction of sub-clinical NE. The predisposing factors being considered are: feed withdrawal, diet, contact with reused litter, genotype of chickens and co-infection with other pathogens in isolation and/ or in combination as well as testing breed sensitivity to disease induction.

2) To identify novel biochemical markers for sub-clinical NE in broiler chickens. It is anticipated that such additional markers will help to identify the presence of sub-clinical NE, and to increase our understanding of the nature of host responses to *C. perfringens* infection.

The major focus of the work described in this thesis is to prioritize the risk factors that purportedly expose birds to an increased risk of sub-clinical NE whilst also contributing to an advance in new preventative strategies to enable control of this billion dollar disease in a post anti-biotic era. This will be through developing a model for induction of sub-clinical NE based on an improved understanding of the involvement and relevance of the many anecdotal and research identified potential risk factors for NE both in isolation and/or in combination.

2 LITERATURE REVIEW

Necrotic enteritis (NE) is an enteric disease caused by overgrowth of a commensal *C. perfringens.* This overgrowth also results in changes not only of the digestive tract environment, but also its bacterial ecology and histopathology. In order to adequately understand (sub-clinical) NE itself, it is therefore essential to briefly review the anatomy of a chicken's digestive tract, its histomorphology and microbial ecology.

2.1 STRUCTURE AND FUNCTION OF THE GASTROINTESTINAL TRACT OF POULTRY

The GIT acts as a gateway for various nutrients to gain access to the circulatory system. The major function of the digestive system is to assimilate nutrients that are required for maintenance, growth, and reproduction of the organism. Intake of feed is accompanied by quick development of the GIT and its associated organs (Uni *et al.*, 1998). Early development of the digestive tract is crucial for achieving better growth and performance in chickens (Tako *et al.*, 2004).

2.2 Different components of Gastrointestinal Tract:

The anatomy of the chicken GIT is unique. Main components of digestive system of birds are beak, mouth, oesophagus, crop, proventriculus or glandular stomach, Gizzard or muscular stomach, small intestine and large intestine (North & Bell, 1990). The general structure of the digestive tract of fowl is illustrated in Figure 2.1.

2.2.1 Mouth and Oesophagus

Chickens have no lips, soft palate, cheeks or teeth, but there are an upper and lower horny mandible referred to as beak (also known as rostrum) to enclose the mouth (Turk, 1982). The oesophagus starts from mouth along the neck enters thoracic cavity and into the glandular stomach (Bradley & Grahame, 1960). The oesophagus acts as passage for food from the mouth to the glandular stomach.

2.2.2 Crop

The crop is a thin-walled storage pouch on the ventral end of the oesophagus (Hill, 1976). While the crop is well known for its storage function, along with some bacterial fermentation (Turk, 1982). Although the crop is a non-secretary organ, it contains enzymes and secretions from saliva, feed and microbes. Bacterial fermentation in the crop results in lactic acid production (Fuller & Brooker, 1974).

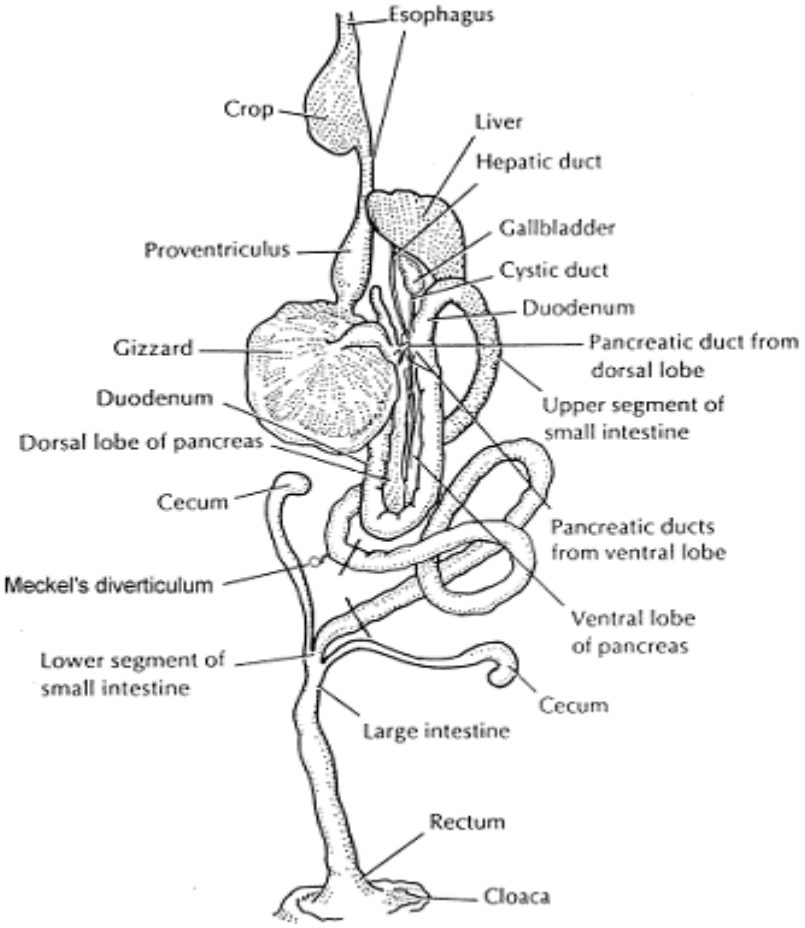

Figure 2.1: The general structure of Chickens digestive tract.
(Sturkie, 1976).

2.2.3 Proventriculus

The crop enters the proventriculus, sometimes called the “glandular stomach” or “true stomach”, that is lined with a glandular mucus membrane. Gastric juices like pepsin (the enzyme for protein digestion) and hydrochloric acid are secreted by

glandular cells of the proventriculus. Ingested feed passes quickly through the proventriculus so there is little digestion of ingested particles here. However, most digestive enzyme action takes place in the next portion of the GIT, "the gizzard", (North & Bell, 1990).

2.2.4 Gizzard (Muscular stomach or ventriculus)

The gizzard lies between the proventriculus and the upper limit of the small intestine (duodenum). In the gizzard larger food particles are ground and reduced to small particles capable of being taken into the intestinal tract for further digestion and absorption (North & Bell, 1990).

2.2.5 Small intestine

The small intestine starts from the posterior end of the gizzard and ends at the ileo-caecal junction. The small intestine is about 62 inches long in the average adult chicken and is divided into the duodenum, jejunum, and ileum. The duodenum is the proximal portion of the intestine, extending from the gizzard to the pancreatic and biliary ducts, and encloses the pancreas to form a U-shaped structure also known as the "duodenal loop". The duodenum is the principal site of nutrient absorption in the chicken (Duke, 1986). Next to the duodenum lies the jejunum, the segment extending from the pancreatic ducts to the Meckel's diverticulum or yolk sac diverticulum (sometimes also known as the yolk stalk). Meckel's diverticulum is used as a land mark to separate the jejunum and ileum (Denbow, 2000). The ileum extends from Meckel's diverticulum to the ileo-caecal junction. Quantitatively, most digestion and absorption takes place in the small intestine (Turk, 1982), where chemical action by pancreatic enzymes aids digestion, also helped by bile, intestinal secretions and various microbes harbouring gut lumen (Darel and Duke, 2004).

The small intestine of newly hatched chickens is relatively immature and undergoes significant morphological development during the first few days of life (Uni & Argov, 2006). The intestine of a newly hatched chicken increases in weight more rapidly than its body mass during the first 10 days (Noy & Sklan, 1999) with the highest growth rate at 5-7 days of age (Dibner *et al.*, 1996; Uni *et al.*, 1999; Uni & Argov, 2006). A two to four fold increase in intestinal length has been observed up to 12 days

of age, while the weight of three intestinal segments (duodenum jejunum and ileum) increased seven to ten fold

2.2.6 Caeca

At the junction of the small and large intestine there are two blind pouches known as caeca (Denbow, 2000). Each caecum is about 6 inches long, it is the largest part of the large intestine and is the main site of bacterial fermentation in poultry. Caeca are also assumed to have a role in the mixing of digesta and absorption of nutrients as well as fermentation (Ewing, 2008). The majority of obligatory anaerobes such as *clostridia* are present in the caeca (Barnes *et al.*, 1972).

2.2.7 Large Intestine or Colon

The large intestine is relatively short in the chicken, being only 4 inches. It extends from the end of the small intestine to the cloaca. The main function of the large intestine is water re-absorption (North & Bell, 1990).

2.2.8 Cloaca and Accessary organs

At the end of the digestive tract of birds there is the cloaca that serves as a common pathway for excretory, reproductive and digestive wastes (Denbow, 2000). The Bursa of Fabricious, one of the main lymphoid organs in poultry is located in the cloaca.

Certain organs, such as the pancreas, liver and gall bladder are directly associated with digestion as their secretions empty into the intestinal tract and aid the digestion of ingested food material (North & Bell, 1990).

Digestion consists of a number of physical and chemical processes. Feed is ingested, broken down into smaller particles, acted upon by digestive secretions, and propelled through the GIT by the muscular activities of the tract. Salivary, gastric, pancreatic, biliary, and intestinal secretions collectively provide mucus for protection and lubrication of the tract. Enzymes from the GIT and its associated organs aid in the hydrolysis of carbohydrates, protein, and lipids into smaller compounds for absorption.

Commensal microflora ferment and break down certain complex carbohydrates that are not digested by endogenous enzymes, thus providing additional nutrients. The microflora of the GIT also synthesizes some amino acids and vitamins essential to the host animal. Bacterial enzymes not only help in digestion of carbohydrates but also encourage digestion of protein and lipids that ultimately contribute to the nutrition of the host (North & Bell, 1990).

2.3 HISTOMORPHOLOGY OF THE SMALL INTESTINE

The whole GIT consists of four layers or tunics as does the intestinal wall. As such, the intestine is a multi-layered tube containing a serosal layer, a longitudinal muscular layer, a sub mucosal layer and a mucosal layer (Turk, 1982; Denbow, 2000).

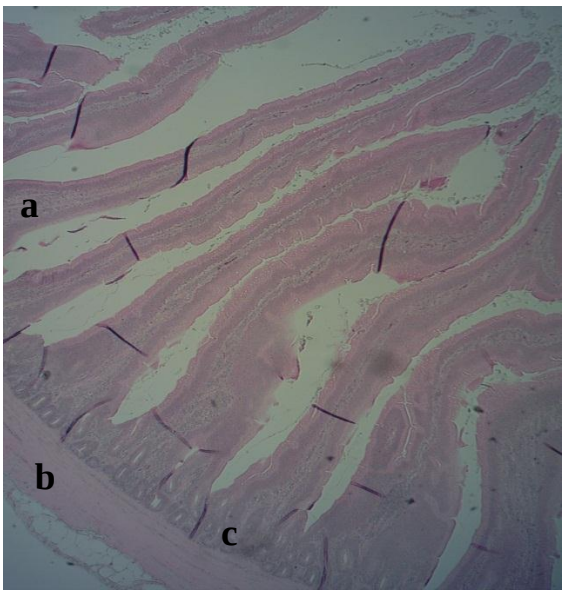

Figure 2.2: Photomicrograph of duodenum showing different layers. (a) Villi (b) Muscular layer (c) crypt. Haematoxylin and Eosin (x4).

2.3.1 Intestinal Mucosa

The mucosa of the GIT is a functional boundary between the environment and the internal physiological compartments of any organism. Intestinal mucosa consist of three layers: epithelium, lamina propria and a muscular layer (Denbow, 2000). The epithelium of the intestine is not only a highly specialized site for absorption but it also provides a good environment for various microbes harboured in the gut (Figure 2.3). Absorption takes place principally through the mucosa of the small intestine. Within the

mucosa the main site of nutrient digestion and absorption is the epithelial layer (Turk, 1982).

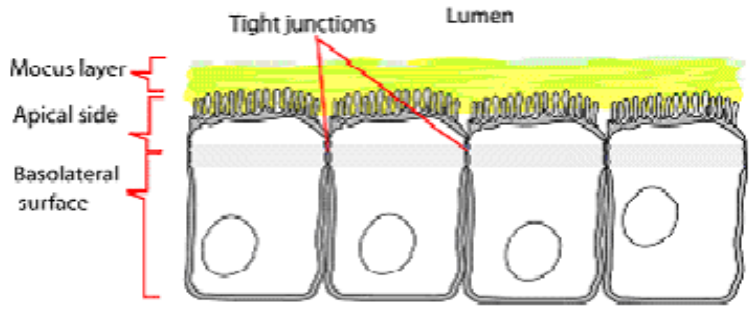

Figure 2.3 : Epithelial layer of intestine (columnar epithelium).

The interior surface of an avian intestine lacks the macroscopic mucous membrane folds that are usually seen in large mammals although it is folded into numerous structures called villi (Figure 2.2; Turk, 1982). Villi increase in size and number as progress down to duodenum giving them a greater absorptive surface per unit of intestine. The duration of villous growth varies: duodenal villous growth is almost complete by day 7, although jejunum and ileum villi development continues beyond 14 days of age (Uni *et al.*, 1995; Uni *et al.*, 1999). Villi are present throughout the length of both the small and large intestines, but length is not consistent between the small and large intestines. There is a steady decrease in the height along the way, being longer in the duodenum at 1.5mm but decreasing in length to 0.4-0.6 mm in the ileum and rectum (Denbow, 2000). Over time, the number of villi increases in both the duodenum and jejunum but not in the ileum (Geyra *et al.*, 2001a).

Between the villi are the Crypts of Lieberkhuhn (Figure 2.2), in which there is a continuous renewal of cell populations called stem cells (crypt cells). These cells have a life cycle of 48 to 96 hours under normal conditions (Uni *et al.*, 1998). Stem cells proliferate by mitosis and then migrate to the top of the villi (Turk, 1982). During this migration process, the enterocytes (intestinal epithelial cells) differentiate into principal (absorptive), or goblet (secretory) cells. Upon reaching the tip of the villus these cells are sloughed into the lumen of the intestine. In this way epithelial cells are continuously replaced by new epithelial cells (Turk, 1982; Uni & Argov, 2006). The crypts contain the undifferentiated cells, goblet cells, endocrine cells and lymphocytes. Paneth's cells and globular leukocytes are present at the base of the crypts (Denbow, 2000). Enterocytes migrate from the crypt up the villi until they are exfoliated into the lumen and are sloughed into the lumen from the villi tip (Turk, 1982). An increase in the total

number of enterocytes is observed with age, due to a dramatic increase in villi length (Uni *et al.*, 1998).

The surface of the villi is covered by enterocytes that have tiny cylindrical projections on their luminal borders. These microvilli further increase the surface area available for absorption. In the inner wall of the villi, just underneath the epithelial cells, is the lamina propria. The lamina propria consists of connective tissue, capillaries, smooth muscle, and nerve fibres. The capillaries bring the blood stream to the base of the epithelial cells so that only one cell layer separates the lumen of the intestine from the blood (Turk, 1982). This facilitates the absorption of nutrients from the lumen of the intestine and their release into the blood stream.

Goblet cells are highly exocrine cells that produce acidic and neutral mucins in similar proportions. There are many goblet cells present between the columnar cells of the surface epithelium and in the superficial parts of the simple glands (Figure 2.4). The goblet cells are scattered over the surface of villi interspersed with absorptive cells. Goblet cells are distributed along the villi (Uni *et al.*, 2003). Goblet cells are short lived and continuously undergo replacement (Uni *et al.*, 2003). They are produced from pluripotent stem cells at the base of the crypt from where they migrate towards the villi tip where they are sloughed off into the lumen. This process takes 2-3 days (Geyra *et al.*, 2001b), and the number of goblet cells increases with chicken age in all regions of the intestine.

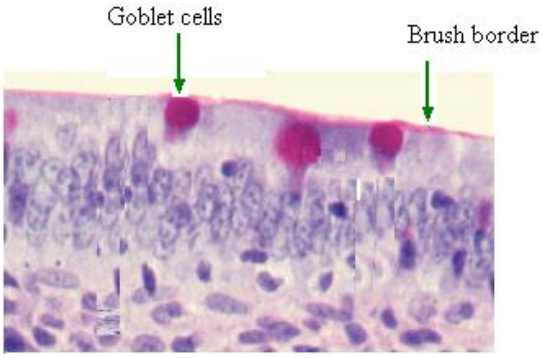

Figure 2.4 : Goblet cells.

The goblet cells synthesize and secrete a mucopolysaccharide called mucin (Turk, 1982). Upon secretion, the mucin hydrates and gels, which generates a protective

mucus layer over the epithelial surface. This layer undergoes continuous degradation and renewal. Other components that reside within the mucus layer include water, electrolytes, sloughed epithelial cells and secreted immunoglobins. This produces a physical and chemical barrier that protects the epithelium from luminal agents such as enteric bacteria, bacterial and environmental toxins and some dietary components that pose a threat to the mucosa (Uni, 2006). The mucus layer also plays a crucial role in nutrient transport between the lumen and brush border membrane (Uni *et al.*, 2003).

It has long been believed that mucin, which is the major component of the mucus layer, has a function that is largely associated with lubrication of bolus movement (Moran, 1985). This mucin layer is also believed to be capable of aggregating several bacterial species and modulating their adherence to the intestinal epithelium, and thus plays an important role in the maintenance of normal microflora. Additional protective factors provided by the mucin are accumulation of bactericidal and bacteriostatic compounds, and secretary immunoglobulin A, all compounds capable of neutralizing or killing toxic bacteria (Robert *et al*., 1991; Thompson & Applegate, 2005). However, mucin also serves a protective function by discouraging the translocation of harmful micro organisms, binding chemical irritants, establishing the unstirred water layer, protecting the underlying epithelial cells, providing a medium for the colonization of favourable microflora, and interacting with the intestinal immune system (Ferket *et al.*, 2005; Thompson & Applegate, 2005). Thus, it is generally agreed that the mucin layer is an important factor in maintaining a strong intestinal barrier against pathogen invasion (Thompson and Applegate, 2005).

Early functionality of the GIT is vital for the growth performance of chickens, making it crucial to achieve optimal intestinal development and functional capacity. The small intestine of a newly hatched chicken is immature and undergoes dramatic changes during the first few days post-hatch. The extensive changes in the morphological development of the small intestine close to immediate-post-hatch includes the differentiation of enterocytes and the definition of crypts as well as the multi- fold enlargement of the intestinal absorptive surface (Uni & Argov, 2006).

Most of the digestion in a bird’s intestinal tract occurs in the lumen of the intestine under the influence of the digestive enzymes secreted by the pancreas and intestinal wall as well as the bile secreted by the liver. Digestion of sugars and peptides

into absorbable monomers, however, takes place within the brush border by the enterocytes, facilitated by membrane bound enzymes (Turk, 1982).

2.4 MICROBIAL ECOLOGY OF THE DIGESTIVE TRACT

The GIT supports an ecosystem that harbours a large and diverse population of bacteria, which create symbiotic relationships with the host (Apajalahti, 2005). There are more bacterial cells residing within the intestinal tract than there are cells of the host organism (Mead, 2000). The microflora of the intestinal tract of broiler chickens influences digestion, gut morphology, immune responses and health. The gut flora is also believed to both protect against intestinal colonization of pathogens and to stimulate the immune response (Mead, 2000). The microbes in the intestinal tract can become attached to mucosal surfaces or food particles, or remain free-living in the lumen (Gabriel *et al.*, 2006). The major sites of microbial activity in the GIT are those in which conditions are relatively stable and the digesta is retained long enough for significant microbial growth to occur (Mead, 2000).

Most of the work on avian gut flora has been done using domestic chickens rather than the commercial poultry currently used in the industry (Fuller, 1984). Intestinal bacteria evolve with age; at hatch, the GIT is a sterile environment after which the flora grows rapidly (Gabriel *et al.*, 2006) and maximum densities of bacteria are reached within the first 5 days post hatch (Apajalahti *et al.*, 2004). During the following weeks the composition of flora changes rapidly, taking 30 or more days in the caeca to develop a stable and dynamic population (Barnes *et al.*, 1972; Lu *et al.*, 2003). Many factors can affect the composition of the avian gut flora, including species, strain, age, diet, sex; environmental stressors, performance enhancers, antibiotic administration and infection with pathogenic organisms (Smith, 1965a; Knarreborg *et al.*, 2002; Hume *et al.*, 2003; Pedroso *et al.*, 2006).

The intestinal microflora is an integral part of the digestive system of all animals, and in common with all living organisms they have nutritional and environmental requirements. Microflora harbouring the gut derives most of its energy for reproduction and growth from dietary compounds that are either resistant to digestion or absorbed too slow (Apajalahti *et al.*, 2004). Since bacterial species differ from each other in relation to their substrate preferences and growth requirements, the

chemical composition and structure of the digesta largely determines the species distribution of the microbial community in the GIT. Therefore, microflora size, composition and activity can be altered by changes in diet (Hume *et al.*, 2003). The structure and function of the digestive tract determines the sites of the intestine in which physiochemical and nutritional requirements for bacterial growth are fulfilled. Despite variations there are no sites in the GIT in which bacterial growth is entirely missing with over-all activity varying in different GIT sections (Apajalahti, 2005). Not surprisingly, different species of bacteria are harboured by different GIT sections on the basis of their preferred predilection sites as various intestinal segments function differently. There are several factors that restrict bacterial growth in the proximal GIT, including chemical inhibition (e.g. acid and bile), highly competitive rate of nutrient absorption (large absorptive surface and active transport), passage rate of digesta, continuous sloughing of both epithelial cells and mucus, and immunological defence mechanisms.

The microbiota residing in the GIT consist of approximately 400 known species, of which 20 - 40 % can be cultured. There are different methods for determining and quantifying the bacteria in digesta or on the epithelial lining. Basic knowledge of intestinal bacterial populations is based on culture-based techniques. Since Muzyer *et al*. (1993) first used denaturing gradient gel electrophoresis (DGGE) to study complex microbial populations, it has become an attractive alternative to culture-based techniques. Many studies have used DGGE as a tool to investigate intestinal microbiota (Hume *et al.*, 2003; Lu *et al.*, 2003; Pedroso *et al.*, 2006). The availability of molecular techniques for identifying non-culturable species has opened new avenues of research into the factors that affect this complex mix of organisms and offers new opportunities for analysing the structure and species composition of intestinal microbial communities (Lu *et al.*, 2003; Apajalahti *et al.*, 2004; Dibner *et al.*, 2007).

2.4.1 The crop

Lactobacilli, enterococci and coliforms are dominant species in the crop, and *Lactobacillus salivarius* is the dominant species during the first week of life. *Lactobacilli* are attached to epithelium and form an almost continuous layer (Fuller, 1978; Dibner *et al.*, 2008). *Lactobacillus* spp is the major species of bacteria that

produces lactic acid, reducing the crop lumen pH to about 5.0 once feeding begins (Fuller, 1977).

2.4.2 The proventriculus and ventriculus (Gizzard)

Both the proventriculus and gizzard appear to be unfavourable for the growth of various bacteria resulting in a bacterial population that is lower than that found in other parts of the GIT (Smith, 1965a; Smith, 1965b). This is likely due to a low pH (range 1 to 4), requiring any surviving microorganisms to show a high degree of acid tolerance (Gabriel *et al.*, 2006). Despite this unfavourable environment, earlier studies have observed bacterial populations in the ventriculus: *Lactobacillus* at (up to 10^8 cfu/g of ventricular contents) and low numbers of *E. coli*, *Streptococcus/Enterococcus* and yeasts have also been found in the gizzard (Smith, 1965a; Smith, 1965b).

2.4.3 Small intestine

Large and diverse populations of bacteria inhabit the lumen of the proximal part of the small intestine. In the duodenum microflora are relatively sparse compared to the lower small intestine (Smith, 1965a). The environment of the ileum is more favourable to bacterial growth because of the lower oxygen pressure, and lower enzyme and bile salt concentrations (Gabriel *et al.*, 2006).

The majority of organisms isolated from the duodenum and ileum are gram positive (Salanitro *et al.*, 1978). The predominant organisms in the small intestine are *E. coli* and *Streptococcus* spp, *Enterococcus* spp, *Staphylococcus* spp and *Lactobacillus* spp at about 10^9cfu/g of ingesta. Lu *et al*. (2003) and Gong *et al*. (2002b) used the molecular analysis technique on 16S rRNA amplification to corroborated the previous culture-based results with lactobacilli predominating in the small intestine as well as smaller numbers of streptococci and enterobacteria.

Anaerobes comprised 39% of the total number of isolates obtained with the greatest range of types occurring in the duodenum (Salanitro *et al.*, 1978). Obligate anaerobes including anaerobic cocci and species of *Eubacterium, Propionibacterium, Clostridium, Gemmiger* and *Fusobacterium* are also present (Salanitro *et al.*, 1978). In

the ileum Apajalahti *et al*. (1998) found bacterial numbers were typically between 10^7 and 10^9 per gram of digesta.

2.4.4 The ceca

In simple stomach animals, bacterial fermentation is concentrated in the upper large intestine. The bacteria in these sections of the GIT utilize dietary compounds that have escaped pre-caecal host digestion and absorption, so these bacteria do not compete with the host when they ferment the entering substrate. In poultry, the most intense bacterial fermentation occurs in the caeca (Apajalahti, 2005). The caeca are evacuated only 1-2 times per day, thus providing relatively stable conditions for microbial proliferation (Gabriel *et al.*, 2006). As a result of these favourable conditions, the resultant microbial community tends to be both large and diverse (Gong *et al.*, 2002a; Lu *et al.*, 2003).

The bacterial population in the caeca is significantly more diverse than that in the ileum, although the bacterial population in both regions is predominantly Gram-positive. This difference between caeca and ileum bacterial distribution is likely to due to the variation of their functional and environmental properties. The main function of the ileum is purely nutrient absorption, while that of the caecum is extensive bacterial fermentation, resulting in further nutrient absorption as well as detoxification of substances harmful to the host (Gong *et al.*, 2002a; Gong *et al.*, 2002b).

At least 38 different types of anaerobic bacteria of different strains have been isolated from chicken caeca (Barnes *et al.*, 1972; Mead, 1989). Using culture based techniques Barnes *et al.* (1972) found strict/obligatory anaerobes such as *Bacteroidaceae*, *Gemmiger formicilis, Fusobacterium* and *Bifidobacterium gallinarum*, all of which are highly sensitive to oxygen, at levels of ~10^{11} cfu/g of caecal content and are predominantly culturable bacteria. Mead (1989) found that 28% of the total caecal culturable bacteria consisted of gram-positive cocci such as *Peptostreptococcus* spp. Other bacteria included *Bacteroidaceae* (20%), *Eubacterium* spp. (16%), *Bifidobacterium* spp. (9%), cocci (6%), *Gemmiger formicilis* (5%), and *Clostridium* spp. (5%). Facultative anaerobes such as *Streptococci* and *Peptostreptococci* spp occur in lower numbers with yeasts, moulds and protozoa generally at low levels or absent (Barnes *et al.*, 1972; Salanitro *et al.*, 1974).

Using new molecular methodologies, based on 16S rRNA bacterial genes analysis, Gong *et al.* (2002a; 2002b) observed that butyrate-producing bacteria (including those related to *Fusobacterium prausnitzii*), ruminococci, *Clostridia* spp and *E. cecorum* were the predominant groups of bacteria on the caecal mucosa. 25% of the sequences cloned from the caecal samples studied were found to be un-reported species, closely related to unidentified butyrate-producing bacteria from the human caecum and bovine rumen (Gong *et al.*, 2002b). Using the same technique, Lu *et al.* (2003) reported that 65% of sequences were related to *Clostridia* spp, *Fusobacterium* spp (14%), *Lactobacillus* spp (8%) and *Bacteriodes* spp (5%). Apajalahti *et al.* (2001) analysed diet-related differences in the caecal intestinal microbial communities using G+C% profiling of 16S ribosomal DNA (rDNA) sequences and found most of the genes detected were not from well-known bacterial species. This finding was later confirmed by Gong *et al.* (2002a) who found that many of the 16S rDNA sequences retrieved from a caecal library exhibited low sequence similarity to the genes of known bacterial genera.

Caecal flora changes from simple to a more complex and increasingly diverse population with the age of the chicken. Hume *et al*. (2003) examined changes in digestive microbial communities by using DGGE and found that caecal amplicon profiles changed from 8 major bands in chicks of 2 days of age to more complex mixtures of 26 major bands in chicks of 32 days of age. The composition of caecal microflora can vary between flocks, between birds within a single flock, and even within an individual bird examined at different times (Salanitro *et al.*, 1974). During the development of chickens and turkeys, microbial populations take longer to develop in the caeca than they do in other parts of the GIT, and may take up to six weeks after hatching to fully develop (Barnes *et al.*, 1972).

It must be noted that variation in the composition of intestinal microbial communities in different published reports may be partly due to the large diversity of gut flora as well as the method being used for enumeration.

2.5 NECROTIC ENTERITIS

One of the most clinically dramatic disease amongst enteric bacterial diseases is necrotic enteritis (NE), first described in broiler chickens by Parish more than fifty

years ago in England (1961). Since then, it has been perceived as a major global threat to the poultry industry, as it is the most common disease throughout all poultry-growing areas of the world (Songer, 1996a). The condition occurs almost exclusively in broilers (Long, 1973; Bernier *et al.*, 1974), although layers are also affected (Kwatra & Chaudhury, 1976; Dhillon *et al.*, 2004) with outbreaks reported in 3-6 month old chickens (Broussard *et al.*, 1986). In addition to broiler chickens it has also been identified in turkeys (Gazdzinski & Julian, 1992). Free living birds like quail and wild crows can also develop NE (Droual *et al.*, 1995; Gazdzinski & Julian, 1992; Asaoka *et al.*, 2004). However NE is of major concern to the poultry industry due to the economical impact of the disease through lost productivity.

2.5.1 Epidemiology

Outbreaks of NE are sporadic, but have a high mortality with severe economic losses (Dahiya *et al*., 1995a). The disease has been reported from various countries, including United Kingdom (Parish, 1961), Canada (Helmbold & Bryant, 1971; Long, 1973), France (Casewell *et al.*, 2003) and Australia (Nairn & Bamford, 1967). Epidemiological data on the incidence of NE are limited although a global survey found NE as a commonly occurring disease throughout the world (Van der Sluis, 2000). A cross sectional survey conducted by Hermans & Morgan (2007) among 857 farms in the UK indicated cases of NE in at least one flock during 2006 with a prevalence of NE in the recently reared flock at 12.3%.

Necrotic enteritis usually occurs in broiler chicks at 2-6 weeks of age and is characterized by a sudden onset of diarrhoea and mucosal necrosis (Long, 1973; Fukata et al., 1991). The disease can occur more than once a year on any particular farm. In Canada it mostly appears in July, August, September and October (Long, 1973), whilst in the UK the frequency of NE is at its highest in the winter months (Hermans & Morgan, 2007). Norway was similar to the UK with a peak incidence of NE during the winter and a lower incidence of NE during the warmer season (Hermans & Morgan, 2007; Kaldhusdal & Skjerve, 1996). It is generally recognised that the disease is not seasonal, although discrepancies in occurrence between different lattitudes that appear to contradict this are as yet unexplained.

2.5.2 Aetiology

The primary cause of NE is *Clostridium perfringens* type A (Long & Truscott, 1976; Alsheikhly & Truscott, 1977b; Alsheikhly & Truscott, 1977a) and type C (Thompson *et al.*, 2006; Prescott *et al.*, 1978b), although reference to type C in the literature is sparse.

2.6 *CLOSTRIDIUM PERFRINGENS*

Clostridium perfringens is a ubiquitous bacterium found in poultry houses and its surroundings, including water, poultry faeces, feed, soil and air (Arbuckle, 1972; Craven, 2000; Arbuckle, 1972), as well as in a wide variety of raw and processed foods particularly meat and poultry (Dahiya *et al.*, 2005a). *C. perfringens* is believed to be the cause of variety of economically significant diseases in domestic animals (Songer, 1996b). Genetically *C. perfringens* is a diverse organism and has successfully been isolated from the GIT of animals and humans. It is a member of the normal gut microbiota and is non-pathogenic in low concentrations (Kondo, 1988; McDevitt *et al.*, 2006a). Particularly in poultry, *C. perfringens* is present throughout almost the full length of the GIT; it has been isolated from the crop, gizzard and caeca (Nauerby *et al.*, 2003; Gholamiandekhordi *et al.*, 2006).

In healthy birds several different types of *C. perfringens* are present, whereas not all isolates of *C. perfringens* from diseased birds are pathogenic (Van Immerseel *et al*., 2009). This indicates that different strains of *C. perfringens* may vary in their virulence and only specific strain(s) have the ability to produce NE, with outbreaks usually caused by a massive proliferation of some of these specific types (Nauerby *et al*., 2003; Cooper & Songer, 2009).

2.6.1 Morphology

C. perfringens was previously known as *Clostridium welchii*. It is a Gram-positive, rod shaped, anaerobic, toxin producing bacterium that is able to form endospores under unfavourable conditions such as environmental stress, nutrient restriction and overpopulation (Stephenson & Lewis, 2005). Although *C. perfringens* is classified as an anaerobe, it can occasionally survive and grow in the presence of

oxygen (Quinn *et al.*, 1994). Colonies of *C. perfringens* are smooth, round and glistening surrounded by an outer zone of complete hemolysis with an inner zone of incomplete hemolysis (Quinn *et al.*, 1994). Optimal temperature for the growth of *C. perfringens* is 43-45ºC (maximum 50-51ºC) with a generation time of 8-10 minutes and an optimum pH of 5-8 (Bryant & Stevens, 1997). However, *C. perfringens* can grow at temperatures between 15-50°C and its endospores can tolerate 100ºC for 2hrs (Williams *et al*., 2003; Williams, 2005). It requires 13 amino acids for its optimum growth (Cato *et al.*, 1986).

2.6.2 Growth characteristics

C. perfringens can grow on a wide variety of culture media (Table 2.1), including Shahidi Ferguson *perfringens* agar (SFPA), Tryptose Sulphite Cycloserine (TSC) agar, blood agar (incubated anaerobically at 37ºC for 24hrs) and cooked meat medium. Byrne *et al*. (2008) compared a range of agars and reported that TSC and reinforced *clostridial* agar (RCA) are the best media for culturing *C. perfringens.* However TSC media with or without the addition of egg yolk is best for culturing C. *perfringens* as black colonies can be observed after 24 hrs of anaerobic incubation. For recovery of *C. perfringens* spores, RCA was the most effective agar (Mead, 1995; Byrne *et al.*, 2008). On the surface of agar plates *C. perfringens* forms large, regular, round, slightly opaque but shiny colonies (Brynestad & Granum, 2002).

Table 2.1: Different types of media used for diagnosis of C*lostridium perfringens*

Media	**Reference**
Blood agar	Long & Truscott, 1976; Kaldhusdal *et al.*, 1995; Branton *et al.*, 1997; La Ragione & Woodward, 2003; Olkowski *et al.*, 2006a; Drew *et al.*, 2004; Nairn and Bamford, 1967
Cooked Meat Medium	Shane *et al.*, 1985
Sulphite polymyxin sulfadiazine agar	Bradley and Radhkrishnan, 1972;
Fastidious anaerobe agar	Engstrom *et al.*, 2003
Lecithin – *Clostridium welchii* agar plates	Baba *et al.*, 1997
Brain Heart Infusion Agar (BHI)	Barbara *et al*., 2007; Kaldhusdal *et al.*, 1999; Cooper & Songer, 2009

2.6.3 Toxins of Clostridium perfringens

C. perfringens produces at least 17 different types of exotoxins, although individual strains only produce a subset of these (Songer, 1996b). Bacterial toxins play an important role in the pathogenesis of NE (Arbuckle, 1972). *C. perfringens* strains are classified into five types A, B, C, D and E (Table 2.2) depending upon the major extracellular toxin produced, i.e. alpha (α), beta (β), epsilon(ε) and iota (ι) (Miclard *et al.*, 2009; Losada-Eaton *et al.*, 2008). Type-A *C. perfringens* produces α-toxin, type B produces α, β and ε toxins, type C produces α and β toxins, type D produces α and ε toxins and type E produces α and ι toxins. This means that α-toxin is produced in varying amounts by almost all strains of C. *perfringens* although different strains of C. *perfringens* produce varying amounts of α-toxin with type-A strain producing the highest amount of α-toxin (Titball *et al.*, 1999). Molecular typing of the major toxins produced by *C. perfringens* has been done by many researchers (Engstrom *et al.*, 2003; Nauerby *et al.*, 2003).

Table 2.2: Major toxins used for typing *C. perfringens* as well as the enterotoxin and their genetic location

Type	**α-toxin**	**β-toxin**	**Epsilon toxin**	**Iota toxin**	**Enterotoxin**	**Major diseases/ Host**
A	+	-	-	-	+	NE in poultry GIT diseases in humans/ human food poisoinig Intestinal clostridiosis in horses
B	+	+	+	-	+	Dysentery in lambs Chronic enteritis in lambs enterotoxaemia in neonatal pigs, calves, goats and foals
C	+	+	-	-	+	NE in poultry, EN in humans
D	+	-	+	-	+	Enterotoxaemia in sheep (pulpy kidney disease)
E	+	-	-	+	+	Enterotoxaemia in lambs, calves and rabbits
Gene	*plc*	*Cpb1* *Cpb2*	*etx*	*iap* *ibp*	*cpe*	
Genetic location	Chromosome	Plasmid	Plasmid	Plasmid	Plasmid/ chromosome	
Biological activity	Cytolytic Haemolytic, Dermonecrotic Lethal	Cytolytic, Lethal	Oedema of various organs kidneys, liver CNS	Disruption of actin cytoskeleton and cell barrier integrity	Cytotoxic, Lethal Causesdiarrhoea by leakage of water and ions	

CNS: Central nervous system; NE: Necrotic enteritis; EN: Enteritis necroticans.
(Modified from (Brynestad & Granum, 2002).

Table 2.3: Different methods for molecular diagnosis and/or Genetic diversity of *clostridium perfringens*

Method	Reference
Pulsed –field gel electrophoresis (PFGE)	Nauerby *et al.*, 2003; Engstrom *et al.*, 2003; Chalmers *et al.*, 2007; Barbara *et al.*, 2008
Polymerase chain reaction (PCR)	Engstrom *et al.*, 2003; Saito *et al*., 1992; Kalender & Ertas 2005
Real time/ Quantitative PCR (qPCR)	Collier *et al.*, 2003; Collier *et al.*, 2008; Zhou *et al.*, 2009
Amplified fragment length polymrphism (AFLP)	Engstrom *et al.*, 2003

2.6.4 Pathogenesis of necrotic enteritis and associated toxins

Despite the clinical and economic significance of NE, the pathogenesis of *C. perfringens* and the associated disease in poultry is still not fully understood (Van Immerseel *et al.*, 2009; Timbermont *et al.*, 2009a). The jejunum is the predilection site for *C. perfringens* (Arbuckle, 1972), although it is normally present in the intestinal tract of healthy chickens (Smith, 1965a; Van Immerseel *et al.*, 2009). However successful progression of the disease may involve a number of different factors as described in section 2.8. It is believed that a key event in the pathogenesis of NE is associated with the adherence of *C. perfringens* to the intestinal villi, as once it has become attached it starts to proliferate and produce the toxins that induce gut necrosis (Alsheikhly & Truscott, 1977a; Alsheikhly & Truscott, 1977b; Cooper *et al.*, 2009). Mostly a sudden onset of the disease is quickly followed by death. The disease usually occurs in broiler chicks at 2-6 weeks of age and is characterized by sudden diarrhoea and mucosal necrosis (Fukata *et al.*, 1991).

2.6.4.1 Alpha -toxin

α-toxin is a multifunctional zinc metalloenzyme produced in varying amounts by nearly all isolates of C. *perfringens* (Songer, 1996b; Sato *et al.*, 1978). It possesses both phospholipase C and sphingomyelinase activities. The α-toxin is believed to be the first bacterial toxin to be identified as an enzyme (Naylor *et al.*, 1998) and is considered as the principal lethal toxin of C. *perfringens* (Songer, 1996b; Sato *et al.*, 1978). The

structure of α-toxin consists of two domain proteins: The α-helical amino-terminal domain (N-terminal domain or N-domain) containing the active site and the β-sandwich carboxy-terminal domain (C- terminal domain or C-domain). The C-domain interacts with the phospholipid content of the cell membrane, thus creating toxicity (Titball *et al.*, 1999; Titball *et al.*, 1993; Flores-Diaz *et al.*, 2004). Alpha toxin has haemolytic, cytotoxic, necrotic properties, so has a direct effect on host metabolism including inhibition of neutrophil chemotaxis, vasoconstriction and platelet aggregation. Alpha toxin causes hydrolysis of the phosphotidylcholine and sphingomyelin portions of the membrane phospholipids, thus promoting membrane disorganization (Titball *et al.*, 1999; Bullifent *et al.*, 1996). Both zinc and calcium ions are essential co-factors required for both the activity and stability of α-toxin. Moreover, zinc makes the α-toxin molecule rigid and stable with little conformational change. α-toxin activity is lost by EDTA, a zinc chelating agent (Sato *et al.*, 1978; Titball *et al.*, 1999).

The gene for α-toxin is known as *cpa* or *plc* and has been both cloned and sequenced / expressed in Escherichia *coli* (Okabe *et al.*, 1989). It is present in all strains of C. *perfringens.* The α-toxin gene is situated near the putative origin of replication, one of the most stable regions within the C. *perfringens* chromosome (Titball *et al.*, 1999). Some variation in the α-toxin sequence has been found but these did not significantly affect its activity (Abildgaard *et al.*, 2009). The quantity of *plc* produced by different strains varies considerably, reflecting differences in the regulation of the *plc* gene. α-toxin is produced during the exponential or growth phase of C. *perfringens* and its mRNA expression is at maximum during this phase. At higher concentrations α-toxin is cytotoxic. At lower concentrations it disturbs phospholipids metabolism and deregulates different signal transduction processes (Bullifent *et al.*, 1996).

The α-toxin has phospholipase C activity and the regulation of α-toxin production is considered to be a critical pathogenicity factor of the type A strain of C. *perfringens* (Engstrom *et al.*, 2003). Although there is controversy (see below) about the role of α-toxin in NE, the α-toxin is believed by many to play a vital role in the severity of NE primarily because of its ability to impair the intestinal mucosal barrier (Alsheikhly & Truscott, 1977b; Alsheikhly & Truscott, 1977a; Rehman *et al.*, 2009). It causes hydrolysis of the phosphotidylcholine and sphingomyelin into diacyglycerol or ceramide respectively (Figure 2.5). These products promote membrane disorganization by activation of the protein kinase C with later stimulation of arachadonic acid cascade.

The activation of arachidonic acid cascade induces production of inflammatory mediators including leukotriens, thromboxane, platelet agglutinating factor and prostacyclin all products that are responsible for membrane damage and the inflammatory process (Van Immerseel *et al.*, 2004; McDevitt *et al.*, 2006a) which results in lysis or other forms of cytotoxicity (Songer, 1997; Titball *et al.*, 1999).

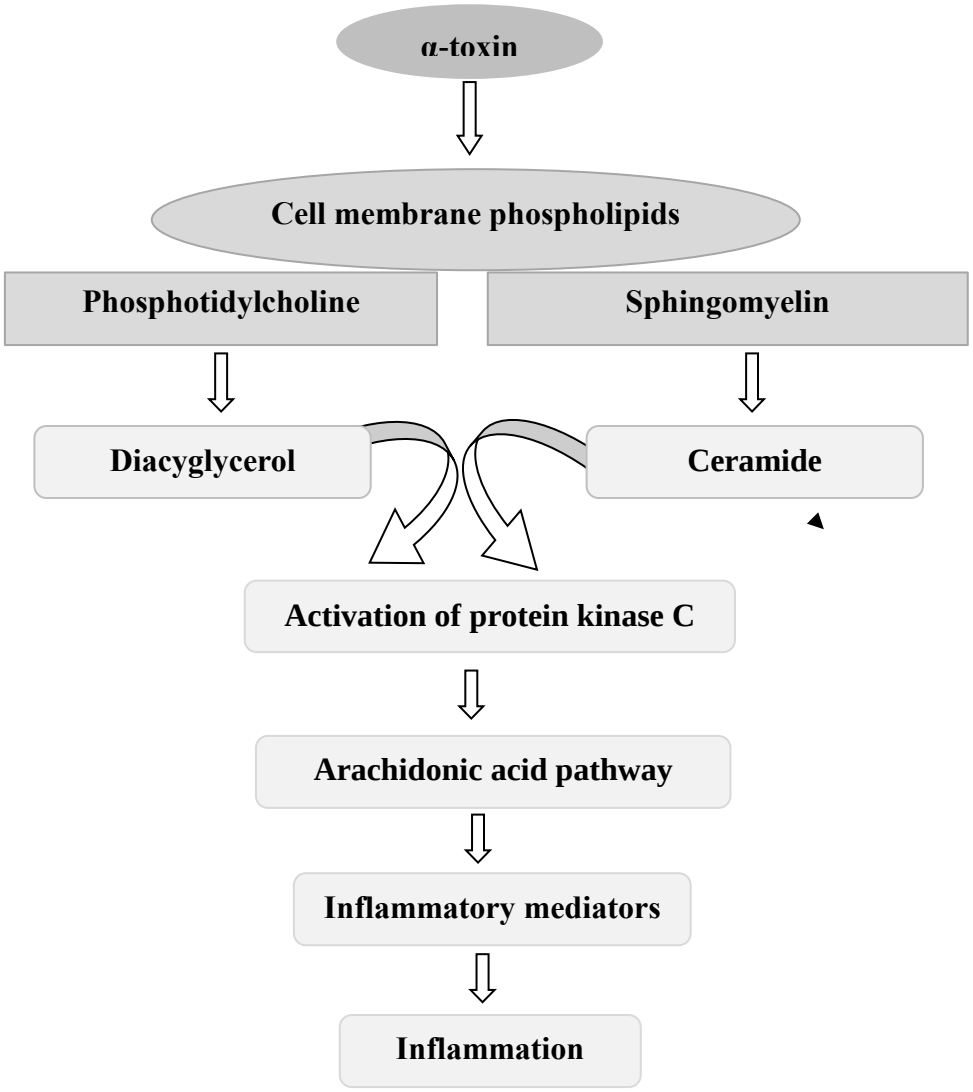

Figure 2.5: Mode of action of α-toxin.
(Modified from McDevitt *et al*.2006a).

For more than three decades α-toxin was considered to be the main virulent factor in the pathogenesis of NE. But, although a number of mechanisms have been described the importance of α-toxin in the pathogenesis of NE is still questionable. Some studies showed that whether α-toxin is given through intra-duodenal infusion or

oral inoculation it induced necrosis of the epithelium of the small intestine (Alsheikhly & Truscott, 1977b). In one *in vitro* study a significantly higher amount of α-toxin was noted in isolates from birds with NE than isolates from healthy birds (Hofshagen and Stenwig, 1992). Against these positive findings a later *in vitro* study demonstrated no difference in α-toxin production between the strains of *C. perfringens* isolated from NE outbreaks and those isolated from clinically healthy broilers (Gholamiandekhordi et al., 2006). Studies have shown that induction of NE lesion was independent of the amount of α-toxin produced *in vitro,* and even that immunization with recombinant α-toxin gave partial protection against experimental challenge with *C. perfringens* (Cooper & Songer, 2009), These findings questioned the proposed role of α-toxin in the disease, demonstrated that α-toxin may have limited implications in NE (Keyburn *et al*., 2006) and was not an essential virulence factor in induction of the disease. In their experiment the inactivation of α-toxin gene (plc) did not affect the virulence of the resultant strain and that an α-toxin negative mutant produced the same type of lesions as that of a wild type strain. In contrast a more recent *in vitro* study concluded that α-toxin can impair the intestinal mucosal barrier function (Rehman *et al*., 2009). Furthermore, immunization with recombinant α-toxin stimulated the production of anti-α-toxin IgG, giving partial protection to broiler chicks against experimental challenge with *C. perfringens* (Cooper & Songer, 2009) suggesting α-toxin does play a possible role in NE.

2.6.4.2 NetB Toxin

In the past few years some studies have cast doubt on the role of α-toxin as the primary cause of NE by demonstrating that instead of conferring complete immunity, vaccination with alpha toxoid partially protected the birds against NE (Cooper & Songer, 2009; Lovland *et al.*, 2004). Some studies indicated that besides α-toxin other proteins can confer immunity against experimentally induced NE (Kulkarni *et al.*, 2007), suggesting that there are additional factors, other than α-toxin, that are important, or perhaps even more important, in NE pathogenesis. Keyburn *et al.* (2006) reported that α-toxin negative mutants of C. *perfringens* type A were able to produce NE in an experimental model. Later a novel pore-forming toxin of C. *perfringens* named NetB was identified in the NE strains of C. *perfringens*. Literature information on Net B is scarce. It has a similar molecular size to β-toxin, hence the name NetB (necrotic enteritis toxin B-like). Its amino acid sequence has limited similarity with other pore forming toxins similar to the β-toxin of C. *perfringens* (38% identity) and the α-toxin of

Staphlococcus aureus (31% identity). It has cytotoxic activity against chicken leghorn male hepatoma cell line (LMH) including karyorehexis and karyolysis (Keyburn *et al.*, 2008). Although the evidence of involvement of NetB toxin in the pathology of NE is convincing there is still ambiguity about the role of NetB toxin in NE (Martin & Smyth, 2009). Keyburn *et al.* (2008) isolated the NetB gene from isolates from chickens infected with NE but did not find the NetB gene in isolates from uninfected chickens. Martin & Smyth, (2009) in contrast found the NetB gene in isolates from chickens both with and without NE. Interestingly, NetB negative isolate was able to produce the disease in experimental model of Cooper & Songer (2009).

Although immunogens other than α-toxin have been identified that give protective immunity against NE (Kulkarni *et al.*, 2006; Kulkarni *et al.*, 2007) this does not necessarily indicate they have a role in the development of NE. These studies also indicated that the alpha toxoid offered significant protection against NE (Kulkarni *et al.*, 2007) suggesting that it does have some role in the pathogenesis of the disease. It is clear that the importance of α-toxin in relation to NE can not be predicted from studies using purified toxin, which reinforces the importance of using the natural infection process to dissect the role of this toxin as an etiological agent of the disease. The sometimes contradictory evidence for the role of α-toxin and NetB toxin argues for the role of another, as yet unidentified, virulence factor(s) that allows C. *perfringens* to compete effectively in the chicken gut to produce the disease.

2.6.5 Disease transmission

During outbreaks of NE the major source of *C. perfringens* may be either contaminated feed or litter (Frame & Bickford, 1986; Songer, 1996a: Branton *et al.*, 1997; Ficken & Wages, 1997). In the 1960s out breaks of NE in Australia were associated with one commercial brand of feed (Nairn & Bamford, 1967). Sometimes feed components contaminated with bacteria may have also been a source of *C. perfringens* in NE outbreaks (Wijewant & Senevira, 1971a; Eleazer & Harrell, 1976) There is a possibility that C. *perfringens* may be able to transmit vertically as bacteria have been found in the yolk sac of an embryonated egg (Shane *et al.*, 1984). Craven *et al.* (2003) suggested that C. *perfringens* may be transmitted within the integrated broiler chicken operation. *C. perfringens* has also been reported to be transmitted mechanically

and or biologically through house flies in poultry houses, resulting in the development of NE (Dhillon *et al.*, 2004).

2.6.6 Types of necrotic enteritis

Under field conditions the disease occurs in two forms: an acute clinical form and a mild subclinical form. The acute form of NE is characterized by visible clinical signs like watery diarrhoea and sudden increase in flock mortality but often without any premonitory signs.

Birds that do not succumb to clinical signs, may develop a sub-clinical clostridial infection. In the sub-clinical form of the disease there is often no peak mortality. Intestinal damage as a consequence of disease leads to a reduction in performance noticeable as reduced weight gain and an increased feed conversion ratio (Kaldhusdal & Hofshagen, 1992; Timbermont *et al.*, 2010). The prevalence of sub-clinical NE is not known, as the lesions can only be revealed by post mortem examination of randomly sampled birds (Engstrom *et al.*, 2003). The sub-clinical form of NE is more important than the acute phase as it persists in broiler flocks without any obvious clinical signs, allowing it to remain undetected and untreated resulting in huge economic losses (Skinner *et al.*, 2010). In the past few years the sub-clinical form of NE has become increasingly prevalent with no overt clinical signs and no peak mortality (Timbermont *et al.*, 2011).

2.6.7 Clinical signs

Sick birds affected with NE are depressed, anorectic with ruffled feathers and huddle together. In the advanced stage of the disease the bird becomes limp/moribund, lying in lateral recumbency as they are unable to stand, relatively immobile, and very quickly die (Long, 1973; Alsheikhly & Alsaieg, 1980; Kaldhusdal *et al.*, 1995). Mostly the course of the disease is short, with no clinical signs before the birds are just found dead (Songer, 1996a; Williams, 2005). Acute symptoms of the disease are severe depression, decreased appetite, reluctance to move, ruffled feathers and diarrhoea (Ficken & Wages, 1997). The period of illness is usually short (1- 2 hrs) so, again, most of the birds are just found dead (Nairn & Bamford, 1967; Helmbold & Bryant, 1971).

Birds that have died of NE have a foetid odour, are usually dehydrated with dark, dry pectoral musculature and pale kidneys (Long *et al.*, 1974). The unopened intestinal wall is darker in colour than normal and distended due to the presence of large amounts of bile stained contents. In an affected flock the mortality rate can be anything from 1% to as high as 50% (Shane *et al.*, 1985; Craven *et al.*, 1999; Craven *et al.*, 2001b).

By definition, sub-clinical NE does not manifest any clinical signs, and mostly under field conditions, only detected at the processing plant(s) by the rejection of carcasses (Lovland & Kaldhusdal, 2001; McDevitt *et al.*, 2006b). It is usually associated with hepatitis and cholangiohepatitis. In some cases bile-stained fluid and some times blood-stained fluid filled the lumen of the intestine with sloughing of the intestinal epithelium, as well as greyish and thickened mucosa particularly in the duodenum and ileum (Lovland & Kaldhusdal, 1999).

2.6.8 Gross lesions

The small intestine is the principal site of tissue damage (Arbuckle, 1972) with gross lesions seen primarily in the jejunum and ileum, although these can also been extended to the duodenum (Helmbold & Bryant, 1971; Long *et al.*, 1974; Broussard *et al.*, 1986; Kaldhusdal & Hofshagen, 1992). Lesions may also be present in the caeca and liver (Long *et al.*, 1974; Alsheikhly & Truscott, 1977a; Alsheikhly & Truscott, 1977b;). Under invasive experimental conditions, such as direct administration of *C. perfringens* or purified toxin administered intra-duodenally, the first appearance of signs of damage to the chickens' intestine have been reported as early as 6 hrs after administration of the challenge (Alsheikhly & Truscott, 1977a; Alsheikhly & Truscott, 1977b).

Typically an intestinal wall is friable and easily torn. There is a marked distension of the intestine with the lumen usually filled with gas and dark brown fluid material. Ulcers can occur either singly or as aggregates in the mucosa of the intestine. In very severe cases, the intestinal mucosa is covered with a layer of fibrino-necrotic material, or a diphtheritic membrane covering the mucosa in segments of variable length usually involving two thirds of the small intestine, the jejunum and ileum (Nairn & Bamford, 1967; Long *et al.*, 1974; Kaldhusdal *et al.*, 1995; McDevitt *et al.*, 2006a).

Lesions of sub-clinical NE are usually present in one or more areas of the intestine (Long *et al.*, 1974). In addition to small intestinal lesions *C. perfringens* is also associated with liver abnormalities; mostly enlargement (Riddell & Kong, 1992; Kaldhusdal *et al.*, 1995; Lovland & Kaldhusdal, 2001). Sometimes severe congestion can be found in the liver (Shane *et al*., 1985).

2.6.9 Histopathological changes

Microscopically lesions of NE from field cases consist of coagulation necrosis at the intestinal villous apices. A clear line of demarcation is visible between necrotic and viable cells. In some cases cellular degeneration may reach the sub-mucosa (Nairn & Bamford, 1967; Ficken & Wages, 1997). An apparently visible psuedomembrane is made up of necrotic/ distorted villi, inflammatory cells and clumps of bacteria (Gholamiandehkordi *et al.*, 2007; Olkowski *et al.*, 2008). From field cases, regeneration of the intestinal tract is characterized by a proliferation of epithelial cells, production of connective tissue network, decreased numbers of goblet, columnar and epithelial cells. Overall regenerative changes leave the affected region with short, flat villi that have a reduced absorptive surface (Long *et al.*, 1974; Ficken & Wages, 1997).

In the sub-clinical form of the disease microscopic changes are mostly seen in the small intestine, sometimes in liver but rarely in the caecum (Kaldhusdal & Hofshagen, 1992). Larger areas of the intestine are affected in the sub-clinical compared to clinical form of the disease. Necrosis starts from the apical villous epithelium (Kaldhusdal *et al.*, 1995). Lesions typically develop from the tips of the villi. Histopathological examination of tissues from the field cases reveal small demarcated areas of degeneration merged with areas of normal unaffected intestine. Degenerative changes begin with congestion of blood vessels followed by diffuse oedema. Later the epithelial cells of the villi become enlarged, with pale vacuolated cytoplasm, and an indistinct homogenous deposit in place of the striated border. The degenerative process progresses down the villi in regular manner that many villi are equally affected. At this stage necrotic tissue is clearly differentiated from normal by a clear sharp line. In some cases stroma of the villi are sloughed off. There is fibrin with amorphous basophilic deposits in the majority of blood vessels.

Other significant changes include dissolution of the nuclei, with nuclear material flowing out of the individual cells in the form of irregular deposits that disappear when all layers of the intestine, from mucosa to serosa, are affected in the final stage of the degenerative process. Regenerative changes of the intestinal tract in field cases are characterized by a proliferation of the epithelial cells of the crypts to replace necrosed cells (Parish, 1961; Long *et al.*, 1974), production of connective tissue in the inflammatory area, and a decreased number of goblet and epithelial cells. In the lumen of an affected intestine, disintegrated necrotic villi, degenerated epithelial cells, goblet and inflammatory cells can be found, leaving the intestinal villi shortened and flattened with a reduced absorptive surface (Long *et al.*, 1974). Large numbers of gram-positive rod-shaped bacteria can be seen in the sloughed epithelium (Parish, 1961; Alsheikhly & Truscott, 1977a; Alsheikhly & Truscott, 1977b; Kaldhusdal *et al.*, 1995).

When NE occurs in association with an *Eimeria* infection, coccidial oocytes are apparent at the base of the intestinal villi (Nairn & Bamford, 1967). Gross and histopathological lesions in turkeys are the same as that of chickens; however in turkeys the duodenal part of the small intestine is more affected than in chickens. Alsheikhly & Truscott, (1977b) observed changes in the morphology of erythrocytes and attributed this to a direct effect of C. *perfringens* toxin on erythrocytes.

2.7 EXPERIMENTAL MODELS OF NECROTIC ENTERITIS

A consistent experimental induction of (sub-clinical) NE has proved difficult. One the one hand, many workers have reported successful experimental induction of NE (Long *et al.*, 1974; Truscott & Alsheikhly, 1976; Alsheikhly & Truscott, 1977b; Pedersen *et al.*, 2008). Approaches to reproduce the disease include intraduodenal inoculation of *C. perfringens* broth cultures (Alsheikhly & Truscott, 1977b; Kaldhusdal *et al.*, 1999), intra-duodenal administration of bacteria free crude toxins of *C. perfringens* (Alsheikhly & Truscott, 1977a), Long & Truscott (1976) used a pure culture challenge model to reproduce NE in broiler chickens. Other methods of introducing *C. perfringens* to induce disease include the mixing of *C. perfringens* cultures in feed (Alsheikhly & Alsaieg, 1980; Pedersen *et al.*, 2008), oral gavage (Olkowski *et al.*, 2006a), and intravenous challenge with *C. perfringens* cultures (Williams *et al.*, 2003), rearing birds on reused litter from NE infected flocks (Hamdy *et al.*, 1983b; Baba *et al.*,

1997) and even use of reused litter from flocks without having any clinical signs of NE (Palliyeguru *et al.*, 2010).

On the other hand, a more detail analysis of the literature shows that the results for experimental induction of the disease are indeed controversial (Table 2.4). Cowen *et al.* (1987) conducted five trials and tried 3 different methods: feeding a *C. perfringens* broth culture-feed mixture, exposure to NE- infected litter and combination of these two yet had variable results and was only able to produce disease in three out of five trials.

Table 2.4: Experimental reproduction of disease

Challenge	**Route**		**Reference:**
Bacteria free crude toxin	Intra-duodenally	Disease produced	Alsheikhly & Truscott, 1977a
C. perfringens (10^7 / gram)	Orally	Disease produced	Long & Truscott, 1976
C. perfringens type A (dose not mentioned)	Feed	Disease produced	Alsheikhly & Alsaieg, 1980
C. perfringens 10^8 cfu/ gram	Feed	Disease produced	Shane *et al.*, 1985
C. perfringens pure culture	Orally Intravenous injection	Disease produced	Bernier *et al*., 1975
C. perfringens	Feed	Unable to produce disease	Cowen *et al.*, 1987
-	Litter	Unable to produce disease	Cowen *et al.*, 1987
4 x 10^6 - 2x 10^9	Feed	Disease produced	Riddell & Kong, 1992
C. perfringens 10^{5}-10^{10} per chicken	Feed		Kaldhusdal *et al.*, 1999
C. perfringens 10^{2}-10^{03}	Drinking water	Unable to produce disease	Takeda *et al.*, 1995
Dose not mentioned	Gavage	Unable to produce disease	Takeda *et al.*, 1995
C. perfringens 10^{01} -10^{10}	Feed	Unable to produce disease	Pedersen *et al.*, 2003
C. perfringens 10^8	Feed	Disease produced	Timbermont *et al*., 2008

NE: Necrotic enteritis; C. *perfringens*: Clostridium *perfringens*

Although *C. perfringens* is major aetiological agent of NE there are other contributory factors (see section 2.6.4) that are required to predispose birds to the disease. Sufficient numbers of *C. perfringens* and damage to intestinal mucosa are two major factors that are known for the production of the disease (Alsheikhly & Truscott, 1977a). Some experimenters have tried to induce NE with concurrent infection of coccidiosis. Shane *et al.* (1985) suggested that infection of broiler chicks with *Eimeria acervulina* prior to inoculation of C. *perfringens* at the levels of 2.5 x 10^8 organisms/ gram of feed produced characteristic lesions of the disease and also resulted in increased mortality, from 8% in birds receiving *C. perfringens* alone, to 35 % in birds receiving *Eimeria acervulina* as well as *C. perfringens*. On the contrary, Baba *et al.* (1992) despite inoculation of higher number of coccidia and *C. perfringens* was unable to induce disease. Therefore inoculation of broiler chickens with *C. perfringens* does not always result in successful induction of disease (Shane *et al.*, 1984; Pedersen *et al.*, 2003).

To facilitate experimental induction of the disease some experimenters used opium to decrease intestinal peristalsis and sodium bicarbonate to neutralize acidity in the gut (Bernier *et al.*, 1977). Some supplemented the diet with dietary Zinc along with *Eimeria* and *C. perfringens* (Baba *et al.*, 1997). McReynolds *et al.* (2004b) used IBD vaccine to induce immunosuppression for successful induction of disease.

Despite these frequent attempts, there is no generally established disease model for the experimental induction of NE in broiler chickens as constant reproduction of the disease is difficult to achieve so there is a need to develop an experimental model of the disease that can be applied to test products known to have a protective effect against *C. perfringens*. Therefore there is urgent need to identify factors that play crucial role in induction and pathogenesis of NE.

2.8 PREDISPOSING FACTORS FOR NECROTIC ENTERITIS

Although *C. perfringens* is documented as a causative agent of NE other contributory factors are essentially required that predispose birds to the disease. A documented key predisposing factor for the development of NE is an intestinal environment that favours the growth of *C. perfringens* in the gut. Favourable conditions

include, a decrease in intestinal motility and a, decrease in intestinal pH (Bernier *et al.*, 1974).

The presence of predisposing factors are as important as the presence of *C. perfringens* and its virulence factors for disease production. Factors that predispose the bird to NE are management, nutrition, overcrowding of birds kept on litter, and inadequate hygiene routines (Kaldhusdal *et al.*, 1999; McDevitt *et al.*, 2006a). Reported factors that predispose the bird to NE can be divided into three major categories (Table 2.5):

- Infectious
- Non-infectious/ Nutritional
- Management

2.8.1 Infectious predisposing factors for NE

Different diseases that commonly occur amongst poultry have been identified as predisposing factors for NE. Among parasitic diseases, coccidiosis and the resultant intestinal damage may be a major predisposing factor for NE (Van Immerseel *et al.*, 2004). Experimental evidence regarding other parasitic infestations as predisposing factors that can lead to NE are scarce: *C. perfringens* was isolated from intestinal scrappings of turkeys from two commercial farms that experienced an outbreak of NE together with *Ascaridia dissimilis* (Norton *et al*., 1992).

2.8.1.1 Coccidiosis

Concurrent intestinal diseases like coccidial infection may be a potential risk factor for the development of NE; especially under field conditions. When sufficient numbers of *C. perfringens* are present coccidial infection can play a crucial role in the occurrence of NE.

The precise mechanism of coccidial infection in the pathogenesis of NE is not clear. There are different theories that suggest coccidia play a major role in inciting the disease, however mucosal damage resulting from coccidiosis is a possible cause that predisposes birds to NE (Gazdzinski & Julian, 1992; Williams *et al.*, 2003). The sexual

stage in the life cycle of the coccidian parasite results in extensive damage to the intestinal mucosa (Shane *et al.*, 1985; Van Immerseel *et al.*, 2004) and it is hypothesized that intestinal damage due to coccidiosis along with sufficient numbers of *C. perfringens* are prerequisites for NE, therefore it is perhaps the best known predisposing factor (Shane *et al.*, 1985; Van Immerseel *et al.*, 2004). In particular the *Eimeria* species that colonize the small intestine, such as *Eimeria maxima* and *Eimeria acervulina,* are known to predispose birds to NE (Alsheikhly & Alsaieg, 1980). Bradley and Radhkrishnan, (1972) observed that the growth of *C perfringens* also increased during infection with *E. tenella*. Baba *et al*. (1997) found that concurrent infection with *E. nacatrix* increased the clostridial population in the intestine of the chickens (Baba *et al.*, 1997).

Mucosal damage in turn facilitates the loss of protein in the intestinal lumen with the subsequent degradation of protein into amino acids by proteolytic enzymes in the GIT that may cause an increase in the acidity of digesta. An acidic pH (5.6 or lower) affects the permeability of the cell membrane and will ultimately suppress intestinal motility all of which favours *C. perfringens* growth. In addition to mucosal damage, coccidial infection also induces a host mucogenic response that results in increased mucus production. *C. perfringens* has a unique ability to utilize mucus as its substrate so increasing the amount of mucus provides a growth advantage for C. *perfringens* (Deplancke *et al.*, 2002; Collier *et al.*, 2008).

Coccidiosis and NE are usually linked as they both have similar symptoms but coccidiosis is usually observed just prior to or during an outbreak of NE (Verleyen, 2009). Some reports suggest that supplementation with anticoccidial preparations may reduce the incidence of NE (Alsheikhly & Alsaieg, 1980; Shane *et al.*, 1985; Riddell & Kong, 1992; Williams, 2005). Some of the experimental evidence does not have reliable results so it has been argued that it was unlikely that coccidial infection plays any significant role in NE. This was because at that time coccidiostats were used during the most susceptible stage of NE (Nairn & Bamford, 1967).

In contrast all the experiments that have successfully claimed reproduction of the disease included an overdose of coccidial vaccine or feeding live coccidia to cause intestinal damage during the progress of the experiments (Pedersen *et al.*, 2003; McReynolds *et al.*, 2005; Pedersen *et al.*, 2008). Alsheikhly & Alsaieg (1980) and

Shane *et al.* (1985) suggested that supplementation with anticoccidials preparations may reduce the incidence of NE. Anticoccidial drugs like narasin, salinomycin lasalocid and maduramicin have been shown to have a beneficial effect on the control of NE in broilers (Elwinger *et al.*, 1992; McReynolds *et al.*, 2007; Timbermont *et al.*, 2009b; Lanckriet *et al.*, 2010; Lee *et al.*, 2011).

2.8.1.2 Viral disease / immunosuppression

Some viral diseases like Infectious Bursal Disease (IBD), Chick Anemia Virus and Marek's Disease have been suggested to promote the development of NE under field conditions (Williams *et al.*, 2003; McReynolds *et al.*, 2007; Timbermont *et al.*, 2009b; Lee *et al.*, 2011). As these diseases can cause immunosuppression, they can increase the severity of NE (Lee *et al.*, 2011). Indeed, in most of the experiments to induce NE, IBD vaccination has been used as predisposing factor (McReynolds *et al.*, 2004a).

Table 2.5 : Various predisposing factors for NE

Risk factor	Reference
Infectious	
Coccidiosis (*Eimeria* infection)	Long, 1973; Alsheikhly & Alsaieg, 1980; Baba *et al.*, 1992; Baba *et al.*, 1997; Hofacre *et al.*, 1998a
Immunosuppression	McReynolds *et al.*, 2004b
Non-infectious	
Fish meal	Wijewant & Senevira, 1971b
Barley	Riddell & Kong, 1992; Kaldhusdal & Skjerve, 1996; Annett *et al.*, 2002
Wheat	Branton *et al.*, 1987; Riddell & Kong, 1992; Kaldhusdal & Skjerve, 1996 Annett *et al.*, 2002
Increased digesta viscosity (NSPs)	Kosher, 2003
Lipids	Dahiya *et al.*, 2006
Dietary protein	Truscott & Alsheikhly, 1977; Kaldhusdal & Skjerve, 1996; Drew *et al.*, 2004; Dahiya *et al.*, 2005a; Wilkie *et al.*, 2005;
Managment	
Litter	Cowen *et al.*, 1987; Droual *et al.*, 1994; Williams, 2005; Hermans & Morgan, 2007; Mikkelsen *et al.*, 2009; Palliyeguru *et al.*, 2010

2.8.2 Non infection predisposing factors for NE

Non infectious predisposing factors for NE include a variety of management/environmental factors, such as poor management, overcrowding, inadequate hygiene routines, wet litter as well as nutritional factors such as inclusion of high levels of wheat, barley, fish meal, imbalance in the diet, the form of feed (see below). Any of these, or a combination have been shown to have a major effect on the incidence of NE (Wijewant. & Senevira., 1971c Long, 1973; Drew *et al.*, 2004; Van Immerseel *et al.*, 2004; Dahiya *et al.*, 2006; Hermans & Morgan, 2007; Hofacre *et al.*, 1998b).

2.8.2.1 Nutrition and dietary factors

It is now widely recognised that diet has a strong influence on the incidence of NE in broilers (Baba *et al.*, 1992; Annett *et al.*, 2002; Drew *et al.*, 2004; Kaldhusdal *et al.*, 1999). Not only the chemical balance but also the physical quality of the diet can influence the microbes present in GIT (Smith & Macfarlane, 1998). Roller-mill ground wheat reduced NE related mortality compared to hammer ground wheat (Branton *et al.*, 1987).

Diets enriched in cereals such as barley and wheat lead to an increased incidence of NE compared with a corn-based diet. This may be due to an increased proliferation of *C. perfringens* in the wheat and barley diets (Riddell & Kong, 1992; Branton *et al.*, 1997; Annett *et al.*, 2002). Cereals like wheat and barley contain high levels of indigestible, water soluble non starch polysaccharides (NSPs) e.g. arabinoxylans and β-glucans, that are not utilized by the bird but can act as a potential substrate for the microflora, so enhancing the growth of pathogenic bacteria within the GIT (Choct *et al.*, 1996; Iji & Tivey, 1998). The NSPs are also hydrophilic, increasing the viscosity of the intestinal contents, predisposing the bird to NE. Van Immerseel *et al.* (2004) gave a possible explanation of this as wheat or barley may contain certain substances that promote the proliferation of *C. perfringens*. Another possible explanation of the association of wheat, meat meal and fish meal with NE could be the higher zinc concentration (Leeson and Summers, 2001), as production of alpha toxin is increased by zinc which has also been shown to protect α-toxin from destruction by trypsin (Baba *et al.*, 1992).

Poor quality wheat at levels above 50% corn-replacement can increase susceptibility to NE (Dekich, 1998). Some researchers have observed an association between NE and a specific brand of poultry feed (Nairn & Bamford, 1967).

The other predisposing factor for NE is a diet with an high protein content, or a diet rich in animal protein such as fish meal or meat/bone meal (Wijewant & Senevira., 1971d; Hafez, 2003). The level of crude protein, the protein source, and the amino acid content of a diet all have a significant affect on the intestinal population of C. *perfringens*. Genome sequence analysis of *C. perfringens* showed that it lacks many enzymes required for amino acid biosynthesis making it necessary to obtain this essential material from its host. Consequently a diet high in protein provides an exceptional amino acid source for *C. perfringens* proliferation thus predisposing the bird to increased risk of NE (Shimizu *et al.*, 2001; Drew *et al.*, 2004). Poorly digested protein diets act as a substrate for the *C. perfringens* in the lower gut, thus enhancing the growth of the organism (Verleyen, 2009). Another possible explanation of the association between wheat or fish/meat meal and NE could be the higher zinc concentration; since production of the alpha toxin associated with *C. perfringens* is not only increased in the presence of zinc but also the zinc protects the toxin from destruction by trypsin (Baba *et al.*, 1992). Recently potato protein has become an additional risk factor due to its higher antitrypsin activity (Palliyeguru *et al.*, 2010). The role of fish meal in the production of NE has also been recognised as being of importance. Most of the experimental models referred to in the literature used fish meal at concentrations from 18% - as high as 50% (Cowen *et al.*, 1987; Cooper & Songer, 2009). It is hypothesized that fish meal contains higher Zinc levels and, as α-toxin is zinc metallozyme it requires zinc for its activity. However, it has also been recognised that higher levels of Zinc in the feed also have a boosting effect on the immune status of the chicken. The dietary fat source is also known to have an effect on the incidence of NE, since animal fat (a mixture of lard and tallow) can lead to higher counts of *C. perfringens* when compared to vegetable/ soya oil (Knarreborg *et al.*, 2002).

Not only the dietary content, but also its actual form can also affect the incidence of NE. An increased risk of NE has been also associated with pelleted feed compared to mash. Some researchers have observed the association of NE with a specific brand of poultry feed although they omitted to mention the name or its composition (Nairn & Bamford, 1967). In addition to all these dietary factors that

indirectly lead to proliferation of *C. perfringens* in the gut of birds, poultry feed is generally considered to be the major source of infected spores (Wijewant and Senevira., 1971).

2.8.2.2 Anti-nutritional factors

Anti-nutritional factors such as protease inhibitors, lectins, tannins and mycotoxins may also affect the incidence of NE. For example, trypsin inhibitors found in soyabean meal reduce the digestibility of proteins, leaving higher concentrations of undigested proteins in the lower gut thus providing more suitable conditions for the growth of proteolytic bacteria like *C. perfringens* (Clarke & Wiseman, 2005; McDevitt *et al.*, 2006a). Trypsin inhibitor activity (TIA) may also play a role in creating conditions favourable to a poor litter quality that indirectly can lead to NE (Clarke & Wiseman, 2010).

Lectins are proteins and glycoproteins present in cereal grains such as wheat, barley, oats and rye as well as in legumes such as soya and kidney beans. Lectins may facilitate bacterial attachment to the gut epithelium which can facilitate the rate and extent of pathogen growth (Giovannini *et al*., 1996; Calderon *et al*., 1997; McDevitt *et al*., 2006a). Tannins are present in many dietary ingredients such as rapeseed meals and beans and can act strongly with protein leading to tissue damage that may predispose birds to NE (Robins & Brooker, 2005; McDevitt *et al.*, 2006a). Although there are numerous diet related factors described in the literature it has not yet been agreed which dietary factors actually influence the disease due to variable results from different experiments.

2.8.3 Management

2.8.3.1 Litter

Last but not least, wet litter has been demonstrated to be further significant risk factor for NE. Wet litter is a frequent condition with prevalence as high as 56% in UK broiler farms (Hermans & Morgan, 2007). There are a wide variety of causes of wet litter, such as climate conditions in the poultry house and leaky drinkers. In recent years the incidence of NE has been increasingly associated with NE. There is a direct correlation between sticky droppings and a poor litter condition with higher nitrogen

levels. Moist and crusty litter allows proliferation of *C. perfringens* (Kaldhusdal *et al.*, 1995). Rough litter has also been suggested as the cause of intestinal damage which, together with increased colonization of *C. perfringens*, may result in a higher incidence of NE. There is conflicting information in the literature whether NE leads to wet litter and/or coccidiosis or whether these two conditions are risks factor leading to NE (Hermans & Morgan, 2007).

Other poorly referenced and ill-defined factors known to increase the incidence of NE are increased farm size, a raised number of chicks placed in the last flock, a greater number of hatcheries supplying the chicks as well as a higher number of feed lorries entering the farm (Hermans & Morgan, 2007).

To summarise the wide ranging literature, it is clear that almost anything that causes stress and results in an imbalance of the microflora of the GIT is a potential risk factor for NE. Despite substantial research efforts there is still a lack of full understanding of the actual mechanisms underlying the effect of the many predisposing factors as experimentation on predisposing factors does not always lead to induced disease.

For many years, poultry producers have prevented and treated NE by routine inclusion of antimicrobial growth promoters (AGPs), such as bacitracin, lincomycin, avoparcin, avilamycin, amoxicillin and tylosin in poultry feed (Ficken & Wages, 1997; Craven *et al.*, 1999; McDevitt *et al.*, 2006a; Lanckriet *et al.*, 2010). There has been growing public concern about the risk of bacterial resistance associated with the routine use of AGP in poultry production, as bacteria may become resistant to antibiotics, and the resistant genes may be transferred easily among and between different bacterial species. This may lead to risk of making antibiotic therapy ineffective in treating animal as well as human diseases. This may be the reason, of European Union wide ban on the use of AGP in poultry feed in January, 2006, (Van Immerseel *et al.*, 2009). In the absence of in-feed antibiotics, alternative strategies require to be explored to protect against the outbreak of NE. However, to assess the efficacy of such strategies an infection model is required, which leads to the aim of this thesis.

PhD OBJECTIVES

As discussed earlier, in the past few decades NE in poultry has been prevented and treated with the addition of in-feed antimicrobials. Rising concerns from consumers, about the negative effects of antimicrobials in animal feed, led many countries to ban their use. This in turn led to a resurgence of NE in poultry, with major economic and social effects globally. The resurgence of NE caused leading researchers to seek alternatives for the initial treatment and ultimately the prevention of this damaging disease.

Despite of the fact that NE has being described almost five decades ago, the key aetiology agent that leads to the pathogenesis of the disease is still controversial, pathogenesis of NE is still poorly understood for a number of reasons. The main virulent factors are debatable as are the predisposing factors of disease, reflecting gaps in our understanding. Another gap in NE research is that to this date no reproducible and reliable disease model has been discovered. Unfortunately NE appeared to be very difficult to induce under control environmental conditions, most likely due to the potential presence of numerous ill-defined predisposing factors, and until now there has been no well established experimental disease model able to effectively and consistently produce the disease.

Different species of broilers can differ in their susceptibility to different infectious diseases. Resistance to diseases could be due to genetic differences between the species (Lamont, 1998; Zekarias *et al*., 2002). There have been anecdotal reports of a variation between different broiler species (Ross vs Hubbard) in their susceptibility to enteric infectious diseases. Any variation in disease-resistance might be the result of underlying inter-species differences (Zekarias *et al*., 2002). Having already experimentally considered a wide range of factors associated with this disease, this researcher felt it was important to determine if there might be any validity in the anecdotal reports of species variation.

The aims of this PhD are therefore:

1. To develop a working infection model that enables experimental induction of sub-clinical NE in broiler chickens with special reference to prioritize nutrition related risk factors that purportedly predispose birds to sub-clinical NE.

2. To test breed sensitivity to disease induction, two chicken breeds, Ross and Hubbard, with varying degrees of susceptibility to the induced infection.

3. To identify the novel biochemical markers for sub-clinical NE.

To achieve these aims, the main focus of the work for this thesis has been:

1. To establish a disease model for sub-clinical NE,

2. Investigate the importance of the various factors that are believed to play a role in the development of sub-clinical NE, such as high proteins levels in diets, co- infection with coccidia, the level of *C. perfringens* in inoculum and contact with re-used litter

3. Use the results to identify novel bio-chemical markers of the host for sub-clinical NE.

Thesis will provide a better understanding of the factors involved in the pathogenesis of sub-clinical NE thereby enabling development of new treatment and preventative strategies for restricting the damage inflicted on the global poultry industry by this terrible disease.

EXPERIMENTS

3 EXPERIMENT ONE:

CAN FEED WITHDRAWAL ASSIST EXPERIMENTAL INDUCTION OF SUB-CLINICAL NECROTIC ENTERITIS?

3.1 Introduction

Necrotic enteritis in poultry was first described by Parish (1961) in a flock of 6-7 weeks old cockerels, since then it has been reported world wide (Nairn & Bamford, 1967; Long, 1973; Kaldhusdal *et al.*, 1999; Brennan *et al.*, 2003; Johansson *et al.*, 2004). Its primary cause is over-growth of *C. perfringens* type A (Alsheikhly & Truscott, 1977b), which is a normal inhabitant of the GIT of healthy birds (Flores-Diaz & alape-Giron, 2003). Necrotic enteritis is most common in broiler chickens 2-6 weeks of age (Helmbold & Bryant, 1971; Bernier *et al.*, 1974). The clinical form of NE result in high mortality rates (Songer, 1996b; Van Immerseel *et al.*, 2004; Thompson *et al.*, 2006) whereas sub-clinical NE remains a major problem in broiler flocks as this form of disease is not manifested by any signs or symptoms (Skinner *et al.*, 2010) and mostly result in reduced weight gain and poor feed conversion (Kaldhusdal & Hofshagen, 1992; Kaldhusdal *et al.*, 1999; Engstrom *et al.*, 2003).

There are numerous suggested predisposing factors of NE, though most of them are ill defined. Possible factors that are associated with higher incidence of NE include increasing the amount of dietary crude protein which may increases the number of *C. perfringens* in caecum and thus predispose birds to NE (Drew *et al.*, 2004). Diets based on rye, wheat oat and barley have been reported to have resulted in higher incidence of NE compared to corn based diets (Riddell & Kong, 1992). Occurrence of NE may also be affected by changes in feeding programmes (Craven *et al.*, 1999; Craven, 2000), including temporarily cessation of feeding and other perturbation resulting from diet change over.

Feed withdrawal has been shown to cause alterations in intestinal morphology and changes in mucus characteristics that in turn affect pathogen colonization in the gut (Deplancke *et al.*, 2002; Thompson & Applegate, 2006). Results of Bilgili (1988) showed that the shear strength of duodenum was reduced in birds with 18 or 24 hrs of feed withdrawal compared to those with only 6 or 12 hrs of feed withdrawal. Later Northcutt *et al.* (1997) confirmed that integrity of intestines declined after 12-14 hrs of feed withdrawal. Feed withdrawal reduces the natural ability of broilers to inhibit the growth of several harmful bacteria like *Salmonella* and *Enterobacteriacae*. This was shown by the increased incidence of *Salmonella* in broiler crops (Corrier *et al.*, 1999) and caeca of market age broilers challenged experimentally with high doses of

Salmonella enteritidis after 18hrs of feed withdrawal (Ramirez *et al.*, 1997). It is known that starvation causes enlargement of goblet cells as well as causing an increase in mucin protein concentration in the small intestine (Uni *et al.*, 2003; Smirnov *et al.*, 2004). Deplancke *et al.* (2002) showed that *C. perfringens* has an acidomucolytic potential, since it utilizes mucin as a substrate, shown by its rapid growth on a mucin containing medium that can subsequently result in NE. Thus, feed withdrawal could result in increased *C. perfringens* proliferation, and thus induce (sub-clinical) NE.

Anecdotal field evidence indicates that NE can occur following a disruption in feeding (for example due to feeder mechanisms failing) as well as following feed change over. Therefore, patterns of occurrence of NE could be affected by dietary restriction or fasting. Moreover preliminary work together with evidential data gathered from a wide range of nutritional studies conducted at our lab supports the view that feed change over could be associated with the occurrence of subclinical NE (un-published work). Recent work has highlighted that a change of feed form (as happens at feed change over) causes birds to temporarily alter, in some cases entirely stop, their eating habits (un-published work). The interval between the end of feeding a starter diet and the beginning of feeding a grower diet is correlated with changes in GIT microflora which in turn may influence occurrence of sub-clinical NE. Therefore the objective of this experiment was to assess whether feed withdrawal assists the induction of sub-clinical NE in broiler chickens inoculated with *C. perfringens*.

3.2 Materials and Methods

3.2.1 Treatment Groups and Experimental design

A total of two hundred and forty, one-day-old male Ross 308 broiler chickens were randomly assigned to one of the three treatments, each replicated four times. All birds were given a standard starter diet until day 10, when they transferred to 12 floor pens adopting stratified randomization on body weight with 20 birds per pen. All the pens were equipped with plastic feeder hoppers and drinkers appropriate for the age and number of birds. On day 10 birds were divided into three treatment groups and grower diet was replaced after variable periods of feed withdrawal, i.e. no withdrawal (Group-1, full fed control), 8 h of withdrawal (Group 2) or 15 hrs of withdrawal (Group 3). In

addition, on day 18, feed withdrawal time for Group-2 and Group 3 were 15 and 24 hrs respectively (Table 3.1).

Table 3.1: Experimental design

Groups	Feed withdrawal Period	
	Day 10	Day 18
1	0 h	0 h
2	8 h	15 h
3	15 h	24 h

Birds were reared in a solid floored pen (1.74m x 1.28 m) containing 10cm thick bedding material of wood shaving in an environmentally controlled house using a standard commercial management programme from day 0 to day 28. Birds were fed nutritionally complete standard commercial starter and grower diets (Table 3.2). No antibiotic growth promoter or anti-coccidial drugs were used in the diets. Both the diets were provided as mash.

The research facility was thoroughly cleaned and disinfected prior to bird placement. Light was provided for 23 hours per day with controlled temperature and humidity. The three treatments were randomly allocated to pens with four positional blocks. Precautions taken to avoid accidental across pen contamination included changing of gloves and use of foot dipping tanks. All experimental procedures were approved by Scottish Agricultural College Animal Ethics committee (AE 15/2009) and carried out under Home Office authorization (PPL 60/3383).

Table 3.2: Ingredients and calculated composition (g/kg) of starter (days 0-10) and grower (days 10-28) experimental diets in experiment one

Ingredient	Starter (g/kg)	Grower (g/kg)
Wheat	621.9	668.9
Soybean meal	216.6	165.6
Rapeseed meal	41	40
Soybean meal (ff)	34.5	38
Vegetable oil	47	50
Choline chloride	0.5	0.5
Monodical	14.5	14
Limestone	12	12
Common salt	2.7	2.7
Lysine	2	1.5
Methionine	3.3	2.8
Sodium bicarbonate	1.5	1.5
Vitamin and mineral premix[1]	2.5	2.5
Calculated nutrient composition		
Metabolizable Energy (MJ/kg)	13.2	13.5
Crude protein	220	201
Crude fiber	36	30
Calcium	8.9	8.7
Phosphorus	8	7.8
Available phosphorus	4.7	4.6

[1]*The vitamin and Mineral Premix (Target Feeds Ltd) contained vitamins and trace elements to meet the requirements specified by the breeder. The premix provided (units* kg^{-1} *diets supplement provided (units* kg^{-1} *diets): Vit A 16,000 iu; Vit* D_3 *3,000 iu; Vit E 75 iu; Vit* B_1 *3 mg; Vit* B_2 *10 mg; Vit* B_6 *3 mg; Vit* B_{12} *15 µg; Vit* K_3 *5 mg; Nicotinic acid 60 mg; Pantothenic acid 14.5 mg; Folic acid 1.5 mg; Biotin 275 µg; Choline chloride 250 mg; Iron 20 mg; Copper 10 mg; Manganese 100 mg; Cobalt 1mg; Zinc 82 mg; iodine 1 mg; Selenium 0.2mg; Molybdenum 0.5mg.*

3.2.2 Bacterial Culture & Administration (Challenge)

To ensure a standardized background level of *C. perfringens* all birds were gavaged at 7 days of age with 1 ml of broth containing 10^4 cfu of *C. perfringens*. The *C. perfringens* strain 56 was isolated from the intestine of a broiler chicken with severe necrotic gut lesions that produce moderate amounts of α-toxin *in vitro*. This was a type A strain (no enterotoxin or beta-2 gene), netB positive and has been used previously to induce NE in an *in vivo* model (Gholamiandehkordi *et al.*, 2007). The strain was stored in the form of frozen beads at -80°C. From the frozen culture, bacteria was grown overnight on Tryptose Sulphite Cycloserine (TSC) agar.

The TSC agar was obtained as dehydrated media (Oxoid Ltd, UK) and reconstituted according to the manufacturer's instructions. 23 gms of powdered TSC agar compound was weighed into a 500ml Schott Duran bottle, and diluted with distilled water to a volume of 500ml and fully mixed. When completed, the bottles of agar media were sterilized in an autoclave at 121°C for 10 minutes before being cooled to 50°C in a preheated water bath where the bottles were held until required for pouring onto plates. All supplements were removed from storage at 4°C about an hour before they were required, to allow acclimatisation to room temperature. Selective antibiotic supplements (SR-0088E, Oxoid Ltd, UK) were reconstituted with 2ml of sterile distilled water. Immediately before pouring, supplements were added to the agar one at a time in the water bath and the bottle mixed gently. Approximately 10-15ml TSC agar with egg yolk emulsion (25ml/500ml: SR-0088E, Oxoid Ltd, UK) was poured into each of the culture plates and allowed to spread evenly. When the agar solidified the plates were labelled.

Frozen beads were streaked out onto the TSC agar plate with the help of sterile disposable loops to ensure an even distribution of the mixture across each agar plate. These plates were incubated invertedly in an anaerobic jar with CO_2 generating gas packs (AnaeroGen™, AN0025A, Oxoid Ltd, UK). The jar was placed in an incubator at 37°C for 24hrs. Before inoculation in the feed, the bacteria were cultured for 24hrs at 37°C in Brain Heart Infusion (BHI) broth (CM 1032, Oxoid, Ltd, UK).

After overnight incubation, 3 colonies (Figure 3.1) were taken from TSC agar plates and mixed with BHI broth. The BHI broth was obtained as dehydrated media and

reconstituted according to the manufacturer's instructions. 37g of powdered BHI was weighed and dissolved in 1 litre of distilled water by mixing. When completed the bottles with the dissolved/mixed BHI were sterilized in an autoclave at 121ºC for 15 minutes. When cooled down, 2ml of BHI broth was taken into sterile bijou bottles.

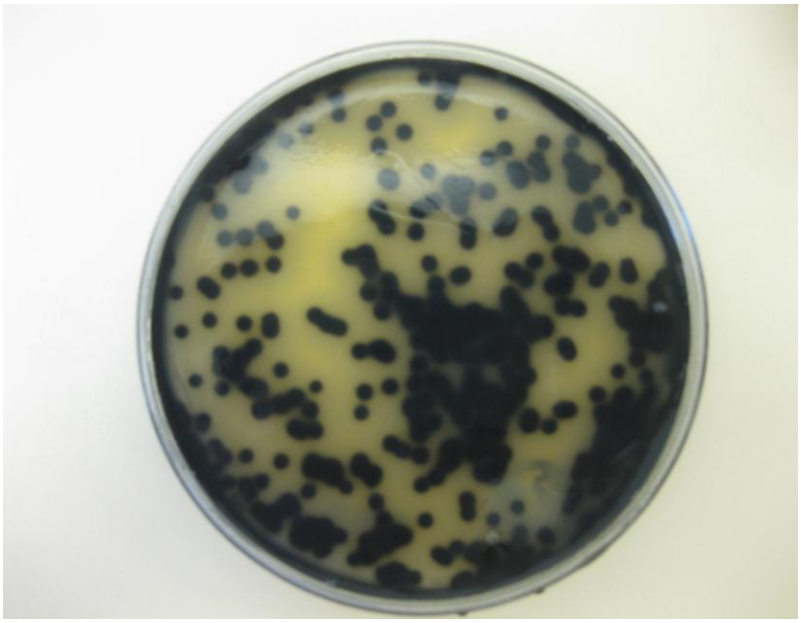

Figure 3.1: Black colonies of *C. perfringens* on Tryptose Sulphite Cycloserine (TSC) agar medium.

3.2.3 Growth performance

Up to 3-4 colonies from overnight cultures of TSC plates were taken and inoculated into bijou bottles with BHI broth. Brain heart infusion broth was incubated at 37ºC, shaking at 60rmp anaerobically overnight (18-24hrs). The concentration of *C. perfringens* in the inoculum was estimated spectrophoto-metrically at 600nm (Spectronic 301, Milton Roy) with the aid of a standard curve. Actual *C. perfringens* concentration in the inoculum was confirmed by plating on TSC agar plates, incubating the plates at 37°C overnight, and counting the number of black presumptive *C. perfringens* colonies. All birds were gavaged on 7 days of age with 1ml of BHI broth containing 10^4 cfu/ml *C. perfringens*. Broth was orally delivered into the crop of each chick once only using a bottle equipped with vinyl tubing about 3-4cm long (Figure 3.2).

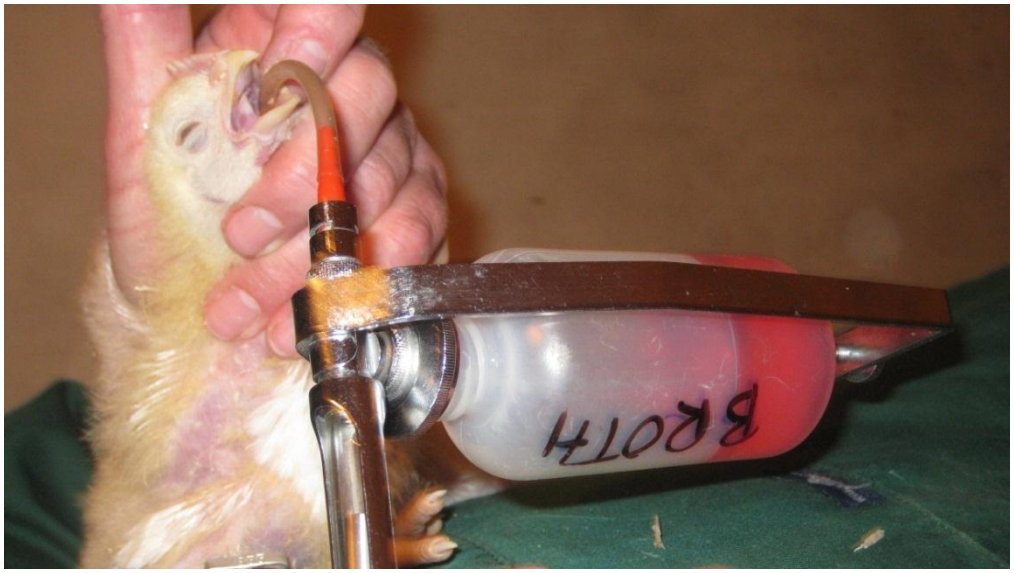

Figure 3.2: Gavaging birds with *Clostridium perfringens* broth.

3.2.4 Vaccinations

All the birds were vaccinated with coccidial vaccine (Paracox® -5) at day old. Each 0.004 ml dose of vaccine contains the following numbers of sporulated oocytes derived from precocious lines of coccidia: *E. acervulina* HP 500-650, *E. maxima* CP 200–230, *E. maxima* MFP 100–130, *E. mitis* HP 1000-1300 and *E. tenella* HP 500–650) (Schering-Plough Animal health, Welwyn Garden City, UK). Birds were vaccinated at 9 days old with infectious bursal disease (IBD) vaccine (Poulvac® Bursine2, Pfizer Animal Health) in drinking water following normal procedure of vaccination i.e. one hour prior to vaccination the water supply to the birds was stopped to ensure every bird was vaccinated.

3.2.5 Data Collection

3.2.5.1 Sampling of birds

Birds were observed daily for any signs and symptoms of NE. On 12, 20 and 28 days of age, five birds from each replicate pen were selected at random and euthanized by an intravenously administered overdose of barbiturate. Total ileum content/digesta were collected in sterile containers to enumerate total *coliforms*, *Lactobacilli* and *C. perfringens*. Samples of digesta were also taken in a separate sterile container for toxin determination.

3.2.5.2 Lesion scoring

Three sections of small intestine (duodenum, jejunum and ileum) were identified from the removed GIT, immediately incised, washed with phosphate buffer saline (PBS) and the whole length of tissue was inspected for evidence of lesions (clostridial or coccidian). A scoring system was used to score intestinal lesions for NE on a scale 0-3 (adapted from Shane *et al.*, 1985) (see Figure 7.2 to Figure 7.4).

- score 0: absence of gross lesions or no lesions;
- score 1: focal necrosis or focal ulceration;
- score 2: focal ulceration coalesced to form discrete patches;
- score 3: extensive diffuse mucosal necrosis;

3.2.5.3 Histopathology

Following post-mortem examination, a 1.5-2cm of tissue sample from small intestine, particularly those showing lesions were taken and washed with phosphate saline to ensure that intestinal samples were completely covered by fixative. Tissue samples were then stored in 10% buffered formalin for histopathology. The amount of fixative was 10 times the volume compared with the amount of tissue sample. Tissues were stored in fixative solution until required for processing.

For processing the intestinal samples were dehydrated by transferring the sections into a series of progressively increasing concentrations of ethanol (50, 60, 70, 80, 90, and 100%) for 2 hrs. Subsequently the sections were immersed in xylene to remove ethanol before being placed in blocks and filled with molten paraffin wax. This is necessary to prevent collapse and distortion of tissue during sectioning. When the wax is cooled or hardened the block of wax containing the processed tissue was sectioned into 3-5µm sections using a microtome. Thin sections were then placed in a warm water bath to remove wrinkles before being taken up onto a glass slide. The slides were place in an oven at 55°C for 15 minutes for the sections to adhere onto glass slide.

The paraffin was removed by quickly immersing the slides through xylene. The deparaffinised tissues were then rehydrated in water. The samples on the slides were stained with Haematoxylin and Eosin (Appendix-A). Haematoxylin was used to stain the nuclear material dark blue, while the eosin was used to stain the cytoplasm pink.

After the staining procedure, the glass slides were carefully blotted dry and mounted by placing a drop of DPX mounting medium (synthetic resin mounting media) onto a cover slip and arranging it over the top of the sections on the slide, Excess DPX was wiped and the slide was allowed to dry. The prepared tissue sections were later examined using a binocular setero-microscope (Olympus BX 41, U-LH100HG, Olympus optical Co. Ltd) connected to a camera (spot idea™ 28.2-5MP) to computer software (Spot idea, Version 4.7), using different magnifications (x4, x10, x40 and x100).

3.2.5.4 Enumeration of *C. perfringens*, total coliforms and lactobacilli

For enumeration of *C. perfringens*, total coliforms and lactobacilli, ileal contents (digesta content from the Meckel's diverticulum to ileo-caecal-colon junction) were taken into sterile screw capped bottles and transferred immediately to our microbiology laboratory. For *C. perfringens* enumeration, dilutions were plated on Tryptose Sulphite Cycloserine agar (TSC, Oxoid UK CM0587) containing supplements (Oxoid, UK, SR0088) and incubated anaerobically overnight. TSC agar plates were prepared as previously described in section 3.2.2 of this thesis. Presumptive lactobacilli and total coliforms were enumerated on Man Rogosa Sharpe agar (MRS, Oxoid CM0361) and Membrane Lactose Glucoronide agar (MLGA, Oxoid CM1031), respectively.

The MRS and MLGA media were especially chosen for their selectivity. Powdered MRS compound (62 g) and MLGA agar compound (88 g) were weighed into one litre Schott Duran bottles, diluted to a volume of 1000ml and mixed well prior to sterilization by autoclaving at 121°C for 15 minutes. The MRS agar was boiled before sterilization. After autoclaving the media were cooled down. Then around 15-20ml volume of media were poured into sterile Petri dishes and allowed to settle. All plates were inverted, sealed and stored at a temperature below 4ºC until required for enumeration of the respective bacteria.

Maximum Recovery Diluent (MRD) was used for serial dilutions of the digesta samples. The media was obtained as dehydrated media (Oxoid Ltd, UK), reconstituted according to the manufacturer's instructions through dissolving 9.5 g MRD powder (CM0733) in 1 litre of distilled water, which was then sterilised by autoclaving at 121°C for 15 minutes.

Each digesta sample was vortexed for 15 seconds in order to ensure adequate mixing. One g of ileal contents were weighed and well mixed with 9 ml MRD. Serial dilutions of 1ml in 10 were prepared for each sample tested down to 10^{-7} to give a total of 7 tubes in the dilution series (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}). From each dilution to be plated, 100µl were applied to the centre of a selected agar plate using a sterile spreader to ensure distribution of the mixture across the agar plate. Time was allowed for the liquid to be soaked up before 15ml of TSC overlay (without egg yolk) was spread. The plate was then incubated invertedly, in an upright position, in a jar providing anaerobic growing conditions. The jar was placed in an incubator at 37°C for 24 hrs. After incubation the number of colonies was counted on the most appropriate dilution, i.e. on plates with 30-300 colonies. This figure was then used to calculate the number of colony forming units per gram of original sample.

For enumeration of *C. perfringens*, dilutions were plated on TSC and were incubated at 37°C in jars containing gas generation kits (Anaerogen: AN0025 and AN0035, Oxoid). *Lactobacilli* were enumerated on MRS after incubation at 37°C for 48 hrs. For enumeration of total *coliforms*, dilutions were plated on MLGA and typical colonies were counted following incubation at 35°C for 24 hrs. Each sample was plated in duplicate. Morphological characteristics of *C. perfringens* were further confirmed with a Gram stain (Appendix B).

3.2.5.5 Quantification of α-toxin in intestinal digesta

For detection of α-toxin in intestinal digesta, *C. perfringens* α-toxin ELISA kit (Cypress diagnostics, Belgium, Ref. VB040) was used according to the instructions of the manufacturer. The test used 96 well micro titration plates sensitized by specific monoclonal antibodies for the α-toxin to coat the well with antigen. Digesta samples were diluted volume per volume with dilution buffer and 100 µl aliquots of digesta samples were added to the duplicate wells. The plates were covered with lids and

incubated at room temperature (18-24ºC) for 1 hr prior to three successive cleanings with a washing solution using the concentrated washing solution provided with the kit that had been diluted 20 fold with a concentrated dilution buffer in distilled water. The plates were further incubated for 1 hr at room temperature with 100 µl of peroxidase labelled anti-alpha-toxin monoclonal antibody. After this second incubation the plates were washed again and a volume of 100 µl of chromogen (tetramethyl benzidine, TMB) was added to each well and incubated for further 10 min at room temperature. The reaction was stopped with 50 µl of stop solution. The optical density (OD) at 450nm was recorded with a microplate reader (Dynex, ICXE0072). Positive and negative controls were included, where positive control consisted of pure α-toxin and negative control of incubation buffer only.

3.2.5.6 Growth parameters

Body weight (BW) was measured on days 10, 14, 21, 28. Average feed intake (FI), average weight gain (WG) and feed conversion ratio (FCR) were calculated for the period days 10-14, 14-21 and 21-28. To determine feed intake, the feed offered was recorded and refusals weighed back on days 10, 14, 21 and 28. Feed conversion ratio was derived by dividing average feed consumed per pen by average weight gain of birds per pen.

The birds were inspected daily within the experimental period and the birds that died or were culled were recorded, weighed and post-mortemed. When calculating feed conversion the body weight of dead birds was taken into account.

3.2.6 Statistical Analysis

The effect of feed withdrawal on counts of different bacteria (*C. perfringens*, total *coliform* and *lactobacilli* and growth parameters (average FI, WG and FCR) were compared using randomized complete block analysis of variance (ANOVA). The pens were treated as the experimental unit, and pen position used as block. Data are presented as means. Days 0-10 was initially used as covariate, but omitted from final model used when it did not contribute significantly in growth parameter analysis.

Because of the skewed nature of their distribution, *C. perfringens*, total *coliforms* and *lactobacillus* counts were transformed according to log (n + 1) to normalize the data before statistical analysis. Comparison contrasts were used to separate treatment means. Significance of the test was determined `at $P<0.05$. All statistical procedures were performed using Genstat 11 for Windows (VSN International Ltd, Hemel Hempstead, UK).

3.3 Results

The birds did not show any signs of clinical abnormalities (i.e. depression and ruffled feathers) in any of the treatment groups subsequent to *C. perfringens* challenge. Only one bird died (0.42%) during the course of the experiment.

3.3.1 Lesion score

On day 12 of the experiment, necropsy findings did not show any gross lesions in the intestines (duodenum, jejunum and ileum) in any of the treatment groups. However on day 20, lesions like mild focal changes and/ or small necrotic patches were seen in two birds from Group-3. On day 28, lesions similar to sub-clinical NE were found in two, three and one birds from Groups 1, 2 and 3 respectively.

The intestinal gross lesions in most of the birds were very mild and inconclusive of NE (e.g. intestinal wall was very friable thin and hyperaemic with mesenteric vessels engorged with blood).

3.3.2 Histopathology

Histopathological examination of formalin fixed intestinal tissue on different days was done to confirm these changes and the relation to sub-clinical NE. Microscopic examination of the tissue sections revealed no lesions specific to sub-clinical NE. There was no necrosis or desquamation of epithelial cells in intestinal villi. There was no evidence of Gram-positive rod-shaped organisms attached to the intestinal mucosa. Polymorphonuclear cells infiltration was not seen in the lamina propria in any of the sections. Although mucosal scrapings did not show any coccidial oocytes

numerous coccidial oocytes were seen scattered in the villi and crypts of intestinal mucosa (Figure 3.3).

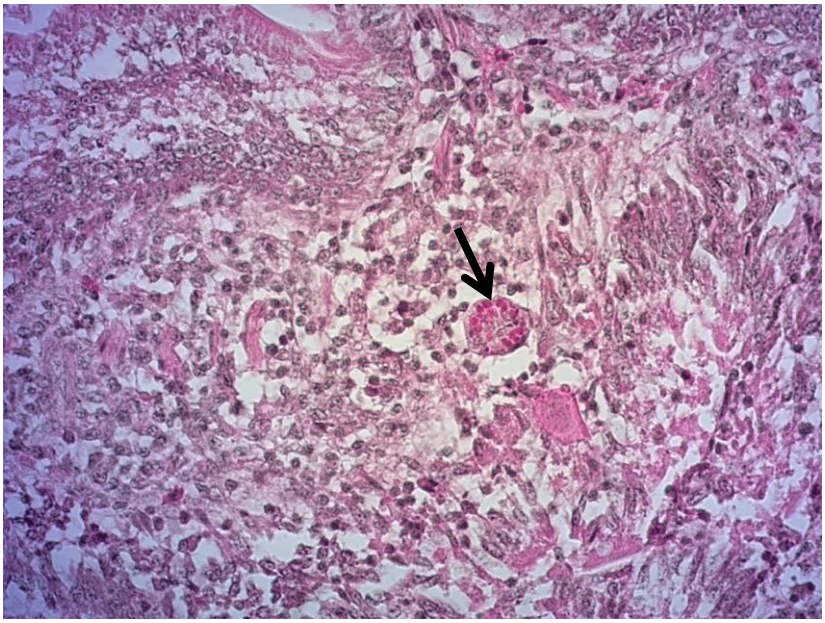

Figure 3.3: Photomicrograph of the intestine of broilers, showing presence of coccidial macrogamete (arrow), the overall architecture of villi are preserved. Haematoxylin and Eosin (x40).

3.3.3 *Quantification of different bacteria and α-toxin in* ileal digesta

Figure 3.4 shows the effect of feed withdrawal on *C. perfringens*, total *coliforms* and *lactobacilli* concentrations in ileal digesta of the birds euthanized on day 28. Group 3 birds had greater concentrations of *C. perfringens* than Group 1 and 2 birds (P = 0.071). Conversely counts of *lactobacillus* were significantly lower in ileal digesta of Group 3 birds compared to Group 1 and 2 birds (P= 0.032; Figure 3.4). Total *coliforms* populations tended to have higher concentrations in digesta of Group 3 birds compared to Group 1 birds (P = 0.061; Figure 3.4). Alpha toxin was not detected in ileal samples of birds from any of the treatment groups on different dissection days.

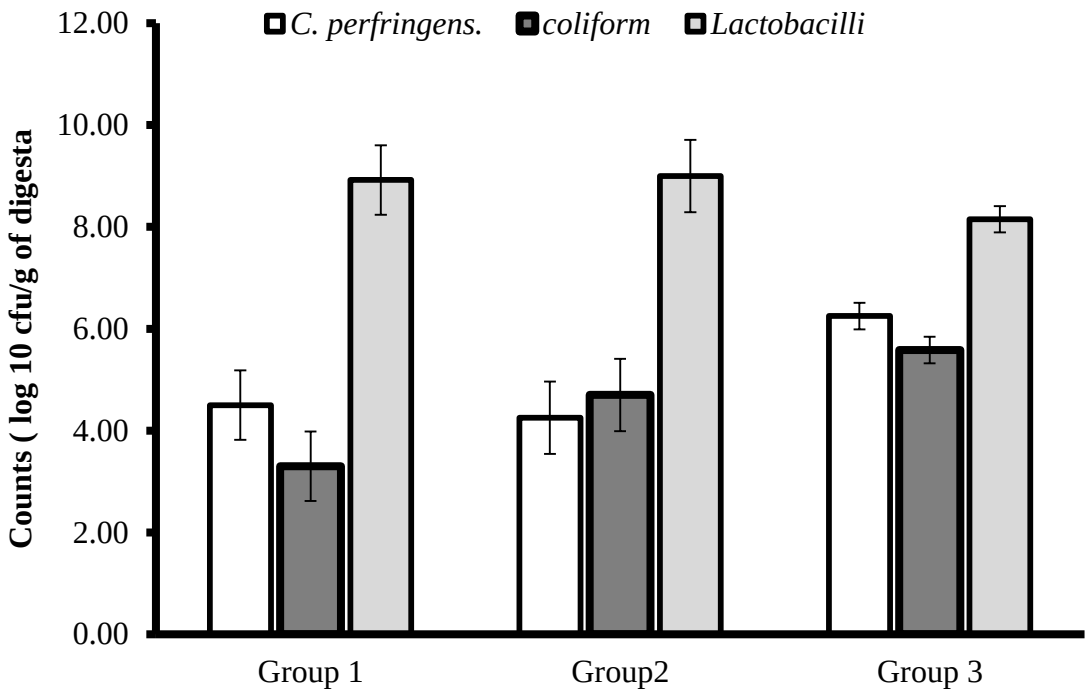

Figure 3.4: Ileal digesta microflora of broilers chickens on day 28 post hatch subjected to different period of feed withdrawal: Group 1: full fed control; Group 2: 8 and 15 hrs feed withdrawal on days 10 and 18, respectively; Group 3: 15 and 24 hrs feed withdrawal on days 10 and 18, respectively. The error bar is the standard error of the mean (SEM).

3.3.4 Growth performance

Table 3.3 shows the effect of feed withdrawal treatment on averaged feed intake (FI) and feed conversion ratio (FCR) during days 0-10, 10-14, 14-21 and 21-28. During days 10-14, Group 1 birds had significantly higher FI than Group 2 and 3 birds, but feed withdrawal did not significantly affect FI during days 14-21 (Table 3.3). However, during days 21-28, Group 2 birds had higher FI than Group 3 birds. Group 2 birds had significantly better FCR compared to Group 1 birds during days 10-14. There was a trend for FCR to be decreased in Group 2 birds during days 14-21 (Table 3.3) but there was no significant effect of feed withdrawal on FCR during days 21-28.

Table 3.4 shows the effects of feed withdrawal on average body weight (BW) of broilers on days 10, 14, 21 and 28 as well as on average weight gain (WG) during days 10-14, 14-21 and 21-28 of birds. On days 14 and 21, Group 3 birds had smaller BW than Group 1 and Group 2 birds. However, on day 28, Group 2 birds had significantly higher BW compared to Group 1 and 3 birds. During days 10-14 and 14-21, Group 2 birds had significantly higher WG than Group 1 and 3 birds. However, feed withdrawal had no effect on the WG during 21-28 (Table 3.4).

Table 3.3: Average feed intake and feed conversion ratio of groups of broilers on different days in different treatment groups

Feed withdrawal			Feed intake (g/bird/period)				Feed conversion ratio			
Groups	Day 10	Day 18	0-10	10-14	14-21	21-28	0-10	10-14	14-21	21-28
1	0 h	0 h	248	293[a]	690	820[ab]	1.30	2.16[a]	1.87	1.89
2	8 h	15 h	247	247[ab]	665	873[a]	1.26	1.64[b]	1.57	1.76
3	15 h	24 h	244	216[b]	663	792[b]	1.23	1.94[a]	1.92	2.02
Probability of treatment effect			0.935	0.025	0.829	0.051	0.499	0.029	0.088	0.362
SEM[1]			9.7	16.3	34.4	18.1	0.03	0.11	0.10	0.12

Means within a column without a common superscript differ significantly ($P<0.05$)

[1]SEM: Standard error of means

[2]Data represents the mean of four pens with 20 birds per pen

Table 3.4: Average body weight and weight gain of groups of broilers on different days in different treatment groups

Feed withdrawal			Average body weight (g)				Average weight gain (g)			
Groups	Day 10	Day 18	10	14	21	28	0-10	10-14	14-21	21-28
1	0 h	0 h	231	367[b]	735[b]	1174[a]	191	135[b]	369[a]	439
2	8 h	15 h	237	387[a]	809[a]	1306[b]	196	150[b]	422[b]	497
3	15 h	24 h	238	349[c]	695[c]	1096[a]	197	111[c]	346[a]	401
Probability of treatment effect			0.266	0.001	<0.001	0.019	0.301	<0.001	<0.001	0.203
SEM[1]			2.8	5.0	11.0	37.1	2.81	3.4	8.4	34.9

Means within a column without a common superscript differ significantly (P<0.05)

[1]SEM: Standard error of means

[2]Data represents the mean of four pens with 20 birds per pen

3.4 Discussion

Although the GIT constitutes only 1.5% of the body weight of chickens, it consumes 6-8% of the dietary energy (Spratt *et al.*, 1990). Therefore short term feed withdrawal can cause rapid alterations in intestinal morphology and mucus secretion which in turn can dramatically reduce the integrity of the intestine (Thompson & Applegate, 2006). Loss of intestinal integrity may leave birds prone to intestinal diseases like sub-clinical NE. Although *C. perfringens* is commonly found in the intestinal tract of poultry, its population can vary considerably from a few to 10^4 cfu/g of digesta and the occurrence of NE is sporadic (Cowen *et al.*, 1987; Engberg *et al.*, 2002; McDevitt *et al.*, 2006b).

During the present experiment none of the treatment groups produced overt clinical signs of NE (depression and ruffled feathers) subsequent to *C. perfringens* challenge in any of the treatment groups. Typical gross lesions of NE consist of marked distensions of the small intestine, with necrotic lesions varying from 0.5-1.5cm in diameter, randomly distributed either singly or in the form of aggregates. In severe cases entire duodenal and jejunal mucosa were covered by a yellow-brown diphtheritic membrane, together with a bile stained fluid or a core of granular debris in the lumen of the intestine (Long, 1973; Shane *et al.*, 1985). On day 12 of the experiment, necropsy findings did not show any gross lesions in the small intestines of any of the feed withdrawal treatment group. However on day 20, lesions like mild focal changes and/or small necrotic patches were seen in two birds from group-3. On day 28, lesions similar to sub-clinical NE were found in two, three and one birds from groups 1, 2 and 3 respectively. In most of the birds gross lesions were very mild and inconclusive for NE (e.g. intestinal wall was very friable thin and hyperaemic with the mesenteric vessels engorged with blood).

Microscopic lesions of sub-clinical NE are characterized by severe necrosis of intestinal mucosa, with fibrin that is mixed with cellular debris adherent to necrotic mucosa, as well as large clusters of *C. perfringens* present in the necrotic mucosa. Marked infiltration of heterophil granulocytes are also observed in the intestinal mucosa (Long *et al.*, 1974; Kaldhusdal & Hofshagen, 1992). However, histopathological examination of microscopic slides confirmed that lesions observed in the current experiment were not typical of NE although some coccidial oocytes were seen in the

intestinal mucosa. Gram staining further confirmed the absence of Gram-positive rod-shaped organisms in the intestinal mucosa. Although mucosal scrapings (done at the time of necropsy only from birds that exhibited lesions on different dissection days) did not show any coccidial oocytes, numerous coccidial oocytes were seen scattered in the villi and crypts of the intestinal mucosa (Figure 3.3). Shane *et al.* (1985) clearly demonstrated that concurrent or prior infection with *E. acerulina* exacerbated the lesion score, the severity of histological lesions and mortality in broiler chicks. coccidiosis has been used as factor that predisposes birds to NE (Alsheikhly & Alsaieg, 1980; Shane *et al.*, 1985).

Although feed withdrawal did not significantly affect the counts of different gut flora, overall the Group 2 birds showed a better bacterial profile in terms of lower numbers of *C. perfringens*, *coliforms* and higher counts of *lactobacilli*. In the present study, numbers of *C. perfringens* ranged between 1.8 x 10^4 and 3.2 x 10^6 cfu/ml of digesta. Some authors have found *C. perfringens* counts ranging from 10^5 to 2 x 10^8/g of small intestine contents of birds suffering from NE (Long *et al.*, 1974; Cowen *et al.*, 1987). Increased number of *C. perfringens* as much as log 4.5 has been observed in ileum in the clinically healthy birds (Dahiya *et al.*, 2005a). Necrotic enteritis is said to occur when the number of *C. perfringens* reaches above 10^8 cfu/g of digesta (Kaldhusdal & Hofshagen, 1992). On the basis of that definition, the levels of *C. perfringens* were found in this study appear to suggest that any lesions observed could have indicated the initiation of sub-clinical NE.

Much of what is currently known regarding the effect of feed withdrawal on gut microflora in broilers has been provided by experiments that have examined feed withdrawal near slaughtering age. Thompson *et al.* (2008) found that as feed withdrawal time increases, the uniformity of the microbial population of the intestine decreases. Many populations of bacteria have been shown to coexist with *C. perfringens*. One of the largest populations of bacteria found in intestine along with *C. perfringens* is *coliform* bacteria (McReynolds *et al.*, 2004b; Collier *et al.*, 2008). In the present study, there was tendency of Group-3 birds to have higher numbers of *coliform* and lower numbers of *lactobacilli* than Group-1 birds (full fed control). These results are similar to Hinton *et al.* (2000) who reported that feed withdrawal periods up to 24 hrs caused increase in counts of *Enterobactericeae* together with a decrease in *lactobacilli* in broilers. Our results also agree with those of Northcutt *et al*. (2003a; 2003b) who did

not observe any significant effect of feed withdrawal on coliform, *E. coli* and *Salmonella* counts on carcasses after 12 hrs of feed withdrawal.

Fukata *et al.* (1991) concluded that when changes in the intestinal environment take place, such as a reduction in major microflora like *lactobacillus*, this can result in an increase in the number of *C. perfringens* which in turn may result in increased production of α-toxin and the resultant breakout of NE. Although not statistically significant, the trend towards a numerical increase of *C. perfringens* should not be ignored. Coupled with other predisposing factors, this increase of *C. perfringens* may contribute to increasing the risk of clinical outbreaks or the development of subclinical NE in broiler chickens under intensive rearing conditions. Thompson *et al.* (2008) also found a reduction in the microbial population and diversity of ileum as feed withdrawal time increased from 0 hr to 24 hrs. Hinton *et al.* (2000) showed that feed withdrawal periods up to 24 hrs caused increases in caecal *enterobacteriacae* population as well as the aforementioned decreases in lactobacilli of broilers. The exact mechanism by which feed withdrawal alters the bacterial population is not fully understood (Thompson *et al.*, 2008). Individual host factors may be contributing to the shifts observed in response to feed withdrawal.

The growth performance of the birds in the experiment was comparable with commercial levels (Aviagen, 2009). In the current study, *C. perfringens* challenge had not caused an inhibition of average BW at day 10 (i.e. compared to commercial expectations). We were expecting that there would be a trend for average FI to be lower during study days 10-14 in group-2 and group-3 birds, since both groups were subjected to feed withdrawal for 8hrs and 15hrs respectively. Unexpectedly, on days 21-28 group-2 consumed more feed (Table 3.3) compared to group-1 (full fed control). During the study period between 10-14 and 14-21, feed withdrawal treatment did not affect the feed intake of the different groups, although feed was withheld for 8hrs and 15 hrs for group 2 and 3 respectively. Similar results were seen on study days 14-28, although interestingly on days 21-28 the birds of group-2 had significantly higher average FI than group-3. The reason for this may be that group-2 over consumed the feed after it had been returned. Short periods of feed withdrawal in fact act as a stimulus to consumption (Washburn & Bondari, 1978). Birds learn quickly to eat normal quantities of food after feed restriction by limiting the time of access to food (Lee *et al.*, 1971). The increase in growth rate of birds of group-2 between 14-21 days is perhaps an example of better

protein utilization and accelerated growth. Birds with restricted access to feed can more efficiently utilize protein from the diet than unrestricted birds when feed is resumed after feed restriction (Fontana *et al.*, 1992). Plavnik & Hurwitz (1990) suggested that growth could be promoted by using a mild feed restriction at an early age. The non-significant results in average WG of birds at 21-28 days of age was expected in response to feed withdrawal on days 10 and 18 only. The numerically increased growth in group-2 at 21-28 days of age was associated with increased feed intake.

Different feed withdrawal periods in the present experiment did not have an effect on the overall incidence of subclinical NE. This disease is not readily reproduced under experimental conditions. Parish, (1961) did not find any effect on the course of NE due to change of diet, including supplements of vitamins and minerals, although some scientists (Shane *et al.*, 1985) were able to successfully produce NE by giving 24 hours of fasting prior to offering infected feed to the experimental birds. However, they gave considerably higher dosages of coccidial oocytes ($3.5x\ 10^5$) orally and very high numbers of *C. perfringens* (2.5×10^8 cfu/g of feed) over many days. In the present study the coccidial vaccine was given at the prescribed dose rate of 3000 coccidial oocytes/0.1ml of vaccine and 10^4 cfu of *C. perfringens* only once orally.

To our knowledge, the current experiment is the first to have assessed the effect of feed withdrawal in isolation on sub-clinical NE in young broiler chickens. Although feed withdrawal has been applied in most of the previous controlled experiments (Pedersen *et al.*, 2003; Jia *et al.*, 2009), even when birds were challenged with very high numbers of *C. perfringens*, these experiments also failed to induce NE (Pedersen *et al.*, 2003). It is known that during short hours of starvation more digestive enzymes, such as trypsin are released with trypsin recognised as inactivating the α-toxin of *C. perfringens* (Baba *et al.*, 1992). It may also be possible that paracox-5® used in the present experiment was unable to induce sufficient gut damage. Although mucin quantity was not measured in the present study it is not impossible that insufficient mucin was produced as a result of starvation.

3.5 Conclusion

The apparent lack of expected results may indicate that, if fasting with *C. perfringens* challenge is indeed predisposing to sub-clinical NE, there must be some other critical variables involved. The findings of the current study appear to indicate that feed withdrawal of the duration used in the present study did not predispose birds to (sub-clinical) NE. Furthermore, as specific lesions were not observed and *C. perfringens* counts in digesta were also low we conclude that feed withdrawal alone does not predispose birds to sub-clinical NE.

4 EXPERIMENT TWO:

INTERACTIVE EFFECTS OF DIET COMPOSITION AND LITTER CHALLENGE ON THE INCIDENCE OF SUB-CLINICAL NECROTIC ENTERITIS IN BROILER CHICKENS

4.1 Introduction

Over the last few years there has been growing interest in utilising soybean meal (SBM) and canola meal (CM) as the major, high quality vegetable protein source in poultry diets. In countries where high prices of various protein sources make their use limited for poultry diets, this results in changes to both the sources and quality of protein in poultry diets (Palliyeguru *et al.*, 2010). A higher than recommended level of dietary protein is believed to be a major predisposing factor affecting the incidence of sub-clinical NE in poultry. High SBM prices limit its use, compared with CM which is available for a much lower cost. However, the nutritional value of CM is limited due to the presence of a number of anti-nutritive factors, including indigestible non-starch polysaccharides and a high phytate (3-6%) content (Bell, 1984; Slominski & Campbell, 1990). Phytate is known to reduce amino acid digestibility (Selle *et al.*, 2000) thus providing increased nutritional opportunities for *C. perfringens* to grow and release α-toxin. On the other hand it also inhibits the activity of digestive enzymes like trypsin and so reduces the degradation of the α-toxin associated with NE. However, whilst there is some information available on the anti-nutritive effects of CM (Bell, 1984; Slominski and Campbell 1990), the literature is lacking detail regarding a possible effect of CM on *C. perfringens* proliferation and concurring incidence of (sub-clinical) NE.

A recently identified dietary factor that may be responsible for NE is the presence of certain anti-nutritional factors, like trypsin inhibitors (TI), in feed. These TIs can vary between different poultry diets, and a concentration of 3µg/ml of TI could possibly occur in practical feed samples (Probert, 2004). Higher than normal levels of TI in a diet may help to stabilise the α-toxin that is responsible for NE, possibly by reducing the surplus of pancreatic trypsin so preventing possible degradation of α-toxin by trypsin, the main factor for induction of NE (Palliyeguru *et al.*, 2011). Recently potato protein concentrate (PPC) has been shown to be an additional risk factor due to its higher antitrypsin activity (Palliyeguru *et al.*, 2010; Fernando *et al.*, 2011). Birds fed diets with a high potato protein content (417.2g/kg) had higher counts of *C. perfringens* when compared with birds fed diets based on other plant proteins (Wilkie *et al.*, 2005). Palliyeguru *et al.* (2010) also found a higher incidence of NE in birds fed potato protein based diets compared to soy protein.

In addition to diet, the condition of litter is another overlooked factor that can be responsible for inducing (sub-clinical) NE. The litter used for the bedding in poultry houses can be composed of wood chips, sawdust, wheat straw, or peanut hulls, and may play a role in possible colonization of microbes in birds' guts since *Clostridia* species have frequently been isolated from poultry litter (Alexander *et al.*, 1968). Poor litter quality often provides an excellent environment for *C. perfringens* spores to accumulate, leading to a potential source of infection (McReynolds *et al.*, 2007). This directly provides an important additional factor by creating suitable conditions for sporulation and growth of C. *perfringens*, but also indirectly facilitates other predisposing factors such as the sporulation of coccidial oocytes (Williams, 2005). In addition, rough litter has been suggested to result in minor gut damage that in the presence of sufficient *C. perfringens* numbers may cause NE (Alsheikhly & Truscott, 1977a).

Therefore the objective of this experiment was to study the effect of three sources of vegetable proteins (SBM, PPC and CM) in nutritionally complete diets, with similar protein contents (crude protein 212 g/kg), on the incidence of NE in male broiler chickens with clean and reused litter. A fourth dietary treatment, with synthetic TI (6µg/ml) added to the SBM control diet, was used to study its impact on onset of sub-clinical NE.

Reused litter was used as challenge to aim to produce sub-clinical NE in order to mimic, as closely as possible, the naturally occurring infectious conditions, and so to avoid the need to frequently dose birds with extremely high levels of the pathogen (Figure 4.1 to Figure 4.4).

Figure 4.1: Broiler chickens reared as single flock during 0-16 days of experiment.

Figure 4.2: Addition of reused litter.

Figure 4.3 : Broiler chickens reared in different experimental pens with clean and reused litter.

4.2 Materials and Methods

4.2.1 Treatment Groups and Experimental Design

A total of 144 one-day-old male Ross 308 broiler chickens were obtained from a commercial hatchery. The birds were reared in a solid floored room following a standard commercial environmentally controlled programme from day 0 to day 32. Adequate feeders and drinkers were provided for the age and number of birds. For the first 16 days birds were reared as a single flock and given a nutritionally complete broiler starter diet (without antibiotic growth promoter or anticoccidials). On day 16 post hatch, the birds were weighed and stratified on body weight (BW) and 6 birds randomly allocated to each of the 24 pens with similarly averaged day 16 BW. The design of the experiment is shown in Table 4.2.

4.2.2 Feeding treatments

Four feeding treatments were designed for this study. Three experimental broiler grower diets were formulated to be nutritionally complete for broiler chickens between 16 to 30 days of age. Diets were wheat based although the major portion of the additional protein supply was provided by one of three protein sources, i.e. SBM, CM and PPC. A fourth dietary treatment consisted of the SBM treatment with added synthetic TI (Sigma-Aldrich; T9003), referred to as SBM+TI. A concentration of synthetic inhibitor at a level of 6µg/g of feed was added to the prepared feed – this is a higher concentration than would normally occur or be possible in practical feed samples (Probert, 2004).

All diets were formulated to have similar contents of calculated metabolizable energy (13.4MJ/Kg) and crude protein (212g/kg) (Table 4.1). Vitamins and minerals either met or exceeded the breed-nutritional recommendations (Broiler Ross 308, 2009). No anticoccidial drugs or antibiotic growth promoter were added to the grower diets. The formulated diets were mixed at SAC, Ayr and provided as mash. In order to formulate diets with similar content of calculated crude protein (212 g/kg) and metabolizable energy (13.4MJ/kg), the diets were varied by adjusting the vitamin and mineral content. This was felt unlikely to have influenced the result(s) since the effect produced was counter to that observed. Experimental grower diets were given from day

16 until the end of the study. Feed and water were provided *ad-libitum* during the experimental period. Light was provided for 23 hours per day and house temperatures and humidity were controlled to provide optimum growing conditions for the age and breed of birds used (Aviagen, 2009).

4.2.3 Litter treatment (Challenge procedure)

The floor area of (1.74m x 1.28m) per pen was covered with wood shavings. For half of the pens, litter consisted of 75% clean wood shavings and 25% reused litter material (reused litter). The reused litter material was obtained from a commercial poultry flock that did not have a history of clinical or sub-clinical NE although sub-clinical coccidiosis was expected. The other half of the pens had 100% clean wood shavings (clean litter). All experimental procedures were approved by SAC Animal Ethics Committee (AE 27/2010) and carried out under Home Office authorization (PPL 60/4097).

Table 4.1: Feed ingredients and calculated chemical composition (g/kg) of the experimental grower diets.

	Dietary treatments		
	SBM[1]	PPC[2]	CM[3]
Ingredients			
Canola	-	-	217
Soybean Meal-48%	207	-	-
Potato protein concentrate	-	110	-
Soybeans full-fat, cooked	143	170	212.2
Wheat	557	620	460
Soybean oil	55	60	69
Dicalcium Phosphate	15	15.4	15.4
Limestone	2	2	2
Defluorinated Phosphorus	17	18.1	17
Common Salt	1.5	1.5	1.5
Vitamin & Mineral Premix[1]	1.8	3.0	3.3
DL-Methionine	1	-	1.5
L-Lysine HCl	-	-	1.5
Calculated Chemical Composition			
Metabolisable energy (MJ/Kg)	13.4	13.4	13.4
Dry Matter	868	872	876
Crude Protein	212	212	212
Calcium	10.6	10.6	11.7
Total Phosphorus	9.7	9.3	11.2
Available Phosphorus	4.4	4.2	4.6

[1] Vitamin and Mineral supplement provided (units kg^{-1} diets): Vit A 16,000 iu; Vit D_3 3,000 iu; Vit E 75 iu (iu=mg); Vit B_1 3 mg; Vit B_2 10 mg; Vit B_6 3 mg; Vit B_{12} 15 µg; Vit K_3 5 mg; Nicotinic acid 60 mg; Pantothenic acid 14.5 mg; Folic acid 1.5 mg; Biotin 275 µg; Choline chloride 250 mg; Iron 20 mg; Copper 10 mg; Manganese 100 mg; Cobalt 1 mg; Zinc 82 mg; Iodine 1 mg; Selenium 0.2 mg; Molybdenum 0.5 mg (Target feed, Whitchurch, Shropshire).

SBM[1]: Soybean meal

PPC[2]: Potato protein concentrate

CM [3:] Canola meal

4.2.4 Experimental design

A 4x2 factorial arrangement of the four feeding treatments and the two litter treatments was used in a randomized complete block design. Three replicate pens were used for each feeding-litter treatment combination with 6 birds per pen (Table 4.2). The 24 floor pens used were within three adjacent environmentally controlled rooms (eight pens per room), and the eight feeding-litter treatment combinations were randomly allocated to the eight pens within each of the three rooms.

Initially it was planned to collect the data for all feeding-litter treatment combinations on days 30, 31, and 32. However, to comply with HO regulations, it was necessary to change from the initial plan since at day 21 the birds in CM feeding treatment groups were found to suffer from stunted growth (see Results), and had to be culled. The birds on all other feeding treatments were kept alive until day 32. This inevitably resulted in two data sets; one for the CM fed birds to day 21 and a second for all other feeding treatment groups.

Table 4.2: Experimental treatments used in the experiment

	Litter treatment	Feeding treatment
1	Clean	SBM
2	Reused	SBM
3	Clean	SBM + TI
4	Reused	SBM + TI
5	Clean	PPC
6	Reused	PPC
7	Clean	CM
8	Reused	CM

SBM: Soybean meal; SBM + TI: Soya with added synthetic trypsin inhibitor; PPC: Potato protein concentrate; CM: Canola meal.

4.2.5 Sampling and data recording

4.2.5.1 Clinical signs and lesion scoring

Experimental birds were assessed daily for clinical signs and symptoms of NE, from the day reused litter was added to the litter pens. At day 21, all the birds from CM dietary treatment with both clean and reused litter were weighed and culled by

intravenously administered overdose of barbiturate. In contrast, for all other feeding treatment groups, two birds from each replicate pen were selected at random, weighed and killed by intravenously administered overdose of barbiturate on days 30, 31, and 32. These days were taken because it was not possible to sample and dissect all the birds within a single day. The intestines of all killed birds were removed and the ileal contents collected individually. The three sections of intestine (duodenum, jejunum and ileum) were identified, immediately incised, washed with phosphate buffer saline (PBS) and the mucosal surfaces inspected for evidence of lesions (*Clostridial* or coccidian). It was intended to use the scoring system established by Shane *et al.* (1985):

- score 0: absence of gross lesions or no lesions;
- score 1: focal necrosis or focal ulceration;
- score 2: focal ulceration coalesced to form discrete patches;
- score 3: extensive diffuse mucosal necrosis;

The livers were also examined for the presence or absence of hepatitis or cholangiohepatitis, which are recognized as consistent with the pathological changes of NE.

4.2.5.2 Histopathology

Following post-mortem examination, samples from the small intestine, particularly those showing lesions were taken for histopathology. A 1.5-2cm tissue sample was taken, washed with PBS and then stored in 10% buffered formalin for histopathology. Histopathology was done according to the procedure described in section 3.2.5.3. Gram staining was also done to confirm the presence of Gram positive bacilli (Appendix- B). The prepared tissue sections were later examined using a binocular stereo-microscope (Olympus BX 41, U-LH100HG, Olympus optical, Co. Ltd) connected by camera (spot idea™ 28.2-5MP) to computer software (Spot idea, Version 4.7) using different magnifications (10x, 40x and 100x).

4.2.5.3 Quantification of *C. perfringens* and α-toxin in digesta

Ileal digesta samples were taken into labelled plastic containers with screw-topped lids. Samples were immediately transferred to the microbiology laboratory and analysed to enumerate *C. perfringens* within 2hrs of collection. Enumeration of *C. perfringens* on Tryptose Sulphite Cycloserine (TSC) agar (Oxide) was performed according to the method described in section 3.2.2.

Quantification of α-toxin in the intestinal digesta was done by an enzyme linked immunosorbent assay (ELISA) using α-toxin *C. perfringens* kit (Cypress diagnostics, Ref. Vb040) following the manufacturer's recommendations as described in section 3.2.5.5.

4.2.5.4 Growth performance

Starter feed was weighed in at day 0 and weighed back at day 16. Then grower feed was weighed in at day 16, and weighed back at day 21 (CM only), and day 30 (SBM, SBM+TI, PPC). Averaged feed intake (FI) was recorded by subtracting feed weighed out from feed weighed in divided over the number of birds in the pen. At day 0, all birds were weighed and individually tagged. At days 16 all birds were weighed individually again. The growth of the birds was subsequently recorded by weighing the birds at day 21 (CM only) or 30 (SBM, SBM +TI, PPC). Average weight gain (WG) was calculated as average live bird weight at the end of the period – average live bird weight at the beginning of the period. Feed conversion ratio (FCR) was calculated by dividing the average feed consumed per pen by the average weight gain of birds per pen.

4.2.5.5 Analysis of litter

Litter was analysed for enumeration of *C. perfringens* and coccidial oocytes at days 16 and 21 (CM only) or 30 (SBM, SBM +TI, PPC). Sterilized gloves and plastic bags were used to collect litter samples from the pen centre, as well as mid way between the centre and each of the four corners of every pen. Litter samples collected were then combined and homogenized.

A representative sample of collected litter was enumerated for *C. perfringens* on TSC agar using the pour plating technique. Litter sample aliquots of approximately 10g were weighed out and suspended in 90ml of Maximum recovery diluents (MRD). From this dilution further 10 fold dilutions were made. From each dilution to be plated, 100µl of mixture was applied to the centre of each agar plate, allowed to be absorbed and then 15ml of TSC overlay was spread before the plate was incubated invertedly in a jar providing anaerobic conditions. The jar was placed in an incubator at 37°C for 24hrs. After incubation the number of colonies was counted to calculate the number of colony forming units per gram of original sample. All samples were analysed in duplicate.

For counting of coccidial oocytes in the litter, 30g of litter was weighed. The litter sample was placed in 200ml of water overnight. The mixture was then shaken and poured into an 8 inch diameter 425µ mesh sieve resting in a round bottomed copper bowl. Debris was then spread over the area of the sieve. Strained fluid was then swirled in the round bottomed bowl to ensure even mixing. The resultant fluid was then poured into a universal tube to almost fill it. Tubes were centrifuged at 1500rpm for 2 minutes. The supernatant was then discarded and the tubes shaken to loosen any sediment. After replacing the lids, the tubes were gently inverted several times. Both chambers (sides) of a McMaster counting chamber were filled using a pipette. The counting chamber was allowed to stand at room temperature for a minimum of 1hour for the oocytes to settle. The number of coccidial oocytes (all species: small, medium and large) were counted at 10 x 10 magnification (Olympus microscope, BX 41, U-LH100HG, Olympus optical Co. Ltd). The sample preparation indicated that the number of oocytes counted needed to be multiplied by 22.5 to obtain the number of oocytes per g of original material.

The litter pH was also determined at the end of the experiment on day 21 (CM dietary treatment group) and 30 (all other dietary treatment groups). Litter pH was determined by using a pH probe with a stainless steel penetration blade attached to Hanna HI 99163 meter (Hanna Instruments Ltd, Bedfordshire, UK). Litter pH was measured directly from the litter, from the centre of each pen.

4.2.5.6 Feed Analysis

Feed samples were ground before chemical analysis. For amino acid analysis, Diet samples were oxidised with a hydrogen peroxide / formic acid / phenol mixture. Excess oxidation reagent was decomposed with sodium metabisulphite. The oxidised sample was hydrolysed with 6M hydrochloric acid for 24 hours. The hydrolysate was adjusted to pH 2.20, centrifuged and filtered. The amino acids were separated by ion exchange chromatography (Biochrom 20 analyser, Amersham Pharmacia Biotech, Pittsburgh, PA) and determined by reaction with ninhydrin using photometric detection at 570nm (440nm for Proline).

Trypsin inhibitor activity of each diet was determined through extraction using dilute sodium hydroxide solution at pH 9.5 and incubating an aliquot of the unfiltered extract with a standard amount of trypsin. The amount of trypsin remaining in the sample was measured by reaction with the synthetic substrate Benzoyl-DL-arginine-P-nitroanilide for a specific time and temperature, causing the formation of the yellow coloured p-nitroaniline. After filtration the colour intensity of the complex was measured spectrophotometrically at 410nm. Within the limits of the test there was a linear relationship between the quantity of p-nitroaniline released and trypsin inhibitor activity.

4.3 Statistical analysis

As mentioned earlier due to stunted growth and some signs of depression the birds from the CM dietary treatment were culled on day 21 of the experiment and their intestinal tracts examined for the presence of lesions. Therefore the CM dietary treatment could not be directly compared with the other feeding treatments. The CM data set was analysed for the effect of litter treatment only (i.e. clean vs reused litter) using analysis of variance (ANOVA) Genstat 11 for Windows, IACR Rothamstead, England). Bacterial enumeration data was converted to $\log_{10}$ cfu/g before analysis.

The effect of other combinations of feeding treatments (SBM, SBM+TI, PPC) and litter treatment (reused and clean) on the data obtained were compared using 3x2 factorial arrangement with a randomised block analysis of variance (ANOVA) (Genstat 11 for Windows, IACR Rothamstead, England). Room was included as a block. The

partitioned sources of variation included treatment, litter and their interaction. An individual pen was treated as the experimental unit.

Because of the skewed nature of the counts of *C. perfringens*, data was transformed according to log (n + 1) to normalize the data before statistical analysis. Duncan's multiple range test was used to separately significantly different means. Effects were reported as significant at $P<0.05$.

4.4 Results

During the experiment no clinical signs of NE were observed following the litter challenge in any of the treatment groups. However, birds from the CM dietary treatment group were stunted, slightly dull, and depressed with ruffled feathers (Figure 4.4). Only one bird during the experiment was found dead, from the CM-reused litter treatment group. This was on day 16 of the experiment and was diagnosed as death from an acute heart failure. Feed analysis showed that the trypsin inhibitor activity of the four diets (SBM, SBM+TI, PPC and CM) was the same, 0.8mg/g of feed.

Figure 4.4: Bird showing signs of depression fed canola meal dietary treatment with litter challenge.

4.4.1 Gross lesions

On day 21, none of the birds had full blown lesions of sub-clinical NE in the small intestine in the CM dietary treatment groups. Some lesions were seen in 14 out of 36 CM chickens, which were all from the reused litter treatment, although lesions were mostly of coccidiosis (Figure 4.5). Lesions observed were distended jejunum and ileum as well as thin, friable intestinal walls. No necrotic lesions were identified in the small intestine of the birds fed a CM diet in clean litter pens.

On days 30, 31 and 32, none of the SBM, SBM+TI and PPC chickens had definite sub-clinical NE gross lesions in their small intestines. However, some lesions of coccidiosis were seen in two SBM+TI birds and one PPC bird, which were all from the reused litter treatment.

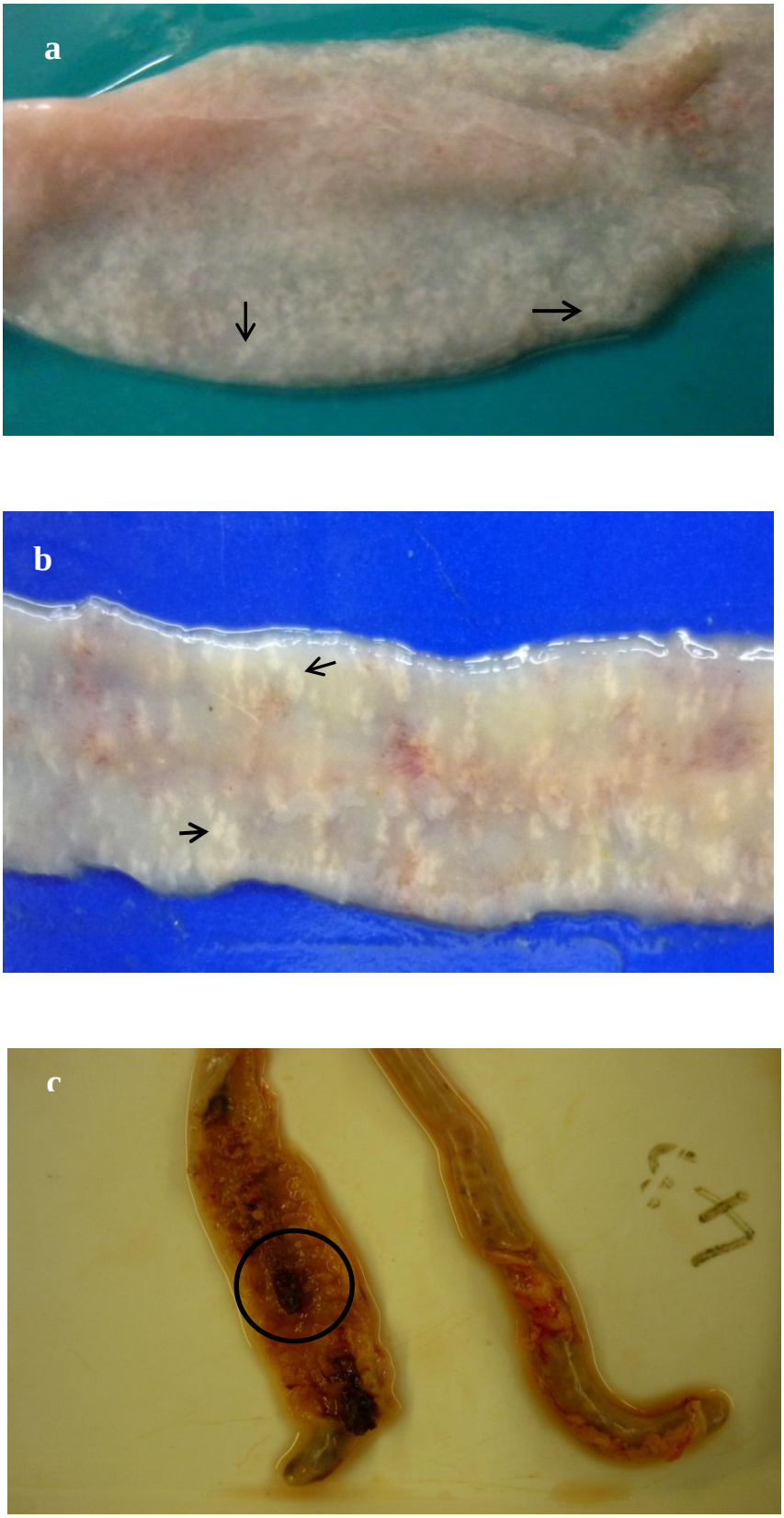

Figure 4.5: Mucosal surface of intestines of broiler chickens fed canola meal dietary treatment group with challenged litter (a) Duodenum (b) Ileum, arrows showing white lesions of coccidiosis (c) caeca, circle showing haemorrhages.

4.4.2 Histopathology

Histological examination of formalin fixed intestinal tissue confirmed that the lesions were not NE specific. There was no necrosis or desquamation of epithelial cells in the villi. Lamina propria did not show infiltration of polymorphs nuclear cells in any section of intestine. There was also no evidence of gram-positive rod-shaped organisms attached to intestinal mucosa. However various sexual and asexual stages of coccidia were found in many intestinal sections from CM birds on the reused litter treatment. Endogenous stages of *Eimeria acervulina* were particularly predominant in their intestinal tissue sections (Figure 4.6).

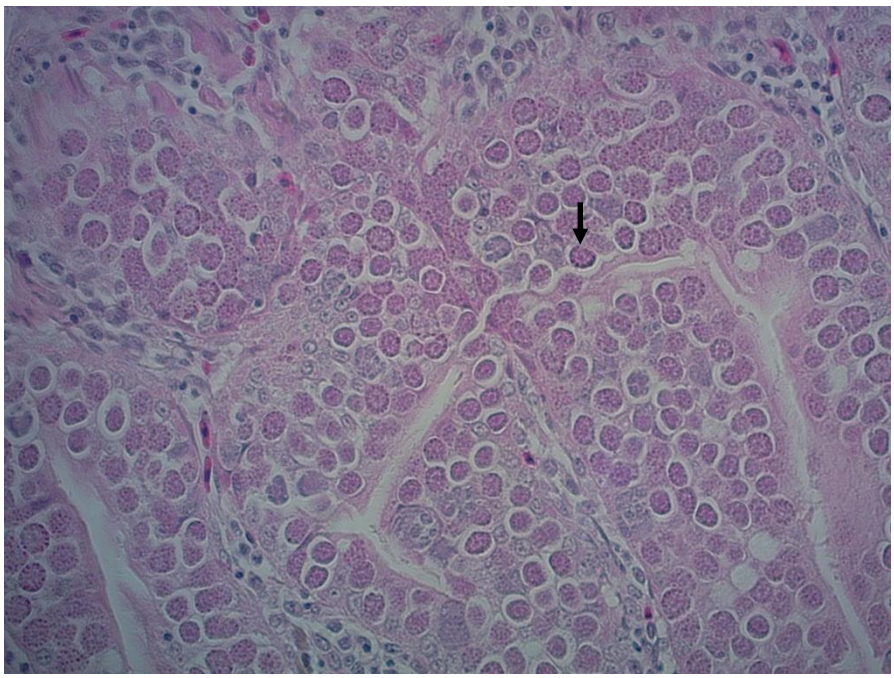

Figure 4.6: photomicrography of intestine of broiler chicken fed canola meal diet with litter challenge showing presence of shizonts of *Eimeria* acervulina (arrow) H and E (x40).

4.4.3 Quantification of C. perfringens and α-toxin in ileal digesta

Table 4.4 shows the effect of litter treatment on the number of *C. perfringens* (cfu/g) in ileal digesta of broilers fed CM diet. There was no significant effect of litter treatment on counts of *C. perfringens* in ileal digesta of these birds. Figure 4.7 shows the effect of feeding and litter treatment on *C. perfringens* concentrations in ileal digesta

of the SBM, SBM+TI and PPC birds. Neither feeding treatment nor litter treatment significantly affected concentrations of *C. perfringens* in ileal digesta (P>0.05). Alpha toxin was not detected in ileal samples of birds from any of the treatment groups on different dissection days

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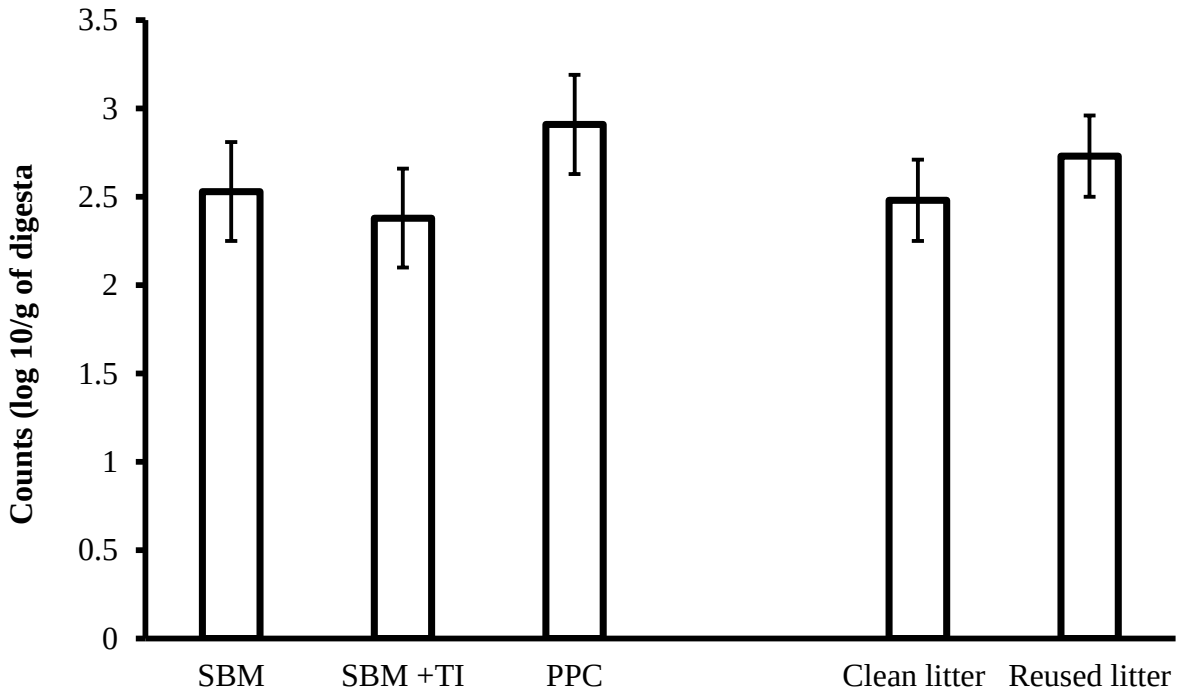

Figure 4.7: Concentrations of *C. perfringens* in ileal digesta of broiler chickens (on days 30-32 post hatch) subjected to different dietary treatments (SBM: soybean meal; SBM + TI: soybean meal with added synthetic trypsin inhibitor and PPC: potato protein) with and without litter challenge (clean and reused).

The error bars are the standard error of the mean (SEM).

4.4.4 Analysis of litter

The reused litter had *C. perfringens* and coccidial oocytes counts of 1.24 x 10^4 cfu/g and 30,686/g respectively, on day 16. Table 4.4 shows the effect of litter treatment on numbers of *C. perfringens* (cfu/g) and pH in the litter of broilers fed CM on day 21. There were no significant differences in *C. perfringens* concentrations and pH between clean and reused litter by day 21. Table 4.3 shows the effect of feeding and litter treatment on pH and *C. perfringens* concentrations in litter of the SBM, SBM+TI and PPC birds at day 30. None of these parameters were affected by feeding treatment or its interaction with litter treatment ($P>0.05$). However, reused litter had significantly increased pH ($P=0.02$) and counts of *C. perfringens* ($P=0.02$) compared to clean litter at day 30 (Table 4.3).

Table 4.3: Litter analysis (day 30) of different dietary treatment groups

	***C. perfringens* (cfu/g of litter)**	**pH**
Diet		
SBM	3.44	6.70
SBM + TI	2.77	6.60
PPC	4.08	6.46
SEM[1]	0.59	0.16
Litter		
Clean	2.48[a]	6.34[a]
Reused	4.38[b]	6.83[b]
SEM	0.49	0.13
Diet x Litter		
SBM x clean	2.49	6.54
SBM + TI	1.65	6.32
PPC x clean	3.29	6.16
SBM x reused	4.38	6.85
SBM + TI x reused	3.88	6.87
PPC x reused	4.87	6.75
SEM	0.84	0.23
Probability of differences		
Diet	0.34	0.59
Litter	0.02	0.02
Diet x litter	0.92	0.81

[1]SEM: Standard error of means

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

Data are means of 3 pens with 6 broiler chickens per pen

SBM: Soybean meal

SBM+ TI: Soybean meal with added synthetic trypsin inhibitor

PPC: Potato protein concentrate

4.4.5 Growth performance

Table 4.4 shows the effects of litter treatment on broilers BW on days 16 and 21 as well as on WG, FI and FCR during days 16-21 of birds fed the CM dietary treatment. On day 21, birds on reused litter had significantly lower BW than birds on clean litter (P=0.02). Birds on reused litter had significantly lower WG (P=0.042) and FI (P=0.03) during days 16-21 compared to birds on clean litter, which concurred with higher FCR (P=0.012; Table 4.4).

Figure 4.8 shows the effects of feeding treatments (SBM, SBM+TI, PPC) and litter treatment on BW on day 30. Final BW was not significantly affected by dietary protein treatments (P=0.11) or its interaction with litter treatment (P=0.17). However, litter treatment significantly reduced final BW (P= 0.01).

Figure 4.9 shows the effect of feeding treatments (SBM, SBM+TI, PPC) and litter treatment on broiler WG between days 16-30. Weight gain was not affected by dietary protein treatments (P=0.14) or its interaction with litter treatment (P=0.11). However, litter treatment significantly reduced the WG (P=0.009).Figure 4.10 shows the effects of feeding treatments (SBM, SBM+TI, PPC) and litter treatment on broiler FI during days 16-30. Feed intake was not affected by feeding treatment (P=0.82), by litter treatment (P=0.39) or their interaction (P=0.27).

Figure 4.11 shows the effects on broiler FCR during days 16-30. Feed conversion ratio was not affected by feeding treatment (P=0.84), by litter treatment (P=0.37) or their interactions (P=0.13).

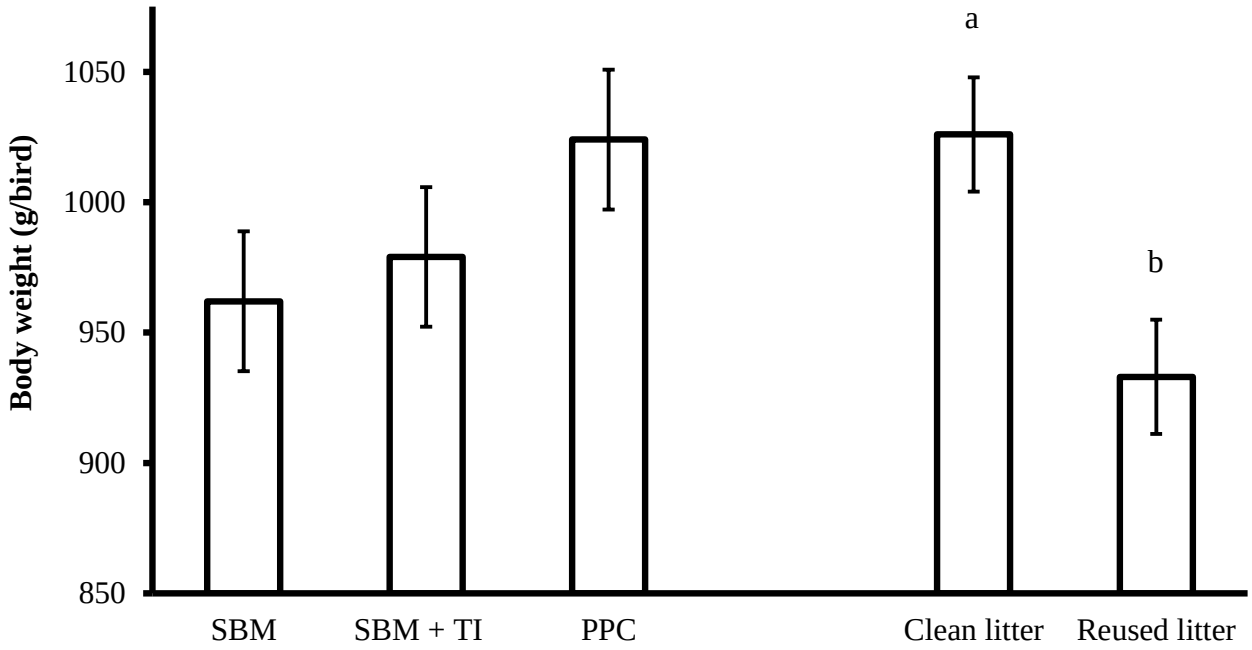

Figure 4.8: Body weight of broiler chickens (on day 30 post hatch) subjected to different dietary treatments (SBM: soybean meal; SBM + TI: soybean meal with added synthetic trypsin inhibitor and PPC: potato protein) with and without litter challenge (clean and reused).

The error bars are the standard error of the mean (SEM).

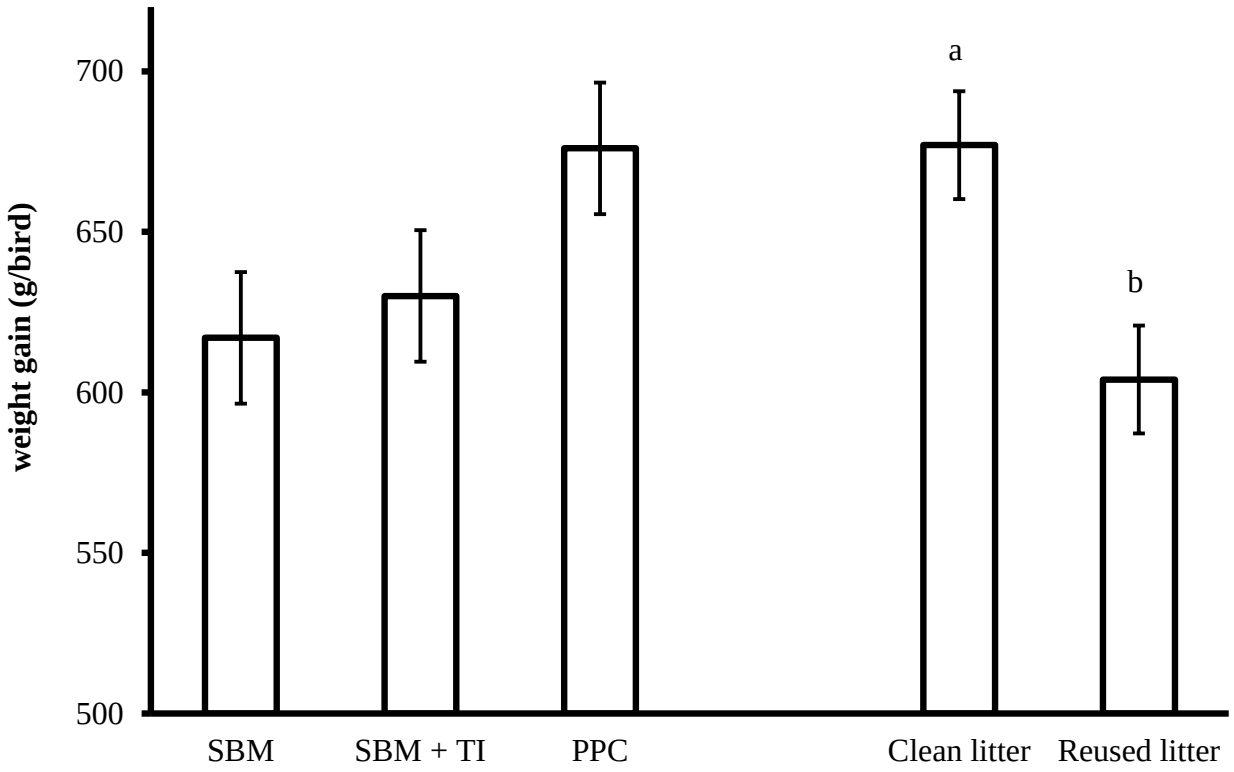

Figure 4.9: Weight gain of broiler chickens (on days 16-30 post hatch) subjected to different dietary treatments (SBM: soybean meal; SBM + TI: soybean meal with added synthetic trypsin inhibitor and PPC: potato protein) with and without litter challenge (clean and reused).

The error bars are the standard error of the mean (SEM).

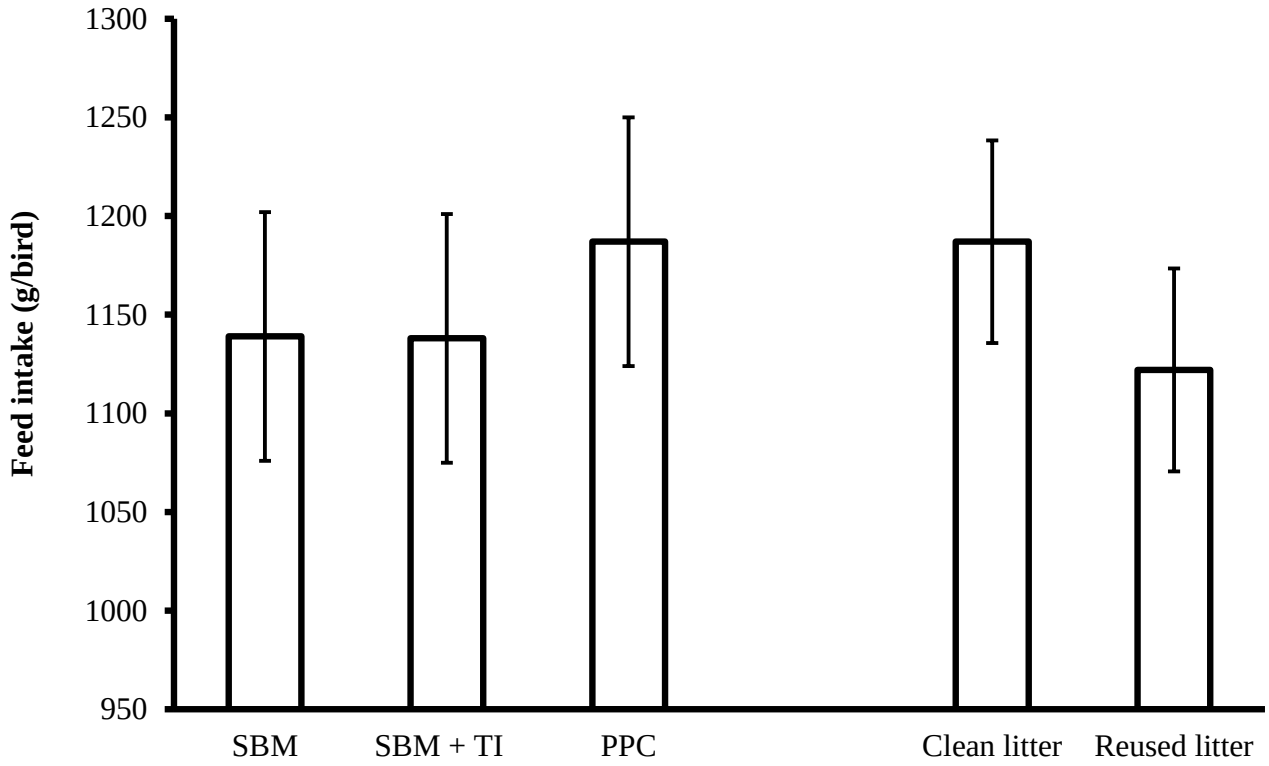

Figure 4.10: Feed intake of broiler chickens (on days 16-30 post hatch) subjected to different dietary treatments (SBM: soybean meal; SBM + TI: soybean meal with added synthetic trypsin inhibitor and PPC: potato protein) with and without litter challenge (clean and reused).

The error bars are the standard error of the mean (SEM).

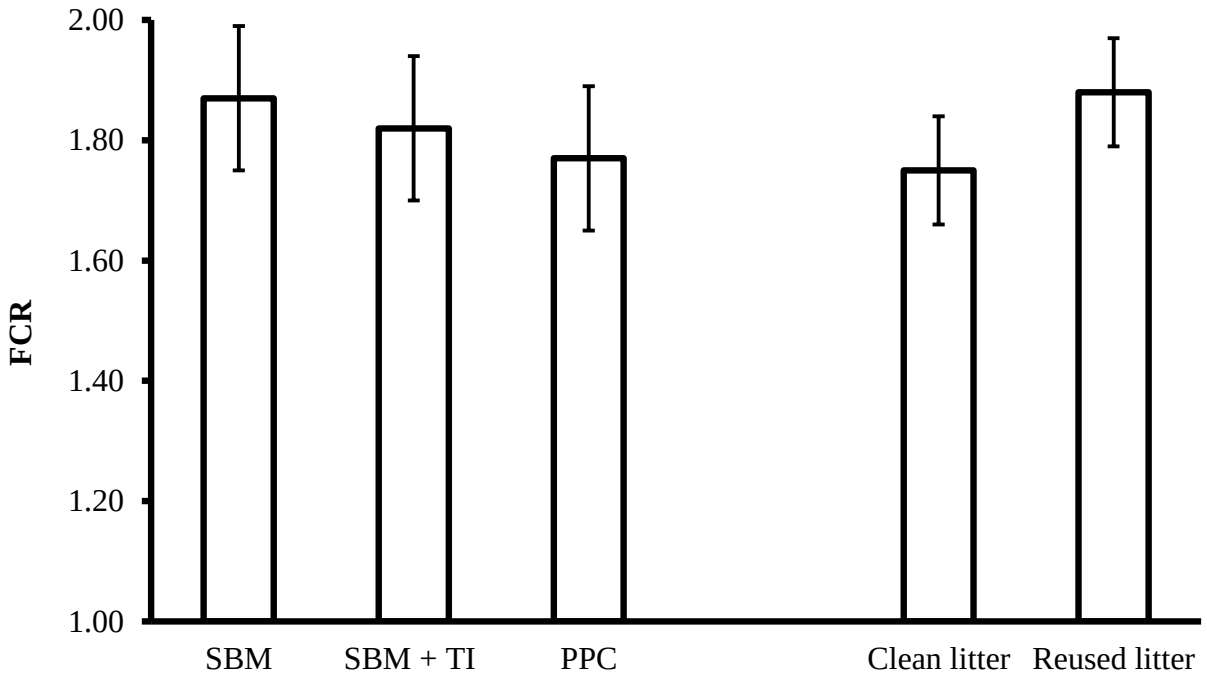

Figure 4.11: FCR of broiler chickens (on days 16-30 post hatch) subjected to different dietary treatments (SBM: soybean meal; SBM + TI: soybean meal with added synthetic trypsin inhibitor and PPC: potato protein) with and without litter challenge (clean and reused).

The error bars are the standard error of the mean (SEM).

Table 4.4: Growth performance, *C. perfringens* counts in ileal digesta and litter and litter pH (on day 21) of male broiler chickens fed canola meal with clean and reused litter.

Variable	Litter		SEM[1]	Probability of treatment effect
	Clean	Reused		
Body weight (day 16) (g/bird)	338	346	2.8	0.20
Body weight (day 21) (g/bird)	473[a]	446[b]	3.1	0.02
Weight gain (16-21) (g/bird)	135[a]	100[b]	5.2	0.042
Feed intake (16-21) (g/bird)	407[a]	377[b]	3.7	0.03
FCR[2] (16-21)	3.08[a]	3.80[b]	0.05	0.012
C. perfringens (log^{10}) (cfu/g of digesta)	3.15	3.91	0.4	0.32
Litter analysis				
pH	6.76	6.92	0.42	0.809
C. perfringens (log^{10}) (cfu/g of litter)	2.49	3.65	0.63	0.32

Data are means of 3 pens with 6 broiler chickens per pen

[1] SEM: Standard error of means

[2] Feed conversion ratio

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

4.5 Discussion

Until 2006 the occurrence of litter related problems, such as wet or poor litter quality, was successfully controlled by the use of in-feed growth promoters (Clarke & Wiseman, 2010). The litter used for bedding in poultry houses may play a role as reservoir for possible intestinal colonization of pathogen microbes, as pathogens such as clostridia have been isolated from poultry litter (Alexander *et al.*, 1968). It has long been suggested that a relationship exists between the type of litter and the incidence of NE under field conditions (Nairn & Bamford, 1967). *C. perfringens* is known to be transmitted to other birds within the same flock through litter ingestion as well as being transmitted to subsequent flocks placed on old litter (Craven *et al.*, 2001b). Literature has documented that an important component in the development of sub-clinical NE is the build up and recycling of *C. perfringens* in the litter (Alexander *et al.*, 1968; Nairn & Bamford, 1967; Craven *et al.*, 2001a; Craven *et al.*, 2001b). Therefore the present study aimed to challenge birds by exposure to reused litter from a commercial farm in order to stimulate the natural conditions for the development of sub-clinical NE.

None of the birds had overt clinical signs of NE following litter challenge. However on day 21, all birds from the CM dietary treatment showed signs of depression and stunted growth and were therefore culled. Necropsy findings showed lesions of coccidiosis with litter challenge in 14 out of 36 birds (39%). No birds on the SBM, SBM+TI and PPC treatments with reused or clean litter had any lesions of coccidiosis or sub-clinical NE, even though these birds were killed 10 days later. Histopathological examination from CM birds showing lesions confirmed that the lesions were typical of coccidiosis. The distribution of lesions and oocytes morphology at microscopic examination showed *Eimeria acervulina* as the primary species involved. The rapid appearance of coccidial lesions in the CM dietary treatment about 1 week after placement of challenged litter suggests that the litter had been seeded with a large, infected dose of coccidial oocytes by the previous commercial flock.

Although CM is increasingly replacing other sources of vegetable protein sources, its nutritional value is limited by the presence of a number of anti-nutritional factors such as a relatively high levels of phytate or phytic acid (3-6%), tannins (1.5-3%), sinapine (0.6-1.8%) and non-starch polysaccharides (NSPs; 18%). Phytate can bind minerals, reduce the digestibility of amino acids and can damage the mucosal layer

of the intestine (Bell, 1984; Bell, 1993) resulting in birds with a higher susceptibility to many intestinal diseases. Recent research has concluded that dietary tannins may not only affect the performance of chickens, but also alter the proper development of immunity against coccidiosis (Mansoori & Modirsanei, 2012). Coccidiosis has been recognized as a crucial factor that predisposes birds to NE. It has also been identified as having synergistic association with *C. perfringens* during the development of experimentally induced NE (Alsheikhly & Alsaieg, 1980; Shane *et al.*, 1985; Broussard *et al.*, 1986; Park *et al.*, 2008; Fernando *et al.*, 2011). As a consequence, coccidiosis is usually found when NE is diagnosed (Nairn & Bamford, 1967; Helmbold & Bryant, 1971; Williams, 2005). A number of studies have shown that chickens infected with *C. perfringens* and coccidial oocytes have higher rates of NE lesions than birds infected with the pathogen alone (Maxey & Page, 1977; Wicker *et al.*, 1977). This suggests that the occurrence of NE has a high correlation with coccidiosis. The reproductive stages of coccidia in the intestinal epithelium initiate gut damage. Mucosal damage is necessary to initiate sub-clinical NE since it provides a base for *C. perfringens* to establish, colonize and cause necrosis of intestinal mucosa. In addition, coccidial infection can also cause immunosuppression that further predisposes birds to sub-clinical NE (McReynolds *et al.*, 2004b). All these studies indicate the importance of coccidiosis in the occurrence of NE.

The overall performance of the birds in the CM treatment in both control and challenged litter groups was relatively poor compared to all other dietary treatment groups. In the present study stunted growth or reduced WG in birds with CM dietary treatment were observed during days 16 to 21. Higher levels of dietary CM (more than 20%) could result in increased liver metabolic activities so could lead to increased utilization of various nutrients for maintenance at the cost of tissue deposition, in turn leading to reduced WG (Woyengo *et al.*, 2011). Higher levels of NSPs tend to increase the viscosity of digesta, resulting in a reduction in both nutrient digestion and absorption, (Annison, 1991; Bell, 1993). This may have contributed to the poor growth performance of CM birds in the current study. Shires *et al.* (1981) reported that inclusion of extracted dehulled canola up to 10% of the diet had no adverse effect on the performance of chicks, but at levels above 20% canola can cause a progressive decrease in FI and WG with an increase in feed/ gain ratio. This concurs with the current study, where CM was used at a level of 21.68% and similar negative results were observed by others (Newkirk & Classen, 2002; Mushtaq *et al.*, 2007; Woyengo *et al.*, 2011). A

further reduction in WG in CM birds on reused litter compared to their clean litter counterparts was expected as these birds developed lesions of coccidiosis. Coccidial infections are known to cause a reduction in growth of birds during acute infection or the first eight days post-infection due to decreased nutrient absorption (Sharma *et al.*, 1973), and indeed, lower WG in birds with coccidiosis has been observed by a number of researchers (Willis & Baker, 1981; Matthews & Southern, 2000; Watson *et al.*, 2005; Woyengo *et al.*, 2011). Similarly Golder *et al.* (2011) found decreased BW and increased FCR in birds infected with both coccidial and clostridial infection.

More than 70% of CM phosphorus is in the form of phytic acid (Summers *et al.*, 1983). Hydrolysis of phytate is necessary to release phosphorus so it is available for absorption from GIT. However poultry lack effective endogenous phytases, so have a limited ability to dephosphorylate and utilize any phosphorus present in the form of phytate (Ravindran *et al.*, 1999; Selle *et al.*, 2000). The reduced growth performance of the birds on the CM dietary treatment in the present study may therefore be due to these higher levels of phytate content in CM. Studies indicate that phytate reduces or inhibits the activity of digestive enzymes such as pepsin, trypsin and α–amylase (Pallauf & Rimbach, 1997; Sebastian *et al.*, 1998), so may help in stabilization of α-toxin, the primary cause of NE in poultry. However, this toxin was not found in this study. In the present study no lesions of sub-clinical NE were observed in any of the birds fed the CM diet. However, lack of development of NE lesions may be due to the restricted time period on the reused litter treatment (5 days).

In the present study one of the dietary treatments was SBM+TI as TIA in the diet may also play a role in the induction of sub-clinical NE. However, post hoc dietary analysis showed that all four diets (SBM, SBM+TI, PPC and CM) had almost the same levels of TI activity (0.8mg/g of feed) so comparison of dietary treatments cannot be made solely on the basis of the TIA content of the diet. To the authors knowledge there has been no study to assess the effect of synthetic TI on the induction of sub-clinical NE in poultry. The absence of higher TI activity in post hoc dietary analysis could indicate the possibility of a degradation of synthetic TI after mixing it with the experimental diet. There is also the possibility that concentration of synthetic TI added was too small. It is also possible that the differences between natural and synthetic TI are sufficiently significant that they cannot be analysed in the same way. However the activity of synthetic trypsin inhibitor as supplied, (Sigma T9003) was not itself tested. Such a test,

had it been applied before use of the trypsin inhibitor, would have determined if it was active when used in the experiment. A high amount of TIA (3.88mg/g) in PPC has been found in other studies (Palliyeguru *et al.*, 2010). The amount of protease inhibitors in PPC can vary considerably depending upon both the quality and variety of potatoes used (Kadam, 1998) and the method of processing of the potatoes (Knorr, 1982).

No effect from dietary treatment and litter challenge was observed on the counts of *C. perfringens* in ileal digesta on any day. Experimental evidence suggests that birds fed with a high content of dietary PPC had higher numbers of *C. perfringens* when compared with birds fed other plant proteins diets, (Wilkie *et al.*, 2005; Palliyeguru *et al.*, 2010), or fishmeal (Palliyeguru *et al.*, 2010). Wilkie *et al.* (2005) found higher numbers of *C. perfringens* in the caecal content of birds fed on a PPC diet and orally gavaged in the crop with 0.5ml of an actively growing culture of *C. perfringens* (~1.0 x 10^8 cfu/ml of broth). However in the present study *C. perfringens* were not significantly increased in the ileal digesta of birds fed on a PPC diet. This may be due to different levels of PPC used in the diet formulation as previous experimenters used a PPC diet containing ~400g/kg of PPC compared to the 110g/kg used in the present experiment. Moreover very low concentrations of *C. perfringens* were present in the litter compared to 10^8 concentrations in the gavage. Some of these studies also documented that diets containing PPC increased the incidence of sub-clinical NE in poultry (Wilkie *et al.*, 2005; Palliyeguru *et al.*, 2008). However Palliyeguru *et al.* (2010) later found no differences in haemorrhagic lesions in the mid-intestines of birds fed a PPC diet, fishmeal or SBM. In agreement with the current study findings of Fernando *et al.* (2011) did not show any significant differences between the NE lesions of the birds on the PPC and SBM diets.

Some of the studies that have successfully induced NE under experimental conditions Hamdy *et al.* (1983a) reared birds in a conventional dirt floor broiler house that contained a build up of litter obtained from a flock that had experienced a severe outbreak of NE, so probably had a higher number of *C. perfringens* than the present study where the number of *C. perfringens* in litter was 1.24 x 10^4 cfu/g. Palliyeguru *et al.* (2010) successfully induced sub-clinical NE with typically 70 birds per pen. The overall NE frequency in the positive control groups in these studies was between 53%-77% based on duodenal haemorrhages. According to Lovland *et al.* (2003) spontaneous *C. perfringens* infection with some precipitating factors, is sufficient to induce sub-

clinical NE in relatively large floor reared flocks. Mikkelsen *et al.* (2009) also successfully induced NE by raising birds in pens with reused litter from a previous NE challenged experiment, in addition to orally gavaging the birds with 2500 sporulated oocytes of different *Eimeria* species. Litter analysis showed that the numbers of *C. perfringens* in Mikkelsen *et al*.'s (2009) pens with reused litter had 6.65 $\log_{10}$ cfu/g of litter, compared to 3.65 and 4.38 $\log_{10}$ cfu/g of litter on days 21 and 30 in the present study. Moreover (Mikkelsen *et al.*, 2009; Mikkelsen *et al.*, 2009)'s diagnosis of NE was made with regard to mortality compared to the present study's analysis using a score of NE typical lesions. Cowen *et al. (*1987) found a 2-10% incidence of NE when giving a *C. perfringens* culture ($1.6x\ 10^8$ to $1x\ 10^9$) in feed along with the addition of reused (used) litter from a flock that had experienced NE. As in the present study, lesions of NE were not observed in bird trials where only NE infectious litter was used. Thus, it can not be excluded that the reused litter treatment used was ineffective in establishing sub-clinical NE due to the absence of sufficient *C. perfringens* challenge.

Alsheikhly & Alsaieg (1980) confirmed that under field conditions, coccidiosis can play a significant role in the incidence of NE when sufficient numbers of toxigenic *C. perfringens* are present. The finding of this study may indicate that if considerable numbers of coccidial oocytes are present in the litter, including of CM in the diet may facilitate the development of coccidiosis-damage to the mucosal layer of the digestive tract as this is considered to be the crucial predisposing factor for the onset of sub-clinical NE in broiler chickens.

Necrotic enteritis was not induced in the present study as no characteristic necrotic lesions were found in any of the birds. Consistent induction of sub-clinical NE has been difficult with variable results even when replicating the same experimental conditions. Palliyeguru *et al.* (2010) were able to successfully induce the disease with the addition of reused litter from a flock without any history of NE whereas Cowen *et al*. (1987) in one experiment were unable to induce the disease with just the addition of NE-infectious litter. Absence of disease induction in the present study may be the result of either inadequate numbers of *C. perfringens* in the reused litter (10^4), and/or the lack of some diet induced intestinal damage failing to replicate suitable conditions for *C. perfringens* to proliferate and release α-toxin. Different researchers have demonstrated the effectiveness of different *C. perfringens* strain on the induction of sub-clinical NE. Only specific strains of *C. perfringens* are capable of producing NE in poultry (Cooper

et al., 2010; Keyburn *et al.*, 2006). It is possible that the litter used in the current study did not have the right strain of *C. perfringens* to induce sub-clinical NE.

4.6 Conclusion

Soybean meal, PPC and CM failed to predispose birds to sub-clinical NE. However, the canola based diet did predispose the birds to coccidiosis. It is postulated that this could have been the result of anti nutrients such as phytate damaging the mucosal layer (Cowienson *et al*., 2004) , making it more susceptible to coccidia. There is evidence that coccidia are a co-factor in NE and as such it would be expected that, although not shown in this study, under commercial conditions canola would also predispose birds to sub-clinical NE.

5 EXPERIMENT THREE:

EFFECTS OF ADDING FISH MEAL TO GROWER BROILER DIETS ON PROLIFERATION OF *CLOSTRIDIUM PERFRINGENS* ON *IN VITRO* DIGESTED DIETS

5.1 Introduction

Clostridium perfringens is an anaerobic, spore-forming, large, gram positive rod that has been identified as major cause of NE in chickens (Alsheikhly & Truscott, 1977a; Flores-Diaz & alape-Giron, 2003; Nauerby *et al.*, 2003). The organism is pathogenic to both humans and animals and is cosmopolitan in nature. The organism is normally present in the gut at levels as high as 10^4 cfu/g of digesta without causing disease (Kondo, 1988; Drew *et al.*, 2004; Dahiya *et al.*, 2006). However, an overgrowth of *C. perfringens* in the gut has been implicated in outbreaks of NE. When the normal population of *C. perfringens* is disturbed, levels can increase rapidly reaching 10^8 cfu/g of digesta with a concomitant production of toxins. The principal toxin produced by *C. perfringens* type A (α-toxin) is believed to be a major virulent factor of NE. Under field conditions outbreaks of NE can occur as early as 17-18 days of age (McDevitt *et al.*, 2006b).

Several dietary factors have been suggested to precipitate outbreaks of NE, including high dietary levels of wheat (Branton *et al.*, 1987; Riddell & Kong, 1992) and/or barley (Kaldhusdal & Skjerve, 1996). Diets rich in protein, particularly from animal sources and fish meal may also contribute to the growth of *C. perfringens* and therefore increase risk of NE (Truscott & Alsheikhly, 1977). It has specifically been suggested that there is a close relationship between fish meal and the incidence of NE in poultry (McDevitt *et al.*, 2006b). Most experimental models of NE rely on the addition of fish meal to diets (Gholamiandehkordi *et al.*, 2007; Pedersen *et al.*, 2008; Timbermont *et al.*, 2010). However, not all experimental models using fish meal were able to reproduce NE under controlled environmental conditions in poultry (Pedersen *et al.*, 2003).

The purpose of the present study was to determine the effect of fish meal addition on *C. perfringens* proliferation on *in vitro* digested grower diets. It was expected this would enable assessment of the role of fish meal as a possible predisposing factor in the development of NE.

5.2 Materials and Methods

5.2.1 Experimental diets

Wheat-soybean based broiler grower diet was formulated (Aviagen, 2007) containing approximately 50% wheat (Table 5.1). No antibiotic growth promoter or anti-coccidial drugs were used in the diets. Diets were produced and mixed at ASRC. Two diets were evaluated: one was a wheat-soybean diet (Table 5.1), the second was the same wheat-soybean diet but with an additional 30% fish meal on top of diet (i.e., 30g of fish meal was added to 70g of basal diet).

Table 5.1: Feed ingredients and calculated chemical composition (g/kg) of the basal grower diet

Ingredient	**Grower (g/kg)**
Wheat	538
Peas	50
Canola meal	60
Fish meal	30
Animal lipids (tallow)	20
Soya oil	30
Soya bean meal (48)	235
Dicalcium Phosphate[1]	14
Limestone	11
Sodium chloride	2
Sodium bicarbonate	2
Methionine	5
V & M mixture[2]	3
Calculated chemical composition	
Metabolisable energy (MJ/Kg)	13.9
Crude Protein	226
Available Phosphorus	1.3

[1] Contained 21.3% Ca, 18.7% P

[2] *V and M supplement provided (units kg^{-1} diets): Vit A 16,000 iu; Vit D_3 3,000 iu; Vit E 75 iu (iu=mg); Vit B_1 3 mg; Vit B_2 10 mg; Vit B_6 3 mg; Vit B_{12} 15 µg; Vit K_3 5 mg; Nicotinic acid 60 mg; Pantothenic acid 14.5 mg; Folic acid 1.5 mg; Biotin 275 µg; Choline chloride 250 mg; Iron 20 mg; Copper 10 mg; Manganese 100 mg; Cobalt 1 mg; Zinc 82 mg; Iodine 1 mg; Selenium 0.2 mg; Molybdenum 0.5 mg (Target feed, Whitchurch, Shropshire).*

5.2.2 In-vitro Digestion of Diets

Diets were i*n vitro* digested according to previously published methods (Zyla *et al.*, 1995; Annett *et al.*, 2002) with minor modifications. All *in vitro* digestion steps were carried out using an orbital incubator (Gallenkamp) with a water bath (Type SB2, Grant instruments) at 40°C to mimic the naturally occurring avian body temperature. Diets were ground in a grinder (ZM 100, Retsch) using a 1mm screen to stimulate the normal grinding action of the gizzard. 10g of each diet was placed in 50 ml polypropylene centrifuge tubes and mixed vigorously with 20 ml of 0.03 M HCl. The pH was measured (range 5.19-5.2) by pH meter (240, Corning), by addition of 1.5M HCl. The tubes were incubated in a water bath at 40°C for 30 minutes. Passage through the proventiculus was simulated by adding 30,000 units of pepsin (P-6887; Sigma-Aldrich Ltd, UK) and 5 ml of 1.5M HCl at pH 1.4-2.0, followed by incubation for a further 45 min. Then, a mixture of 68.3 mg of pancreatin (Sigma-Aldrich Ltd, UK) in 6.5 ml of 1.0M $NaHCO_3$ was added drop wise with constant stirring, at pH 6.3-6.7 through 1.0M $NaHCO_3$ addition. Volumes of mixture in the tubes were equalized by adding distilled water. The samples were incubated for a further 2 hrs. The samples were centrifuged for 30 minutes at 2000g and then filtered through gauze. The supernatant was immediately dispensed in sterile screw-capped tubes and frozen at –20°C to be used the next day.

5.2.3 Preparation of inoculum

Vegetative cells of fresh overnight cultures were used for inoculation of *in vitro* digested diets. *C. perfringens* strain 56 was isolated from the intestines of broiler chickens with severe necrotic gut lesions producing moderate amounts of α-toxin *in vitro*. This was a type A strain (no enterotoxin or ß-2 gene), that is netB positive and has been previously used to induce NE in an *in vivo* model (Gholamiandehkordi *et al.*, 2007; Pedersen *et al.*, 2008; Timbermont *et al.*, 2010). The strain was kindly provided by Dr. Leen Timbermont (Ghent University, Belgium), stored in the form of frozen beads. From the frozen culture, bacteria were grown overnight on TSC agar plates under anaerobic conditions as described in section 3.2.2. Following incubation a colony from the TSC plate was streaked on fresh TSC agar, incubated overnight and inoculated into a sterile BHI broth (Oxoid, UK). BHI was dispensed into an already autoclaved sterile flask with a cotton plug for 15 minutes. 1ml of BHI was taken into a small bijou bottle and 3 colonies of *C. perfringens* from the TSC plate was mixed with the BHI. From this

inoculated BHI, 0.2ml was added to the flask with 100ml of BHI and further incubated overnight in a shaking incubator at 60rpm. Before inoculation into the *in vitro* digested diets the concentration of *C. perfringens* in the BHI was determined by spectrophotometer (Spectronic 301, Milton Roy) before serial dilution and plating onto TSC agar according to the procedure described in section 3.2.2.

5.2.4 Proliferation of C. perfringens on in vitro digested diets

The fluid thioglycollate (CM0173) was obtained as dehydrated media (Oxoid Ltd, UK) and reconstituted according to the manufacturer's instructions. 29.75gms of powdered agar compound was weighed into a 1litre Schott Duran bottle, and diluted with distilled water to a volume of 1000ml. Fully mixed bottles of liquid media were boiled to completely dissolve the powder. On completion the liquid media was distributed into final containers that were sterilized in an autoclave at 121°C for 15 minutes. Finally the fully mixed TG fluid was cooled at room temperature for later use.

The frozen diets were rapidly defrosted under cold running water. For growth assessment of *C. perfringens* 1ml of BHI (CM 1135, Oxoid, UK) (7.2×10^6 colony-forming units) were inoculated into four treatment (1-4) (see below). Four replication tubes were used per sample:

1. Containing 9 ml thioglycollate medium served as control.

2. Containing 9 ml TG plus enzymes (0.22 mg/ml pancreatin and 0.63 mg/ml pepsin) that have been added during *in vitro* digestion of diets, served as enzyme added control .

3. Containing 6 ml TG with 3 ml supernatant from digested grower diet.

4. Containing 6 ml TG with 3 ml supernatant from digested grower diet with 30% fish meal.

The samples were incubated anaerobically at 40°C for 3hours, 6 hours, 12 hours and 24 hours at 200 rpm. At the end of incubation, the tubes were vigorously agitated to

ensure even distribution of bacteria throughout the media, and 1ml of sample was withdrawn to make serial dilution (10^{-1} to 10^{-7}) in maximum recovery diluents (MRD). The presumptive identification and enumeration of *C. perfringens* was done on TSC agar as described in section 3.2.2. 100µl of each dilution was transferred aseptically into three replicates. Tryptose Sulphate Ccylcoserine agar plates with the help of a sterile spreader, with gentle rotation of the petri plates. An additional layer of 15ml TSC agar without egg yolk was then poured onto the plates. When the agar was solidified, the plates were placed in an upright position in an anaerobic box with anaerobic packs (AN0025 and AN0035). The jar was kept at 37°C for 24 hours. Plates with 20-200 black colonies were counted and cfu/ml for each sample was determined.

5.3 Statistical Analysis

The treatment means were compared using 4x4 factorial arrangements with analysis of variance (ANOVA). The partitioned sources of variation included treatment, time and their interactions. Because of the skewed nature of the counts of *C. perfringens* data was transformed according to log (n + 1) to normalize the data before statistical analysis. Differences were reported as significant at $P<0.05$. All statistical procedures were performed using Genstat 11 for Windows (VSN International Ltd, Hemel Hempstead, UK).

5.4 Results:

Plate counts on BHI were used to confirm the levels of *C. perfringens* in inoculum following overnight incubation and were 7.2 $x10^6$ cfu/ml of BHI. Initially all the supernatants (*in vitro* digested diets) were assessed for the presence of *C. perfringens* and were found to be negative.

Figure 5.1 shows the effect of controls (TG medium, enzymes) and *in vitro* digested diet supernatants on the growth of *C. perfringens* over different time periods. The *C. perfringens* counts averaged 8.51, 8.68, 7.84, and 7.30 $\log_{10}$ cfu/ml after 3, 6, 12 and 24 hours of incubation, respectively (S.E.D. 0.10 $\log_{10}$ cfu/ml; $P<0.001$). *C. perfringens* levels increased in TG medium during the 6 hour incubation period and then start declining until 24 hrs of incubation being averaged 8.49, 8.81, 7.53, and 6.95 $\log_{10}$ cfu/ml after 3, 6, 12 and 24 hours of incubation, respectively (S.E.D. 0.10 $\log_{10}$

cfu/ml; P<0.084). However *C. perfringens* growth in TG with added enzymes did not show much dramatic change, it remained steady throughout the incubation time being averaged 8.28, 8.33, 8.08, and 7.99 log_{10} cfu/ml after 3, 6, 12 and 24 hours of incubation, respectively (S.E.D. 0.10 log_{10} cfu/ml; P<0.084).

Levels of *C. perfringens* increased in the supernatants with fish meal during the 6 hour incubation period. In particular, *C. perfringen*s proliferation tended to be greatest in the medium containing supernatant from the digested grower diet with 30% added fish meal, compared with the digested grower diet alone (Figure 5.1; 8.26 vs 7.93 log_{10} cfu/ml; S.E.D 0.18 log_{10}; P= 0.084) Whilst the interaction between diet and time was not formally significant (P=0.13), fish meal effects were most pronounced after 24 hours of incubation (7.60 vs. 6.65 log_{10} cfu/ml; S.E.D 0.36 log_{10} cfu/ml; P<0.05). At 3, 6 and 12 hours of incubation (S.E.D 0.26 log_{10}) concentrations of *C. perfringens* were 8.52, 8.82, and 8.11 log_{10} cfu/ml in the diet with added fish meal compared to concentrations of 8.75, 8.78 and 7.56 log_{10} cfu/ml in the digested grower diet (Figure 5.1)

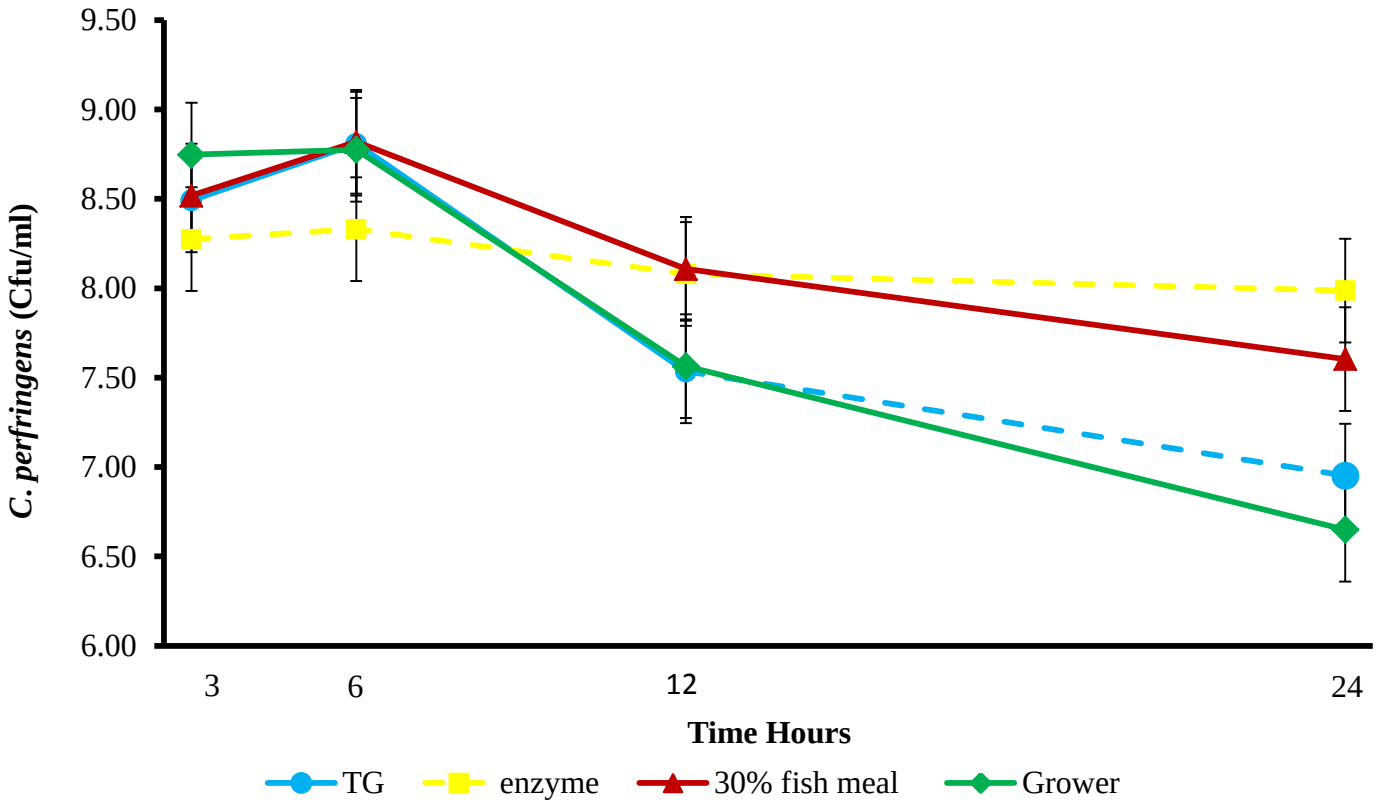

Figure 5.1: Proliferation of *C. perfringens* in controls, supernatants from grower diet with and without 30% fish meal. TG: Thioglycollate; +30% Fish meal: supernatant from grower diet with added 30% fish meal; Grower: supernatant from simple grower diet.
The standard bars are the standard error of means (SEM).

5.5 Discussion

Previously published evidence on the role of fish meal on NE incidence is conflicting. Some authors concluded that diets rich in fish meal predispose birds to NE (Kocher, 2003). Others used elevated levels of fish meal in diets to aid experimental induction of NE (Alsheikhly and Truscott, 1977). However, some concluded that high dietary fish meal neither increased the risk of NE nor the number of *C. perfringens* in the ileum or caeca of broiler chickens (Olkowski *et al.*, 2006). Indeed, high levels of dietary fish meal have been used during experimental production of NE in poultry (Truscott & Alsheikhly, 1977; Pedersen *et al.*, 2008; Wu *et al.*, 2010).

Annet *et al.* (2002) found that supernatant obtained from digested wheat and barley based diets resulted in higher proliferation of C. *perfringens* A compared to corn-based diets. In the present study the growth of *C. perfringens* type A was similarly investigated although grower diets with and without 30% added fish meal were used. Prior to inoculation, all the supernatants were analysed for *C. perfringens*, however none were recovered at the 10^1 level indicating that diets used unlikely had detectable numbers of *C. perfringens*. The present study found that *C. perfringen*s proliferation tended to be higher in *in vitro* digested grower diet with added fish meal, especially towards the end of the study. Enzymatic exposure to pancreatin and pepsin in the grower diet with added fish meal may have yielded more nutrients (e.g. amino acids) that would have been available for *C. perfringens* utilization, resulting in increased proliferation. Drew *et al.* (2004) examined the effect of fish meal and soy protein on intestinal populations of *C. perfringens* and found that fish meal (400g/kg) significantly increased the counts of *C. perfringens* in the ileum and cecum of broiler chickens. Drew *et al.* (2004) attributed this increase to a better amino acid profile in terms of higher amounts of methionine, histidine, glycine and alanine in the fish meal diet.

In the present study, the presence of fish meal appeared to aid the survival of *C. perfringens* as at 12 hrs there was 0.55 log difference between grower and added fish meal diets. By 24 hrs this difference was almost full log (0.95) indicating that additional growth nutrients were released and/or made available for the growth of *C. perfringens*. Increased clostridial proliferation in the fish meal diet compared with the simple grower diet is in agreement with the previously reported *in vivo* studies. Wu *et al.* (2010) assessed the effect of high levels of fish meal on the incidence of NE in broiler chickens

and found higher *C. perfringens* in birds with fish meal compared with the non-fish meal control group. However, Olkowski *et al*. (2006a) were unable to find any effect of fish meal on the occurrence of NE, although higher levels of dietary fish meal significantly increased the number of *C. perfringens* in the caecum and ileum of the birds. This strongly suggest that higher levels of *C. perfringens* alone are unlikely responsible for inducing (sub-clinical) NE. The levels of *C. perfringens* observed decreased throughout the incubation period in all cases for both simple grower and grower with added fish meal, likely due to nutrient exhaustion of the medium used.

The possible reason for the higher growth of *C. perfringens,* after 24 hrs in TG media with added enzymes compared to treatments with *in vitro* digested grower diets and simple grower diet with added fish meal, may be the higher amount of TG used for this treatment (9ml vs 6ml), thus providing more media for *C. perfringens* growth. Although both enzymes (pepsin and pancreatin) were added during the *in vitro* digestion of the diets, it is assumed that some of the enzymes would have been used during the process. Further since it was impossible to run and analyse all tests simultaneously, all supernatants were kept frozen overnight which is likely to have resulted in diminished enzyme activity in those diets due to their proteinous nature. Xuan Wang (2008) found that proliferation of different strains of C. *perfringens* was inconsistent and he attributed this inconsistency to variables in strain metabolism and growth requirements by different strains.

In vitro assays do not fully recreate the same conditions as *in vivo* due to absence of factors such as feed passage in the gut. Other factors like food passage time, gut flora, that also influence the extent of digestion are difficult to reproduce *in vitro.* Despite these differences, *in vitro* techniques have been utilized for prediction of broiler intestinal viscosity when fed rye based diets in the presence of exogenous enzymes, and these studies found a good relationship between *in vitro* and *in vivo* assays (Bedford & Classen, 1993). Zyla *et al.* (1995) described *in vitro* methods for feed digestion as accurate, rationally fast, cost-effective, simple and robust when compared with *in vivo*. Welfare concerns relating to animal experiments mean that generally *in vitro* methods are preferred (Weurding *et al.,* 2001). However the various draw backs of *in vitro* approaches may impact results *i.e*. failure to take account of competition from other gut flora that may affect the growth of *C. perfringens in vivo*. Despite these obvious

limitations, *in vitro* assays have been shown to be useful when budgetary limitations make *in vivo* studies too expensive.

5.6 Conclusion

The overall findings of the present study support the view that high levels of dietary fish meal may assist survival of *C. perfringens*, suggesting that the role of fish meal as a predisposing factor for (subclinical) NE cannot be excluded.

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6 EXPERIMENT FOUR:

INDUCTION OF SUB-CLINICAL NECROTIC ENTERITIS THROUGH IN-FEED CHALLENGE WITH LOW AND HIGH DOSES OF CLOSTRIDIUM *PERFRINGENS*

6.1 Introduction

Sub-clinical NE is an economically important disease of poultry with global significance since it is usually undetected so often remains untreated (Skinner *et al.*, 2010). Although *C. perfringens* is the primary etiological agent of sub-clinical NE, other contributory factors are also required to predispose birds to this disease. These precipitating factors are both numerous and mostly ill-defined, but a number of previous researchers have shown these to include diet composition, other intestinal diseases and management related stress (Shane *et al.*, 1985; Kaldhusdal & Lovland, 2000; McDevitt *et al.*, 2006b). In order to investigate, strategies for controlling sub-clinical NE, it is crucial to create a model that enables repeatable experimental induction of sub-clinical NE under controlled environmental conditions. However it has proved difficult to reproduce the disease and to date there is no standardized model. The published models vary in *C. perfringens* strains, challenge procedures, challenge dose, challenge days, and incorporation of predisposing factors (Truscott & Alsheikhly, 1976; McReynolds *et al.*, 2004b; Palliyeguru *et al.*, 2010).

As a result, despite much research in many countries, the exact conditions that precipitate out-breaks of NE under field conditions continue to be ambiguous. Numerous factors have been identified that promote the development of sub-clinical NE. These predisposing factors are mainly dietary in nature, although another risk factor for the onset of sub-clinical NE is concurrent intestinal diseases like coccidial infection. Under field conditions, particularly when there sufficient numbers of *C. perfringens* are present, coccidial infection can play a crucial role in the occurrence of NE. In particular the *Eimeria* species that colonize the small intestine such as Eimeria *maxima* and *Eimeria acervulina* may predispose birds to NE (Alsheikhly & Alsaieg, 1980). Bradley and Radhkrishnan (1972) observed an increased growth of *C. perfringens* in the caecum during infection with E. *tenella*. However Baba *et al.* (1997) suggested that concurrent infection with E. *nacatrix* and *C. perfringens* has a synergistic effect and increases clostridial population in the intestine of the chickens.

From experiment three (Chapter 5; Saleem *et al.*, 2011) it was concluded that fish meal (30% added on top of grower diet) may enhance *C. perfringens* proliferation It was therefore decided to use fish meal as a predisposing factor in subsequent experiments. The current experiments were to develop experimental model of sub-

clinical NE with low in-feed doses of *C. perfringens*, together with higher doses of coccidial vaccine in broiler chickens fed wheat-based diets incorporating high amounts of fish meal (Experiment 4a) and with higher and repeated in-feed doses of *C. perfringens*, together with higher doses of coccidial vaccine (Experiment 4b).

The targeted in-feed *C. perfringens* level was 10^2 cfu per g of feed in Experiment 4a. As sub-clinical disease was not observed, this target increased to 10^9 cfu in Experiment 4b. Experiment 4b was also set out to compare two breeds (Ross 308 & Hubbard) in their resistance to induction of sub-clinical NE under the same experimental conditions, as different broiler strains can differ in susceptibility to infectious diseases (Lamont, 1998; Zekarias *et al.*, 2002).

6.2 Materials and Methods

6.1.1 Treatment groups and Experimental Design

6.1.1.1 Experiment 4a

A total of 48 day-old male Ross 308 broiler chickens were obtained from a commercial hatchery, reared in solid-floored pens in an environmentally controlled house from day one to up to day 21. Two treatments (see below) were compared using three replicate pens with 8 birds per pen. Six floor pens, in three positional blocks, were used in an environmentally controlled room.

6.1.1.2 Experiment 4b

A total of 45 day-old male (30 Ross 308 and 15 Hubbard) were obtained from a commercial hatchery, reared in solid-floored pens in an environmentally controlled house. Three groups of birds (2 Ross and 1 Hubbard) were subjected to one of three different treatments (see below). All three treatments were randomly allocated to pens in three positional blocks. A total of nine floor pens, with 5 birds per pen were used in an environmentally controlled room. At this time only one Hubbard treatment was included due to limitations, imposed by the Animal Ethics committee, to use low number of animals whereever possible - particularly in studies putting the birds under challenge. This effectively limited the researcher to a small scale pilot study. If this experiment had achieved greater success, more birds would have been used.

Table 6.1 : Experimental design for experiment 4a and 4b showing the days of age at which the challenges and vaccines were given and the days for lesion scored

Experiment	Treatment	No of replicates (pens)	No of birds per pen	*C. perfringens* Challenge	Coccidial Vaccination (days)	Lesion scoring (days)	*C. perfringens* Challenge dose[a]	Number of birds sampled per pen[b]
4a	Control (Ross)	3	8	-	18	21	-	8
4a	Challenge (Ross)	3	8	17,18,19	18	21	1.00×10^2	8
4b	Control (Ross)	3	5	-	18	21	-	5
4b	Challenge (Ross)	3	5	17,18,19	18	21	1.54×10^9	5
4b	Challenge (Hubbard)	3	5	17,18,19	18	21	1.45×10^9	5

[a] Calculated number of *C. perfringens* in average inoculum in feed

[b] Birds were randomly sampled at day 21

In both experiments the birds were reared as a single flock from day 0 to day 7. On day 7, the birds were weighed and randomly allocated to each pen with similarly averaged day 7 BW. The designs of experiments 4a and 4b are shown in Table 6.1. Adequate feeders and drinkers were provided for the age and the number of the birds with commercial wood shavings provided as bedding.

6.1.2 Diet and feed mixing:

The same starter and grower diets were used for both of these experiments. Two nutritionally complete diets, wheat-soybean based broiler starter and grower were formulated (Aviagen, 2007). Both feeds contained approximately 50% wheat. No antibiotic growth promoter or anti-coccidial drugs were used in the diets. Diets were produced and mixed at the ASRC, SAC, Ayr and fed as mash. Both starter and the grower diets had the same formulation for all the birds involved in both study 4a and 4b (Table 6.2).

All the groups were fed the starter diet from day 0 to day 7, followed by the grower diet from day 8 until day 15. Thereafter all birds were fed the grower diet, mixed 3:1 with fish meal until day 21. Challenged groups were given a *C. perfringens* culture in their diet on days 17, 18 and 19. In contrast, the birds of the control group were given feed mixed with sterile brain heart infusion broth (BHI) only. The challenge-feed was mixed in the Microbiology Laboratory, SAC, Ayr each day during the challenge period. Feed and water were given *ad libitum* throughout the study period. Pen dimensions in both experiments were 1.74 x 1.28 m. A solid 45cm high plywood barrier at bird level separated adjacent pens. A wire fence was located on the top of all barriers up to the ceiling. Fresh wood shavings to the depth of 10cm were provided as bedding at the start of each study. Each pen was provided with a single food hopper and bell drinker.

Table 6.2: Ingredients and calculated composition (g/kg) of starter and grower diets used in experiments 4a and 4b

Ingredient	**Starter (g/kg)**	**Grower (g/kg)**
Wheat	523	538
Peas	50	50
Canola meal	60	60
Fish meal	30	30
Animal lipids (tallow)	23	20
Soya oil	30	30
Soya bean meal (48%)	245	235
Dicalcium phosphate[1]	14	14
Limestone	12	11
Sodium chloride	2	2
Sodium bicarbonate	2	2
Methionine	5	5
Vitamin & mineral mixture[2]	4	3
Calculated chemical composition		
Metabolisable energy (MJ/Kg)	13.8	13.9
Crude Protein	217	226
Available Phosphorus	1.5	1.3

[1]Contained 21.3% Ca, 18.7% P

[2] V and M supplement provided (units kg^{-1} diets): Vit A 16,000 iu; Vit D_3 3,000 iu; Vit E 75 iu (iu=mg); Vit B_1 3 mg; Vit B_2 10 mg; Vit B_6 3 mg; Vit B_{12} 15 µg; Vit K_3 5 mg; Nicotinic acid 60 mg; Pantothenic acid 14.5 mg; Folic acid 1.5 mg; Biotin 275 µg; Choline chloride 250 mg; Iron 20 mg; Copper 10 mg; Manganese 100 mg; Cobalt 1 mg; Zinc 82 mg; Iodine 1 mg; Selenium 0.2 mg; Molybdenum 0.5 mg (Target feed, Whitchurch, Shropshire.

The birds in the experimental pens were reared following a standard commercial environmental control programme from day 0 to day 21. All treatment groups were housed in the same room. To avoid contamination with *C. perfringens*, the birds were reared in alternate pens, thereby creating an empty pen between each pen of birds. The empty pen was disinfected every second day during the study. There were foot dips in front of each challenge pen. Additional precautions, such as changing gloves and foot dipping tanks between treatment pens, were taken to avoid accidental contamination of unchallenged pens with *C. perfringens*. Light was provided for 23hrs with controlled temperature and humidity. Birds were individually tagged. All birds that died or were culled were recorded, weighed and post-mortemed. All experimental procedures were approved by the SAC Animal Ethics committee (AU AE 22/2009 (Exp 4a) and AU AE 1/2010 (Exp 4b)) and carried out under Home Office authorization (PPL 60/3383 (Exp 4a) and 60/3398 (Exp 4b)).

6.1.3 Preparation of inoculum and challenge procedure

Vegetative cells of fresh overnight cultures were used for inoculation of chickens. *C. perfringens* strain 56 was isolated from the intestines of broiler chickens with severe necrotic gut lesions producing moderate amounts of α-toxin *in vitro*. It is a type A strain (no enterotoxin or beta-2 gene), strain is netB positive and has been used previously to induce NE in an *in-vivo* model (Gholamiandehkordi *et al.*, 2007; Pedersen *et al.*, 2008; Timbermont *et al.*, 2010). The strain was kindly provided by Dr. Leen Timbermont (Ghent University, Belgium), stored in the form of frozen beads. From the frozen culture, bacteria were grown overnight on Tryptose Sulphite Cycloserine (TSC) agar plates. Before inoculation in the feed, the bacteria were cultured for 24hrs at 37°C in BHI broth (Oxoid, UK) as describe in section 3.2.2. After overnight incubation on TSC agar plates, colonies of *C. perfringens* were taken, mixed with BHI, incubated at 37ºC shaken at 60rmp anaerobically overnight. After incubation, 0.2ml of BHI was mixed with BHI broth in flasks for final mixing with the feed. Before preparation of each batch of challenge-feed the concentration of *C. perfringens* in the BHI was determined by spectrophotometer and then serially diluted and plated onto TSC agar according to the procedure described in section 3.2.2. On days 17, 18 and 19 birds on the challenge treatments were offered inoculated feed only. Feed and BHI broth containing *C. perfringens* were in a ratio of 1:1. Fresh cultures and mixtures of culture and feed were prepared daily. The mixture, which had a paste-like consistency, was

placed in feed trays. Birds were fed with challenge feed once daily and had continual access to the challenge feed. Uneaten feed was removed and the feeders thoroughly cleaned before the next feeding. The birds of the non-challenged control group were given grower diet mixed 1:1 w/v with sterile BHI broth once a day, also on days 17, 18, and 19.

6.1.4 Coccidial Vaccination

On day 18, all birds received anticoccidial vaccine "Paracox-8™" (Schering-Plough Animal Health, Welwyn Garden City, UK). This vaccine contains live attenuated oocytes of *Eimeria acervulina Eimeria brunetti, Eimeria maxima* (two lines), *Eimeria mitis, Eimeria necatrix Eimeria praecox,* and *Eimeria tenella* and was given by oral gavage at 10 times the dosage prescribed by the manufacturer. This level of coccidial vaccine was chosen because at this dose slight reddening of intestinal mucosa was observed (Pedersen *et al.*, 2008).

6.1.5 Sampling and data recording

6.1.5.1 Enumeration of *C. perfringens* in BHI and feed

A sample of BHI mixed with *C. perfringens* culture was used for enumeration of *C. perfringens* by the spread plate method on TSC agar plates. After mixing with BHI, representative feed samples were collected and immediately examined for enumeration of *C. perfringens*. Feed sample aliquots of approximately 10g of feed were weighed out and suspended in 90ml of Maximum Recovery Diluent (MRD). From this dilution further 10 fold dilutions were made. From each dilution 100µl of mixture was inoculated onto TSC plates. After incubation the number of colonies was counted on the most appropriate dilution (Plates with 30-300 colonies). This figure was then used to calculate the number of colony forming units per gram of original sample.

6.1.5.2 Clinical signs and lesion scoring

Birds were assessed daily for clinical signs and symptoms of NE, from the day feed was inoculated with C. *perfringens* until termination of the experiment. On day 21 all the birds were humanely killed by intravenous administration of an overdose of

barbiturate. The entire gastro-intestinal tract (GIT) was inspected for the presence of lesions/pathological changes associated with sub-clinical NE or evidence of lesions of coccidiosis. The disease frequency was measured per treatment, as the fraction of randomly selected birds with gross lesions consistent with sub-clinical NE. Three different segments of GIT (duodenum, jejunum and ileum) were identified, immediately incised, and washed in Phosphate Buffer Saline (PBS). Then the mucosal surfaces were inspected and scored for any lesions of clostridia, necrotic and haemorrhagic lesions. A modification of the scoring system used by Shane *et al*. (1985) was planned to be used to score intestinal lesions. However, in experiment 4b, only a small proportion of birds were found with lesions. All these were observed as focal necrosis with 5-10mm diameter. Therefore only two scores were used, i.e. lesions present (1) or absent (0).

6.1.5.3 Histopathology

Samples from the small intestine, particularly those showing lesions, were taken for histopathology. A 1.5-2cm of tissue sample was taken, washed with phosphate buffer saline (PBS) and stored in 10% neutral phosphate-buffered formalin for histopathology as described in section 3.2.5.3. Specimens for histopathology were embedded in paraffin, processed routinely, cut at 5-6µm and stained with haematoxylin and eosin (Appendix A). Gram's staining was also done to confirm the presence of Gram positive bacilli (Appendix B). The prepared tissue sections were later examined using a binocular stereo-microscope (Olympus BX 41, U-LH100HG, Olympus optical, Co. Ltd) connected by camera (spot idea™ 28.2-5MP) to computer software (Spot idea, Version 4.7) using different magnifications.

6.1.5.4 Quantification of *C. perfringens* and α-toxin in digesta

Ileal content (digesta content from the Meckel's diverticulum to ileo-caecal-colon junction) of all the birds from each replicate pen were taken into sterile screw-capped bottles and immediately transferred immediately to the Microbiology laboratory, for analysis of C. *perfringens.* The presumptive identification and enumeration of *C. perfringens* was done on TSC agar as described in section 3.2.5.4. Each of the digesta samples was vortexed for 15 seconds in order to ensure adequate mixing. One g of digesta was weighed and serially diluted in MRD. From each dilution to be plated, 100µl was applied to the centre of each agar plate, allowed to be absorbed and then

15ml of TSC overlay was spread before the plate was incubated invertedly in an jar provided with anaerobic conditions as described in section 3.2.2. The jar was placed in an incubator at 37°C for 24hrs. After incubation the number of colonies was counted to calculate the number of colony forming units per gram of original sample.

Quantification of α-toxin in the intestinal digesta was done by Enzyme linked immunosorbent Assay (ELISA) using α-toxin *C. perfringens* kit (Cypress diagnostics, Ref. Vb040) following the manufacturer's recommendations as described in section 3.2.5.5.

6.1.5.5 Growth performance

At days 0, 7, 16 and 21 all birds were weighed individually, to calculate bird weight gain (WG) during the experimental periods. Starter feed was weighed at day 0 and weighed back at day 7. The grower feed was weighed back at days 16 and 21. Feed intakes (FI) were calculated by weighing the initial feed inputs against the uneaten feed over the experimental period. Feed conversion ratio (FCR) was calculated by dividing average feed consumed per pen over average weight gain of birds per pen.

6.1.5.6 Feed analysis

Amino acid contents of grower feed with added fish meal were determined as described in section 4.2.5.6. Analysis of feed samples was done in duplicate.

6.2 Statistical Analysis

The effect of challenge on digesta counts of *C. perfringens*, FI, BW, WG and FCR were compared using randomized complete block analysis of variance (ANOVA). The pens were treated as an experimental unit, and pen position used as a block. Data are presented as means.

Because of the skewed nature of the counts of *C. perfringens*, data was transformed according to log (n + 1) to normalize the data before statistical analysis. Differences were reported as significant at $P<0.05$. Day 16 BW was initially used as a covariate, but omitted from the final model used when it did not contribute significantly.

The data obtained for the incidence of intestinal lesions were compared using a non-parameteric Fisher's exact test. In experiment 4b, comparison contrast was used to separate treatment means. Significance of the test was determined at $P< 0.05$. All statistical procedures were performed using Genstat 11 for Windows (VSN International Ltd, Hemel Hempstead, UK).

6.3 Results

In both experiments, none of the control or challenged birds showed clinical abnormalities of NE. Moreover no mortality was observed in any of the treatment groups during both studies.

6.3.1 Enumeration of C. perfringens in feed

During the days when the birds were challenged with *C. perfringens* in their feed, a fresh feed and *C. perfringens* broth culture mixture were prepared daily. Analysis of feed mixed with BHI containing the culture of *C. perfringens* showed that average inoculums for the birds on days 17, 18 and 19 for the challenged group were 1.00×10^2 cfu/g feed (SEM 5.7 cfu/g) offered in experiment 4a and $\sim 1.5 \times 10^9$ cfu/g feed (SEM 4.29×10^8 cfu/g) offered in experiment 4b (Table 6.1). *C. perfringens* was not detected in the feed of the control groups.

6.3.2 Lesions score

Table 6.3 shows the occurrence of sub-clinical NE lesions in the small intestine on day 21 in experiments 4a and 4b. In experiment 4a, no lesions of sub-clinical NE were observed in birds who had received the C. *perfringens* challenge (P=1.00). In experiment 4b, slight reddening of the intestinal mucosa was observed in most of the birds receiving *C. perfringens* challenge. In addition, lesions typical of sub-clinical NE were observed in two challenged Ross birds and one challenged Hubbard bird (P= 1.00; Table 6.3). Thus, overall, sub-clinical NE lesions were observed in three out of 30 challenged birds (10%) but there were no treatment differences (P=1.00) All lesions were focal necrosis, 5-10 mm in diameter. In one Ross bird focal necrotic areas were almost confluent. No bird in the control group developed lesions in either of the experiments.

Table 6.3: Occurrence of lesions of sub-clinical NE in the small intestine of broiler chickens on day 21 in experiments 4a and 4b

Experiment	Treatment	Bird	No of pens	No. of birds per pen	Sub-clinical NE lesions	Total %
4a	Control	Ross	3	8	0/24	0
4a	Challenge	Ross	3	8	0/24	0
4b	Control	Ross	3	5	0/15	0
4b	Challenge	Ross	3	5	2/15	13.3
4b	Challenge	Hubbard	3	5	1/15	6.66

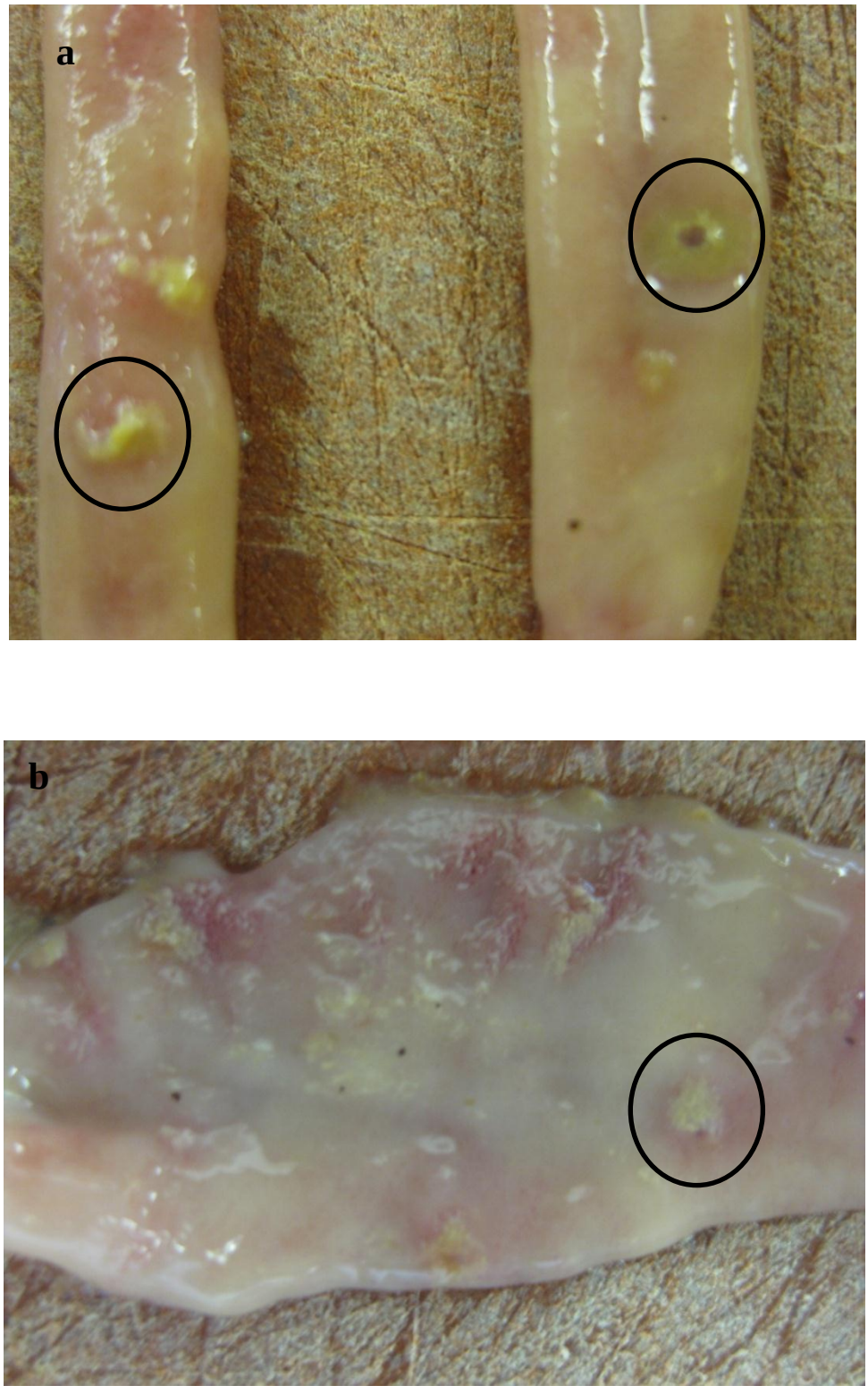
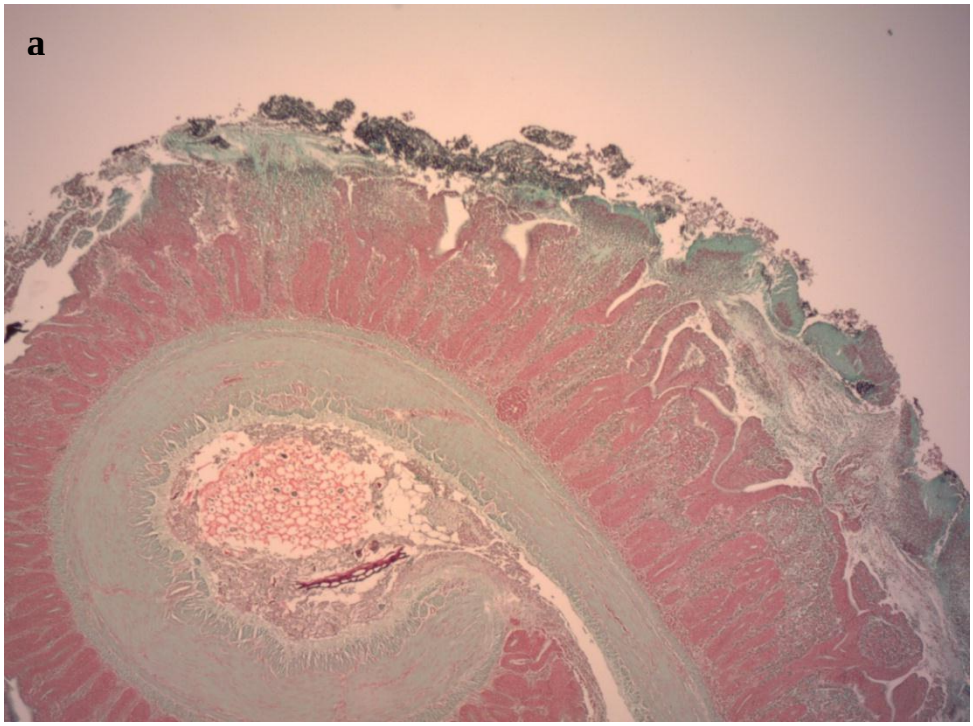


Figure 6.1: Focal necrosis (black circles)in the mucosa of intestine of broiler chickens receiving higher concentration of *C. perfringens* (approximately 10^9) and higher dose of coccidial vaccine with high fishmeal diets (experiment 4b).

6.3.3 Histopathology

Histopathological examination of formalin fixed intestinal tissue from experiment 4a revealed no lesions of sub-clinical NE. However in experiment 4b, histopathological sections from the three birds that had visible gross lesions exhibited microscopic changes typical of sub-clinical NE. There was severe necrosis of the intestinal mucosa, with an abundance of fibrin admixed with cellular debris adherent to necrotic mucosa. Marked infiltration of heterophilic granulocytes was also observed. The tissue sections with Gram stains from these birds demonstrated the presence of Gram positive bacilli with characteristic *C. perfringens* morphology in the form of large clumps primarily around necrotic areas (Figure 6.2). Some colonies of *C. perfringens* were also seen in the vicinity of non-necrotic areas. Many haemorrhagic cells were also visible in intestinal villi in some tissue sections from these birds. The lesions like villous atrophy, as well as blunting and necrosis of villi tips were also seen in some microscopic sections. There was no evidence of coccidial oocytes or schizonts in any of the intestinal tissue sections examined either with or without gross lesions.



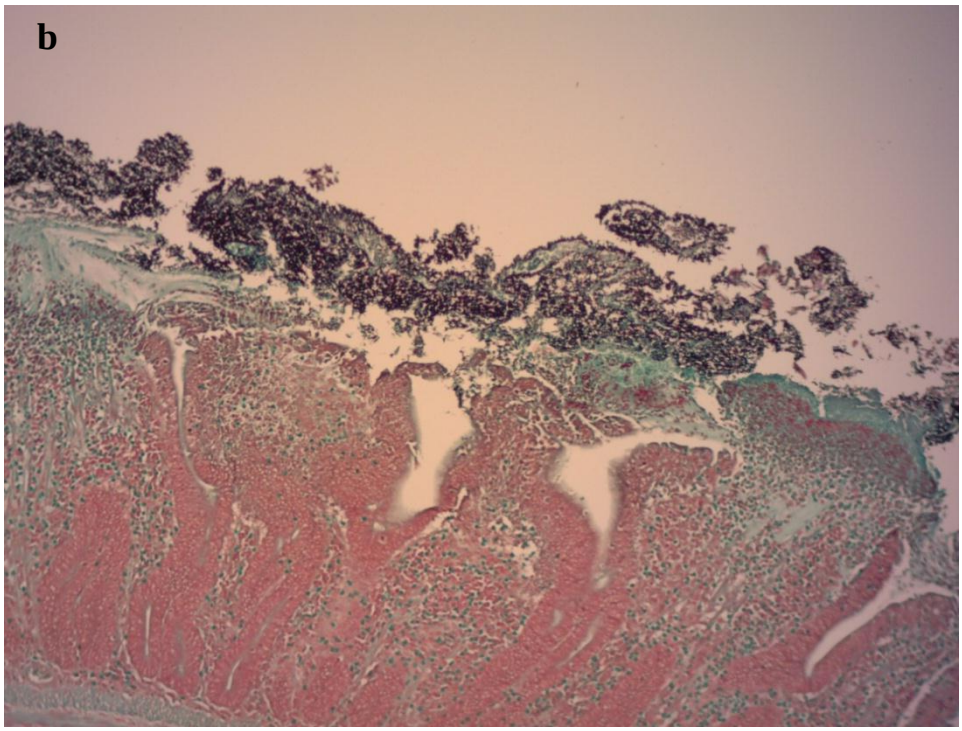


Figure 6.2: Photomicrography of the intestine of broilers exhibited gross lesions of sub-clinical NE in experiment 4b (a) necrotic villi and presence of fibrin like material in the lumen, Gram stain (x10) Figure 2 (b) same section showing aggregation of Gram positive bacilli (arrow) thickly clustered around necrotic villi, Gram stain (x40).

6.3.4 Quantification of *C. perfringens* and α-toxin in the ileal digesta

The average log transformed counts of *C. perfringens* in experiment 4a and 4b are shown in Table 6.4 and Table 6.5 respectively. In experiment 4a, counts of *C. perfringens* in ileal digesta from control and challenged birds did not differ. In experiment 4b, unchallenged control birds had a significantly lower numbers of *C. perfringens* on day 21 compared to *C. perfringens* challenged Ross and Hubbard ($P<0.001$; Table 6.5). However, counts of *C. perfringens* did not differ between challenged Ross and Hubbard birds (2.87 vs 3.35 $\log_{10}$ cfu/g of digesta). Alpha toxin was not detected in the ileal samples of birds from any of the treatment group, with or without lesions.

6.3.5 Growth performance

Table 6.4 and 5 shows the effect of challenge on BW on days 16 and 21, and WG, FI and FCR during day 16 to 21 in experiment 4a and 4b respectively. Challenge did not significantly affect final BW, WG, FI and FCR in both experiments 4a and 4b ($P>0.05$).

Table 6.4: The growth performance and *C. perfringens* counts (day 21) in ileal digesta of broilers chickens in challenge and control treatment groups in experiment 4a

Variable	Control	Challenge	SEM[1]	Probability of treatment effect
Body weight (day 16) (g/bird)	445	436	17.2	0.721
Body weight (day 21) (g/bird)	747	731	17.1	0.569
Weight gain (days 16-21) (g/bird)	302	295	6.70	0.539
Feed Intake (days 16-21) (g/bird/day)	68	68	0.96	0.922
Feed conversion ratio[2]	1.13	1.15	0.02	0.470
C. perfringens (Log_{10} cfu/g of digesta)	1.76	1.33	0.44	0.499

Data represent the mean of 3 pens with eight broiler chickens per pen.
[1] SEM: Standard error of means.
[2] Feed conversion ratio = feed intake: weight gain (g/g).

Table 6.5: The growth performance and counts of *C. perfringens* (day 21) in ileal digesta of broiler chickens in control and challenge treatment groups in experiment-4b.

Variable	Control (Ross)	Challenge (Ross)	Challenge (Hubbard)	SEM[1]	Probability of treatment effect
Body weight (day 16) (g/bird)	461	492	478	31.8	0.804
Body weight (day 21) (g/bird)	806	797	794	8.8	0.662
Weight gain (days16-21) (g/bird)	327	322	317	8.75	0.761
Feed intake (days 16-21) (g/bird/day)	75	77	75	1.69	0.623
Feed conversion ratio[3] (days16-21)	1.14	1.19	1.18	0.02	0.456
C. perfringens (Log_{10} cfu/g of digesta)	0.30^{b}	2.87^{a}	3.35^{a}	0.48	<0.001

Means within a row without a common superscript differ significantly ($P < 0.05$).
[1] SEM: Standard error of means.
[2] Data represent the mean of 3 pens with five broiler chickens per pen.
[3] Feed conversion ratio = feed intake: weight gain (g/g).

6.4 Discussion

Necrotic enteritis and its sub-clinical form under field conditions are very complex with outbreaks dependant upon innumerable factors such as the nature of the diet, gut environment, overall poultry management and co-infection with other enteric diseases (McDevitt *et al.*, 2006b). When faced with such a multi-factorial disease, a working reproducible experimental model is an essential tool if the industry is to be able to determine a variety of control strategies such as effective, long lasting vaccination, novel feed additives or other nutritional strategies. However, successful and consistent reproduction of NE has proved to be difficult. Limited data is available from attempts to reproduce NE under controlled experimental conditions, with even less data relating to reproduction of sub-clinical NE (Truscott & Alsheikhly, 1977; Alsheikhly & Truscott, 1977b; Kaldhusdal *et al.*, 1999; Pedersen *et al.*, 2003; Wu *et al.*, 2010).

Experiments 4a and 4b included various suggested predisposing factors to induce sub-clinical NE in a model of challenged broiler chickens, in the presence of both low and high concentrations of *C. perfringens* through oral gavage. All birds were administered with coccidial vaccine (Paracox-8®) at a dose 10 times higher than that prescribed by manufacturer as Eimeria co-infection is known to be the important predisposing factor provoking NE lesions (Van Immerseel *et al.*, 2004). Our work (Experiment 2) together with studies conducted by Prescott *et al.* (1978a) has shown that fish meal supports the growth of *C. perfringens*. Therefore a high level of fish meal (30% on top of basic diet) was used during days 16 to 21 to further facilitate sub-clinical NE. In addition to these two factors, diets contained approximately 50% of wheat, as higher levels of wheat have also been shown to excerbrate the incidence of NE (Truscott & Alsheikhly, 1977; Branton *et al.*, 1987; Riddell & Kong, 1992).

In both experiment 4a and 4b, birds were closely monitored for clinical signs of NE. However, neither clinical signs of the disease were observed nor was any mortality recorded. For examination of gross lesions day 21 of age was chosen because this is the age at which outbreaks of NE often occurs (Long, 1973; Wilson *et al.*, 2005). In experiment 4a no disease lesions were seen whereas in experiment 4b lesions were seen in some challenged birds only. As none of the birds showed clinical signs specific to NE the lesions observed in experiment 4b must be classified as sub-clinical NE. However,

the proportion of inoculated birds that developed such lesions was low (3 out of 30, or 10%).

In experiment 4b, histopathological examination of birds with lesions confirmed that these were typical of sub-clinical NE, revealing damaged intestinal villi with multifocal necrosis along with the presence of necrotic debris. The apical parts of the villi were most affected. There was also marked infiltration of heterophilic granulocytes. Colonies of bacterial rods were frequently noted within the necrotic areas. Similar histopathological findings have been observed in birds with sub-clinical NE by other researchers (Branton *et al.*, 1987; Kaldhusdal & Hofshagen, 1992; Palliyeguru *et al.*, 2010).

In experiment 4b, on day 21, concentrations of *C. perfringens* in ileal digesta of the control group were significantly lower compared to the challenged Ross and Hubbard group, clearly demonstrating an association between in feed *C. perfringens* and NE (Figure 6.3). Alpha toxin is believed to be the major virulent factor of NE (Van Immerseel *et al.*, 2004). However in the present studies α-toxin was not detected in ileal digesta of any of the broiler chickens, with or without lesions. Quantification of α-toxin may not be a reliable parameter since a clear relationship between α-toxin production by different strains of *C. perfringens* and induction of NE has yet to be established. Moreover α-toxin production by *C. perfringens* isolates from diseased and healthy broiler flocks has been shown to be similar (Gholamiandekhordi *et al.*, 2006), although this would not explain the absence of α-toxin from our studies.

In addition to gross lesions, the sub-clinical form of NE is often associated with damage to intestinal mucosa leading to a retarded growth rate and increased FCR (Kaldhusdal & Hofshagen, 1992; Liu *et al.*, 2010; Elwinger *et al.*, 1992). However, in the present studies *C. perfringens* challenge did not affect weight gain or the FCR of groups of broiler chickens from days 16-21. Similar results were found by Pedersen *et al*. (2008) who did not find any difference in the weight gain between challenged and unchallenged birds. In experiment 4a no intestinal lesions of sub-clinical NE were observed whereas in experiment 4b the proportion of birds with lesions was too low so judged unlikely to have had an impact on growth parameters.

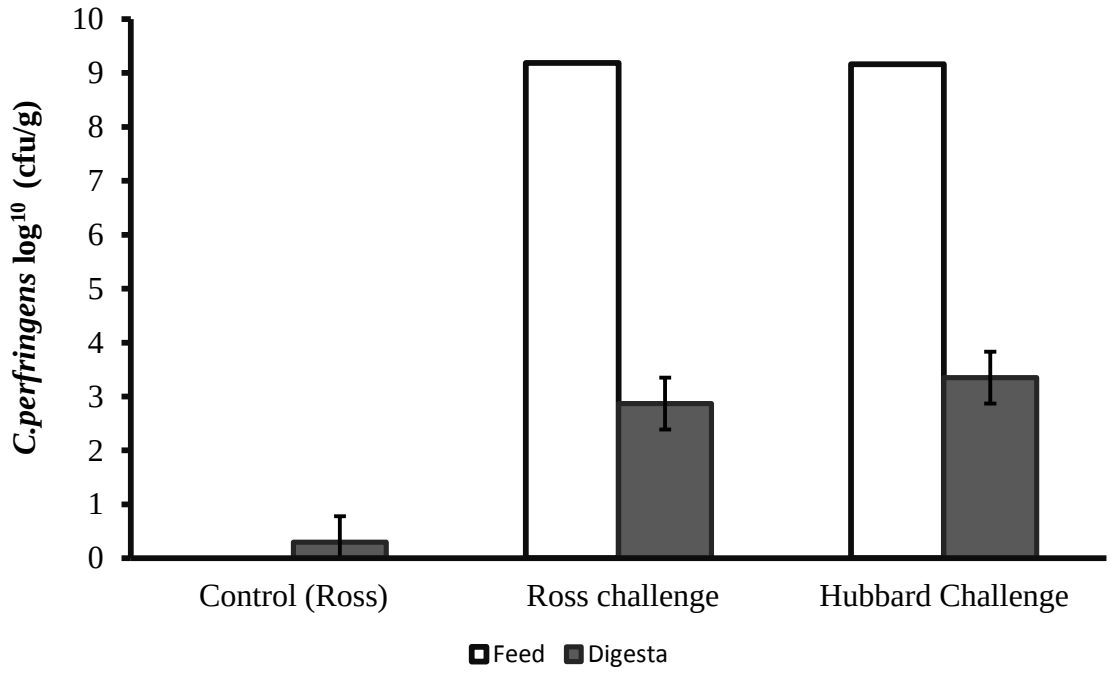


Figure 6.3: Counts of *C. perfringens* (cfu/g) in feed and ileal digesta of different treatment groups.

It was not possible from Experiment 4a to establish an infection challenge model for *C. perfringens*. Although some of the predisposing factors were applied, such as incorporation of more than 50% of wheat in the diet (Branton *et al.*, 1987; Riddell & Kong, 1992), high levels of fish meal (30% on top of basic diet) in grower diet from days 7 to 16 (Prescott *et al.*, 1978a). Diet was offered in the form of mash rather than pellets as Engberg *et al*. (2002) found higher numbers of *C. perfringens* in the caecum of birds fed a mash compared to pelleted diet. Lack of successful induction of the disease in experiment 4a indicates that some factors other than diet are also critical for disease induction.

There could be many reasons for the failure of the challenge model in experiment 4a to reproduce NE, thought the most likely explanation is that the number of *C. perfringens* cfu in inoculum was very low (10^2), as this was even lower than that found in GIT of healthy chickens (10^4 (Kondo, 1988; Drew *et al.*, 2004)) These results suggest that the oral dosage of *C. perfringens* was insufficient to produce lesions even in presence of higher doses of coccidial vaccine and a diet high in fish meal. Challenge inoculum was prepared once, and stored for use on the three inoculation days. It can not be excluded that by day 3, some bacterial cells may have died in this older broth before it was administered to the birds. Alsheikhly & Truscott (1977b) inoculated fresh

cultures of *C. perfringens* directly into the duodenum of the chickens and were able to detect changes in intestine within 1hr. There is also a possibility that when broth was mixed with feed some of the vegetative cells converted into spores that could not find suitable conditions for growth in the lower gut. It was therefore decided to make significant changes in the subsequent model *i.e*. experiment 4b, when in-feed challenge was repeated giving higher numbers of *C perfringens* (10^9). An additional change was instead of preparing inoculum once for adding to the feed on each subsequent day, the inoculum was freshly prepared every day prior to being added to that day's feed only.

The literature records many attempts to reproduce NE in poultry, all using different methods but most using recognised predisposing factors to induce NE. The most well known factors are coccidial infection and the inclusion of high protein levels in diets. Various approaches for challenge have been applied such as use of a bacterial culture (Alsheikhly & Truscott, 1977b; Branton *et al.*, 1997; Cowen *et al.*, 1987; Long & Truscott, 1976) and contaminated litter (Cowen *et al.*, 1987; Hamdy *et al.*, 1983b). However, these published reports indicate only limited success to experimentally reproduce NE, let alone sub-clinical NE (Nairn & Bamford, 1967; Bernier & Filion, 1971; Davis *et al.*, 1971). Interestingly, although presence of *Eimeria spp*. and high levels of fish meal in the diet proved essential in inducing of NE in some studies (Miller *et al*., 2010), other studies very high levels of *C. perfringens* inoculation (10^{10} cfu) along with higher doses of coccidial vaccine and fish meal (grower diet 3:1 fish meal) failed to induce NE in broiler chickens (Pedersen *et al.*, 2003; Timbermont *et al.*, 2010). In the present studies wet mount preparations at the time of necropsy from intestinal scrapings showed no evidence of any *Eimeria* spp. Previous studies with higher doses of coccidia (up to 50,000 sporulated oocytes orally along with feed containing *C. perfringens* (approximately 10^7 cfu/g of feed) also failed to produce NE lesions (Baba *et al.*, 1992). A later series of experiments (Baba *et al.*, 1997) only showed oedema of the intestine, in spite of giving higher coccidial infection and higher dose of *C. perfringens* (10^8-10^9) for 5 consecutive days.

In experiment 4b, some birds developed NE lesions. Although the number of sub-clinical NE positive birds in experiment 4b was small (3 out of 30, or 10%), birds from the challenge groups had developed gross NE lesions without clinical signs and mortality, which is typical of sub-clinical NE. According to Olkowski *et al*. (2006a) gross lesions are not always observed in experimental trials. The incidence noticed in

experiment 4b was considerably lower than the 79% incidence reported elsewhere in commercial flocks (Cooper *et al.*, 2009). However, several challenged studies with high doses of *C. perfringens* have failed to reproduce (sub-clinical) NE under controlled conditions, though intestinal lesions like hyperaemia and haemorrhages with high colonization of *C. perfringens* have been documented (Craven, 2000; Pedersen *et al.*, 2003; Dahiya *et al.*, 2007a; Liu *et al.*, 2010). The 10% incidence observed in experiment 4b is roughly consistent with many other studies. Cowen *et al*. (1987) found 1.3-10% incidence of NE in three out of five trials. Long & Truscott (1976) found 12% and 26% incidence, based on mortality, after feeding *C. perfringens* infected feed for 24 hrs or 5 days, respectively. The amount of *C. perfringens* in culture was 1.6 x 10^8 to 1x 10^9 cfu per ml of broth and a incidence of NE (10%) was found in trials where, in addition to *C. perfringens* culture, birds were reared on litter from a flock with NE. Incidence of sub-clinical NE has been shown by Kaldhusdal & Hofshagen (1992) at only 3.64%, with only seven out of 192 birds exhibiting sub-clinical NE lesions. In contrast, later trials by the same group found 10% of birds with NE lesions found that response to *C. perfringens* challenge differs significantly from one experiment to another (Kaldhusdal *et al*., 1999). According to Long & Truscott (1976) lesions typical of NE could be produced in 11-24% of broiler chickens consuming feed containing approximately 10^7 per gram of *C. perfringens*. Thus, in conclusion, though the incidence of sub-clinical NE in experiment 4b could be considered low, its level is within the wide range reported in the literature.

6.5 Conclusion

In conclusion, lower concentrations of *C. perfringens* in feed (10^2), predisposing factors like higher doses of coccidial vaccine and high levels of fish meal in the diet were unable to produce sub-clinical NE. The results also show that on high levels of fishmeal diets, even excessively high challenges of *C. perfringens* may not result in consistent production of gross lesions of sub-clinical NE. Whether the lower number of Hubbard birds with gross lesions (1 out of 15) compared to Ross birds (2 out of 15) indicates a higher level of resistance that requires further study. Further study is also needed to determine whether continued feeding of challenged feed beyond day 3 and other predisposing factors are also critical for disease production.

7 EXPERIMENT FIVE:

INDUCTION OF SUB-CLINICAL NECROTIC ENTERITIS WITH HIGH DOSES OF *CLOSTRIDIUM PERFRINGENS* THROUGH GAVAGE CHALLENGE IN THE PRESENCE OF A COMBINATION OF PREDISPOSING FACTORS

7.1 Introduction

Some dietary factors, such as high protein content, or high levels of animal protein sources (e.g. fish meal, meat and bone meal) can all significantly increase intestinal *C. perfringens* concentrations (Hafez, 2003; Kocher, 2003) so have been identified as risk factors for NE. However, previous experiments (detailed in Chapters 3, 4 and 6) have shown that the provision of each of the following predisposing factors, when applied alone, does not always result in the induction of sub-clinical NE.

Litter condition is an important factor as it not only creates suitable conditions for sporulation and growth of C. perfringens but also indirectly facilitates another predisposing factor, the sporulation of coccidial oocytes (Williams, 2005). However Experiment 2 (Chapter 4) was unable to induce NE by manipulating the diet together with using reused litter. Although sub-clinical NE was not induced, there were lesions of coccidiosis together with a failure to gain sufficient weight in birds that were given canola meal as the major protein source. A possible reason for this may have been insufficient viable and/or vegetative *C. perfringens* present in the reused litter.

Experiments 4a and 4b, detailed in Chapter 6 demonstrated that, despite having high levels of fish meal included in the experimental diets along with higher doses of coccidial vaccine, sub-clinical NE was not induced in the presence of low numbers of *C. perfringens* (experiment 4a) or high numbers of *C. perfringens* (experiment 4b). This shows that higher levels of fish meal in diet even when combined with high doses of coccidial vaccine may not be sufficient to induce sub-clinical NE.

Analysis of the results of all the previous experiments, when viewed together appear to suggest that challenging birds with *C. perfringens* in the individual presence of previously known, predisposing factors (i.e. extra fish meal, coccidial vaccination and IBD vaccination) is not sufficient to reliably induce sub-clinical NE. The experiment described here seeks to draw on the lessons learnt from these previous experiments by combining all the predisposing factors that have, in isolation not induced sub- clinical NE.

The objective of the present experiment was to induce sub-clinical NE in broiler chickens by combining all the predisposing factors that have previously been used in isolation (i.e. diet; fishmeal, canola meal, feed withdrawal, coccidial and IBD vaccinations detailed in Chapters 3, 4 and 6) following oral gavage with high doses of *C. perfringens* to determine if, together, this can induce sub-clinical NE.

7.2 Materials and Methods

7.2.1 Treatment groups and Experimental Design

A total of 112, one-day-old mixed sex Ross 308 birds were obtained from a commercial hatchery and reared in a single solid-floored pen from 0 to 7 days of age in an environmentally controlled house. Two treatments (challenge and sham challenged) were compared using 8 replicate pens. For the first 7 days the birds were given a commercial starter diet. On day 7 the birds were randomly allocated to one of 16 pens, resulting in 7 birds per pen with similarly averaged day 7 body weights. The study was conducted using a randomized complete-block design.

7.2.2 Diets and feed mixing.

Two nutritionally complete feeds were formulated, a wheat-soybean based broiler starter and a grower (Aviagen, 2009). Both feeds contained approximately 46-50% wheat (Table 7.1). None of the experimental diets contained antibiotic growth promoter or anti-coccidial drugs. Diets were produced and mixed at SAC and fed as mash. All the birds were fed the starter diet from day 0 to day 7, followed by the grower diet from day 8 until day 15. Thereafter all birds were fed the grower diet containing canola meal (155 g/kg; Table 7.1), mixed 3:1 with fish meal until the end of the study. Throughout the experimental period birds were fed and watered *ad libitum* except for a 20 hour feed withdrawal on day 16, prior to commencing experimental challenge through gavage (see below).

Table 7.1: Feed ingredients and calculated chemical composition (g/kg) of the experimental diets

Ingredients	**Starter**	**Grower**
Wheat, White	515	464
Gluten Meal	50	-
Soybean oil	26	-
Rye	40	80
Soybean Meal -48%	284	200
Tallow	43	63
Canola	-	155
Di-Calcium Phosphate[1]	20	20
Common Salt	4.5	3.5
Vitamin/Mineral Premix[2]	3	3
DL-Methionine	1.4	2
L-Lysine HCl	0.5	2
Limestone	-	7.5
Calcium carbonate	12.5	-
Calculated Chemical Composition		
Dry Matter	868	868
Metabolisable energy (MJ/Kg)	13.2	13.5
Crude protein	227	211
Calcium	10	10
Total Phosphorus	7.5	8.0
Potassium	8	8

[1]Contained 21.3% Ca, 18.7% P.

[2]*Vitamin and Mineral supplement provided (units kg^{-1} diets): Vit A 16,000 iu; Vit D_3 3,000 iu; Vit E 75 iu (iu=mg); Vit B_1 3 mg; Vit B_2 10 mg; Vit B_6 3 mg; Vit B_{12} 15 µg; Vit K_3 5 mg; Nicotinic acid 60 mg; Pantothenic acid 14.5 mg; Folic acid 1.5 mg; Biotin 275 µg; Choline chloride 250 mg; Iron 20 mg; Copper 10 mg; Manganese 100 mg; Cobalt 1 mg; Zinc 82 mg; Iodine 1 mg; Selenium 0.2 mg; Molybdenum 0.5 mg (Target feed, Whitchurch, Shropshire).*

The birds were reared following a standard commercial environmental control programme from day 0 to day 25 in the experimental pens. The research facility was thoroughly cleaned and disinfected prior to bird placement. Adequate feeders and drinkers were provided for the age and number of the birds with commercial wood

shaving provided as bedding. Pen dimensions were 0.87 x 0.64 m. A solid 45cm high plywood barrier at bird level separated adjacent pens with a wire fence located topping all barriers to the ceiling. Fresh wood shavings to the depth of 10cm were provided as bedding at the start of the study. Each pen was provided with a single food hopper and bell drinker. To avoid accidental cross-pen contamination with *C. perfringens*, the birds were reared in every second pen, creating an empty pen between each pen with birds. The empty pen was disinfected every second day of the study. Precautions such as changing of gloves and use of foot dipping tanks were also taken. Light was provided for 23hrs with controlled temperature and humidity. Birds were individually tagged. All experimental procedures were approved by Scottish Agricultural College Animal Ethics Committee (AU AE 19/2011) and carried out under Home Office authorization (PPL 60/3898).

7.2.3 Preparation of inoculum and challenge procedure

Vegetative cells of fresh overnight cultures were used for inoculation of the chickens. *C. perfringens* strain 56 was isolated from the intestines of broiler chickens with severe necrotic gut lesions producing moderate amounts of α-toxin *in vitro*. It is a type A netB positive strain (no enterotoxin or beta-2 gene), and has been used previously to induce NE in an *in vivo* model (Gholamiandehkordi *et al.*, 2007; Pedersen *et al.*, 2008; Timbermont *et al.*, 2010). The strain was kindly provided by Dr. Leen Timbermont (Ghent University, Belgium), stored in the form of frozen beads. From the frozen culture, bacteria were grown overnight on TSC agar plates. The bacteria were cultured for 24hrs at 37°C in BHI broth (Oxoid, UK) as described in section 3.2.2 of this thesis. After overnight incubation on TSC agar plates, colonies of *C. perfringens* were taken, mixed with BHI, incubated at 37ºC and shaken at 60rmp anaerobically for 8-12hrs (Appendix C). The concentration of *C. perfringens* in the inoculum was estimated spectrophoto-metrically at 600nm (Spectronic 301, Milton Roy) with the aid of a standard curve. Actual *C. perfringens* concentration in the inoculum was confirmed by plating on TSC agar plates, incubating the plates at 37°C overnight, and counting the number of black presumptive *C. perfringens* colonies as described in section 3.3.3. of this thesis.

All the birds in the challenge group were orally gavaged daily (on days 17, 18, 19 and 20) with 1.5ml of inoculum (BHI broth containing actively growing culture of *C. perfringens*) using a 10ml bottle equipped with vinyl tubing about 3-4cm long. The *C. perfringens* inoculum was gavaged twice a day and freshly prepared each time as described in Appendix- C. The chickens in the control treatment were orally gavaged on the same days with 1.5ml of freshly prepared sterile BHI twice a day. Bacterial counts were performed on the culture every time prior to gavaging (see below).

7.2.4 Infectious Bursal disease vaccination

On day 16, all birds were vaccinated with infectious bursal disease (IBD) vaccine (Poulvac® Bursine2, Pfizer Animal Health) in the drinking water following normal vaccination procedure i.e. one hour prior to vaccination the birds' water supply was stopped to ensure every bird was vaccinated.

7.2.5 Coccidial vaccination

On day 18, all the birds received anticoccidial vaccine "Paracox- VIII (Schering-Plough Animal Health, Brussels, Belgium). The vaccine contains live attenuated oocytes of *Eimeria acervulina* (two lines), *Eimeria maximum* (two lines), *Eimeria mitis, Eimeria necatrix, Eimeria praecox* and *Eimeria tenella*. Vaccine was by oral gavage at 10 times the dosage prescribed by the manufacturer as described in section 3.2.4.

7.3 Sampling and data recording

7.3.1 Enumeration of C. perfringens *in BHI and feed*

A sample of BHI mixed with *C. perfringens* culture was used for enumeration of *C. perfringens* by the spread plate method on TSC agar plates. Samples of BHI were approximately 1ml suspended in 9ml of Maximum Recovery Diluent (MRD). From this dilution further 10 fold dilutions were made. From each dilution 100µl of mixture was inoculated onto TSC plates as described in section 3.2.2. After incubation the number of colonies was counted on the most appropriate dilution (Plates with 30-300 colonies).

This figure was then used to calculate the number of colony forming units per gram of original sample.

7.3.2 Clinical signs and lesion scoring

Birds were assessed daily for clinical signs and symptoms of NE from the day of oral inoculation of *C. perfringens* through gavage till termination of the experiment. On days 21 (one day post-challenge), 25 (four days post-challenge), and 26 (five day post-challenge) of the experiment, two birds from each replicate pen were selected at random, weighed and killed by electric stun followed by exsanguinations. The entire GIT was inspected for the presence of lesions/pathological changes associated with sub-clinical NE or evidence of lesions of coccidiosis. Three different segments of GIT (duodenum, jejunum and ileum) were identified, immediately incised, and washed in PBS. Then the mucosal surfaces were inspected and scored for any lesions of clostridia, necrotic and haemorrhagic lesions. A modification of the scoring system used by Shane *et al*. (1985) was used to score intestinal lesions:

- score 0: absence of gross lesions or no lesions;
- score 1: focal necrosis or focal ulceration;
- score 2: focal ulceration coalesced to form discrete patches;
- score 3: extensive diffuse mucosal necrosis;

On all dissection days the livers of killed birds were also examined for the presence or absence of hepatitis *or* cholangiohepatitis, both of which are recognized as consistent with the pathological changes of NE.

7.3.3 Histopathology

Following post-mortem examinations on days 21, 25 and 26, samples from the small intestine, particularly those showing lesions, were taken for histopathology. A

1.5-2cm tissue sample was taken, washed with phosphate buffer saline (PBS) and stored in 10% neutral phosphate-buffered formalin for histopathology as described in section 3.2.5.3. Specimens for histopathology were embedded in paraffin, processed routinely, cut at 5-6µm and stained with Haematoxylin and Eosin (Appendix A). Gram's staining was also done to confirm the presence of Gram positive bacilli (Appendix B). The prepared tissue sections were later examined using a binocular stereo-microscope (Olympus BX 41, U-LH100HG, Olympus optical, Co. Ltd) connected by camera (spot idea™ 28.2-5MP) to computer software (Spot idea, Version 4.7) using different magnifications.

7.3.4 Quantification of C. perfringens *and α-toxin in digesta*

One bird on day 16 (pre-challenge) and two birds on days 21, 25, and 26 (post-challenge) were selected *at random* from each pen and euthanized. Ileal content (digesta content from the Meckel's diverticulum to ileo-caecal-colon junction) of all killed birds from each replicate pen were taken into sterile screw-capped bottles and immediately transferred to the Microbiology laboratory, for analysis of C. *perfringens.* The presumptive identification and enumeration of *C. perfringens* was done on TSC agar as described in section 3.3.3. Each of the digesta samples was vortexed for 15 seconds in order to ensure adequate mixing. One gm of digesta was weighed and serially diluted in MRD. From each dilution to be plated, 100µl was applied to the centre of each agar plate, allowed to be absorbed and then 15ml of TSC overlay was spread before the plate was incubated invertedly in a jar providing anaerobic conditions. The jar was placed in an incubator at 37°C for 24hrs. After incubation the number of colonies was counted to calculate the number of colony forming units per gram of original sample.

On days 21, 25 and 26, four birds from each treatment were selected at random and ileal digesta were collected for determination of α-toxin. Quantification of α-toxin in the intestinal digesta was done by Enzyme linked immunosorbent Assay (ELISA) using α-toxin *C. perfringens* kit (Cypress diagnostics, Ref. Vb040) following the manufacturer's recommendations as described in section 3.2.5.5.

7.3.5 Growth performance

At days 0, 7, 16, 21 and 25 all birds were weighed individually, to calculate weight gain (WG) during the experimental periods. Starter feed was weighed at day 0 and weighed back at day 7. The grower feed was weighed back at days 16, 21 and 25. Feed intakes (FI) were calculated by weighing the initial feed inputs against the uneaten feed over the experimental period. Feed conversion ratio was calculated by dividing average feed consumed per pen over average weight gain of birds per pen. Feed intake, WG and FCR were calculated for the period days 7-16, 16-21 and 21-25. The birds were inspected daily within the experimental period.

7.3.6 Feed analysis

Amino acid contents of grower feed with added fish meal were determined as described in section 4.2.5.6 of this thesis. Analysis of feed samples was done in duplicate.

7.4 Statistical Analysis

The effect of challenge on sub-clinical NE lesion score, growth performance and α-toxin antibodies in ileal digesta were compared using a randomized block analysis variance (ANOVA). The interactive effects of challenge (infected vs sham-infected), gender (male and female) and post-challenge days (21, 25 and 26) on the enumeration of *C. perfringens* in ileal digesta were compared using split plot design under generalized analysis of variance (ANOVA). The data obtained for the incidence of intestinal lesions were compared using a non-parameteric Fisher's exact test.

Due to the skewed nature of the numbers of *C. perfringens* and α-toxin antibodies in ileal digesta, the data was log transformed (n+1) to normalize the data before statistical analysis. Effects were reported as significant at $P<0.05$ and trends were noted when the P-value was near to 0.1. All procedures were performed using Genstat 11 for windows (VSN International Ltd, Hemel Hemstead, UK).

7.5 Results

Following gavage, birds were examined at least twice daily for any clinical abnormalities. During the experiment there were no clinical signs of disease requiring medical treatment in any of the groups (challenged or control). No mortality was observed in any of the treatment pens.

7.5.1 Enumeration of C. perfringens in BHI

A fresh *C. perfringens* broth culture mixture was prepared each time the birds were challenged through gavage. Analysis of BHI containing the culture of *C. perfringens* showed that on average inoculums contained 6.5 x 10^9 cfu/ml (SEM 9.5 x 10^8) of BHI on day 17, 7.9 x 10^9 cfu/ml (SEM 6.55 x 10^8) of BHI on day 18, 3.2 x 10^9 (SEM 1.45 x 10^8) of BHI on day 19 and 1.3 x 10^9 cfu/ml (SEM 4.5 x 10^7) of BHI on day 20 respectively. *C. perfringens* was not detected in the BHI broth of the control groups.

7.5.2 Lesions score

Figure 7.1 shows the effect of challenge and post-challenge days (21, 25 and 26) on intestinal lesion scores of sub-clinical NE. Lesions occurred in several birds receiving *C. perfringens* challenge and were similar to those of sub-clinical NE as described previously (Shane *et al.*, 1985). Chickens with *C. perfringens* challenge had significantly higher sub-clinical NE lesion scores compared to the unchallenged control group ($P<0.001$). Sub-clinical NE lesions scores were significantly higher in challenged groups on days 21 ($P<0.001$) and 25 (0.023), but not on day 26 ($P=1.00$).

In chickens with sub-clinical NE lesions the small intestine was not only distended with gas but also was thin walled. Most lesions were focal necrosis (Figure 7.2). In some of the birds, despite random distribution of lesions, these were so extensive as to appear almost confluent in certain areas. In most cases, there were more than 5 small necrotic foci. Most of the lesions were located in the jejunum mainly proximal to Meckel's diverticlulm. However, in birds having severe cases (Figure 7.4), lesions extended to both the duodenum and ileum. Unexpectedly, although lesions were seen on

days 21 and 25 (31.2% and 35.9% respectively), on day 26, no birds in the *C. perfringens* challenged treatment had lesions.

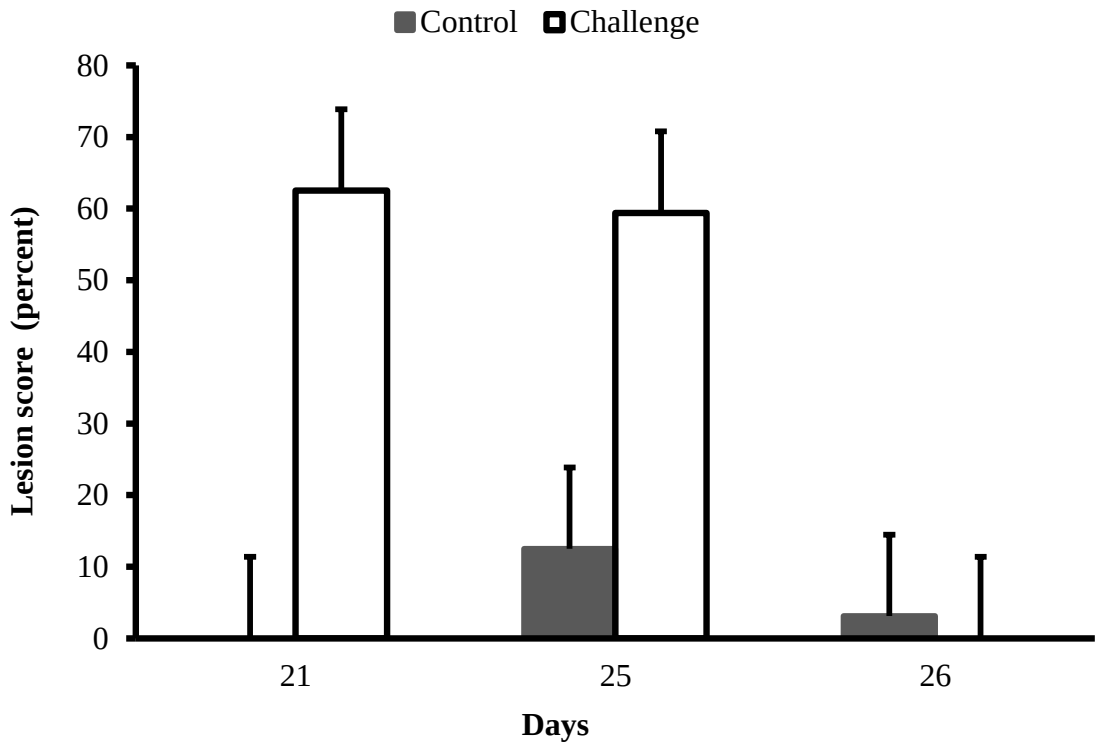


Figure 7.1: Evaluation of sub-clinical NE lesion score (percentage) in broilers subjected to different treatments (control and challenge) on different days of age (21, 25, and 26).

The error bars are standard error of the means (SEM).

Although none of the birds had clinical coccidiosis, lesions of coccidiosis were seen in most of both the challenged and unchallenged control birds (Table 7.3). Birds with coccidial lesions showed transverse white foci. No lesions were detected in the livers of any of the birds with or without lesions in their small intestine. During necropsy it was particularly noticeable that birds with NE lesions also had more watery digesta compared to birds without any lesions. None of the birds had liver lesions on any dissection days in any treatment group.

Table 7.2: Occurrence of lesions (sub-clinical NE and coccidiosis) in small intestine of broiler chickens on different days (pre-challenge and post-challenge).

Sampling days	Sub-clinical NE		Probability of difference
	control	Challenge	
16	0/8	0/8	
21	0/16	10/16	<0.001
25	2/16	9/16	0.023
26	1/16	0/16	1.00

Table 7.3: Occurrence of coccidial lesions in small intestine of broiler chickens on different days (pre-challenge and post-challenge with *C*. *perfringens* gavage).

Sampling days	Coccidial lesions	
	Control	challenge
16	0/8	0/8
21	15/16	16/16
25	15/16	16/16
26	15/16	13/16

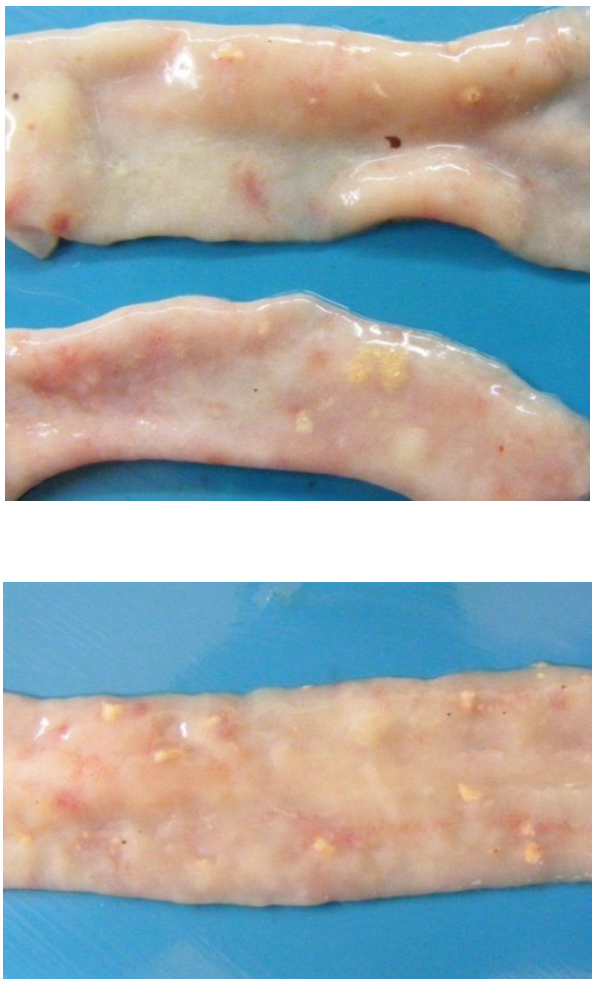


Figure 7.2: Focal necrosis in the mucosa of jejunum of broiler chickens receiving a challenge with *C*. *perfringens* through gavage

(a-b, lesions score 1+).

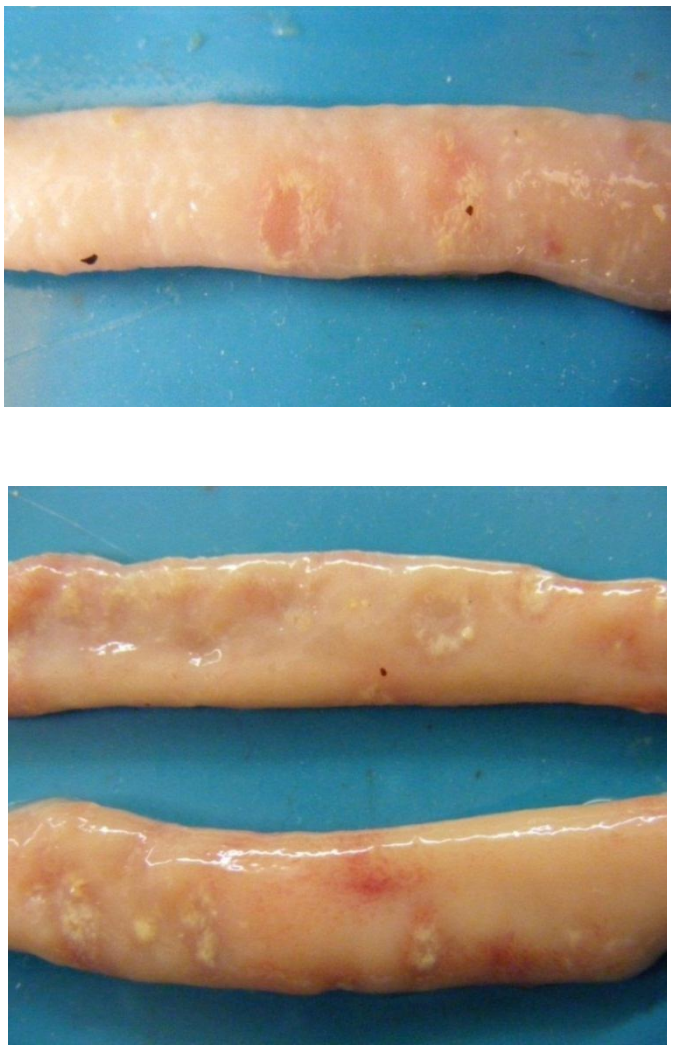


Figure 7.3: Focal ulceration on mucosal surface of small intestine of broiler chickens coalesced to form discrete patches

(a-b, lesions score 2+).

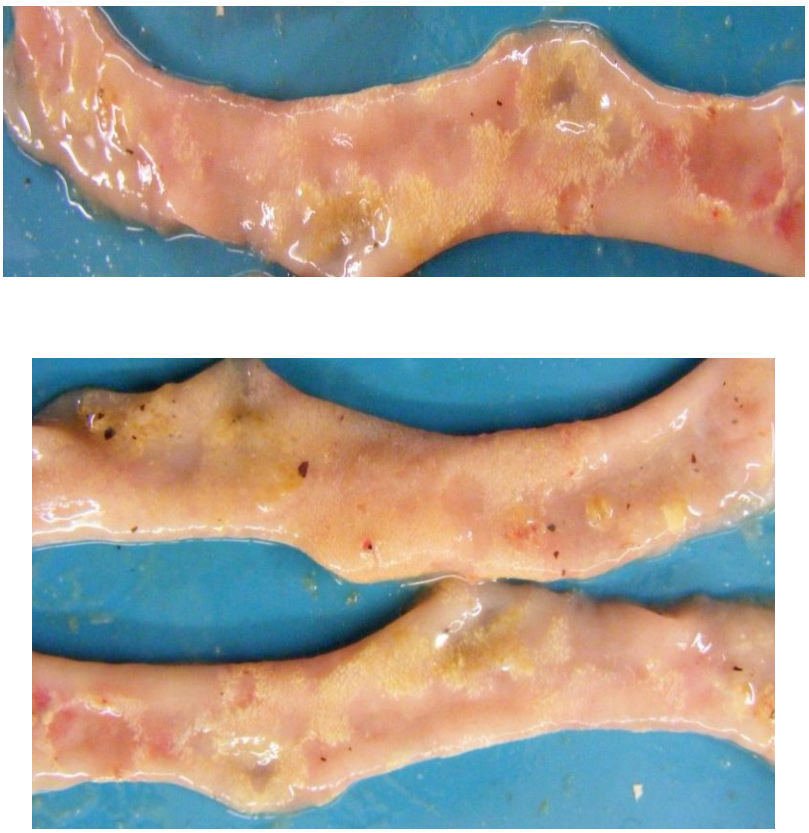


Figure 7.4: Extensive diffuse mucosal necrosis on mucosal surface of small intestine of broiler chicken. Note the appearance of pseudo membrane (lesion score 3+).

7.5.3 Histopathology

Microscopically, most of the challenged birds showed diffuse coagulative necrosis of the mucosal layer of the intestine typical of sub-clinical NE. Lamina propria were hyperaemic and infiltrated with numerous inflammatory cells. There were intense nuclear basophilia, pyknosis, karyorrhexis and karyolysis, showing a complete break down of nuclear material. Mild to moderate haemorrhages or congestion was present throughout the mucosal layer. Some of the tissue sections showed distended, irregular crypts sometimes filled with necrotic debris. Necrotic debris contained degenerated epithelial cells, remnants of lamina propria and fibrin. There was a marked congestion of blood vessels in both the lamina propria and sub- mucosa.

Histopathological slides with Gram stain showed the presence of numerous rod-shaped bacteria with *C. perfringens* morphology forming large clumps and sloughed epithelium. These were primarily around necrotic areas, but also on the epithelial surface of the villi (Figure 7.4). Gram positive bacilli can also sometimes be found in the vicinity of non-necrotic tissue (Figure 7.6). Although lamina muscularis and serosa were not affected the diphtheric membrane was composed of erythrocytes, desquamated epithelial cells, heterophils, fibrin and Gram positive bacilli. In addition rod shaped bacteria could be seen in the sub-mucosa of the small intestine. Various sexual and asexual stages of coccidia were found in most of the intestinal segments; large coccidial schizonts were particularly seen in regions of the lamina propria and in the crypt region. Occasionally tissue sections from challenged birds showed food particles in this debris.

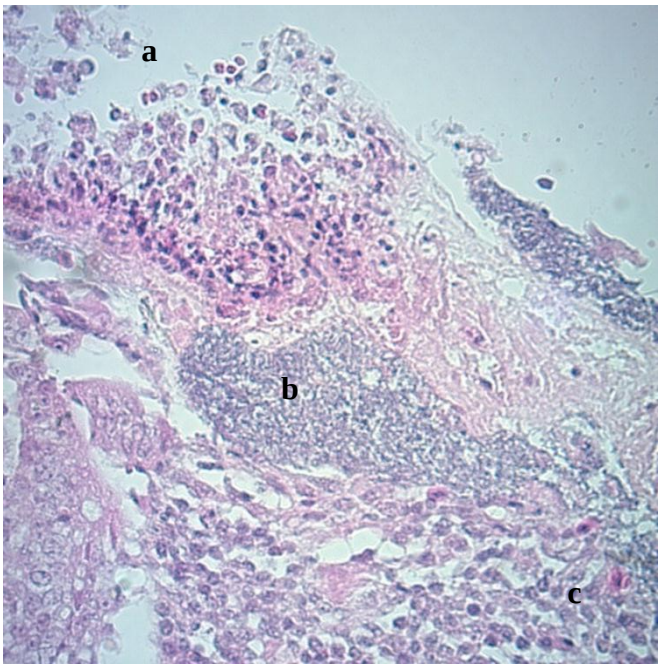


Figure 7.5: Intestine of broiler chickens showing (a) epithelial detachment (b) separation of epithelial cell layer from underlying lamina propria (c) note base of normal intact cells at base separated from necrotic tissue by clusters of bacteria (H & E; 40x).

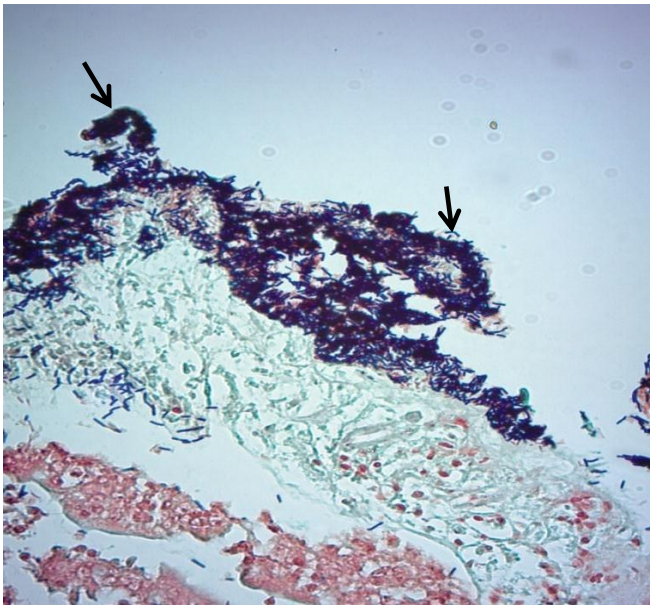


Figure 7.6: Gram stained *C*. *perfringens* on tip of villi (black arrow).

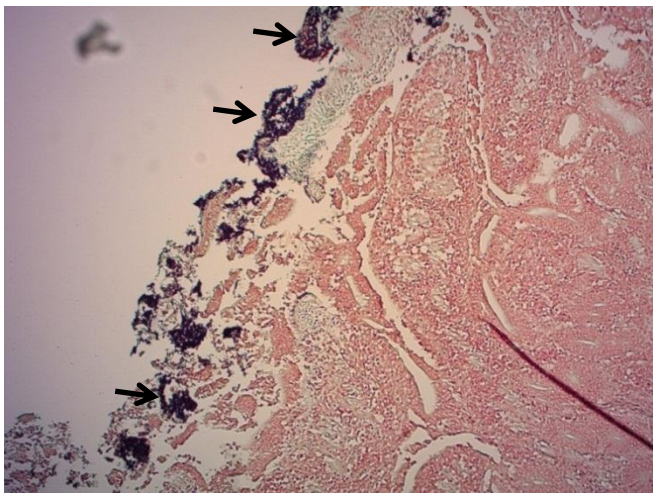


Figure 7.7: Formation of pseudo-membrane on villous surface. Note the presence of *C. perfringens* (black arrows) on desquamated villi (x 10).

7.5.4 Quantification of C. perfringens and α-toxin in the ileal digesta

Baseline values of *C. perfringens* in the ileal content were assessed prior to challenge on day 16 of the experiment. No significant difference was observed in the number of *C. perfringens* between control and challenged birds on day 16 (3.70 vs 3.50 $\log_{10}$ cfu/g; P= 0.425). Table 7.4 shows the effect of challenge (infected and sham infected), gender (male and female) and post-challenge days (21, 25, 26 days) on *C. perfringens* concentrations in ileal digesta of the birds. Counts of *C. perfringens* were significantly affected by challenge treatment and post-challenge days and their interaction (Table 7.4). The *C. perfringens* counts (cfu/g of digesta) were found to be highest on day 25 and the lowest on day 26. Concentrations of *C. perfringens* were affected by a gender x treatment interaction (Table 7.4), female broiler chickens had a greater increase in concentrations of *C. perfringens* compared to male broiler chickens (57% vs 38%).

Table 7.4 : Counts of *C. perfringens* in ileal digesta of control and challenged broiler chickens on different post-challenge days

Treatment	Gender	days	*C. perfringens* count (log cfu/g of digesta)
	Male	21	2.32
		25	5.41
Control		26	4.42
	Female	21	1.61
		25	4.41
		26	3.67
	Male	21	8.27
		25	7.19
Challenge		26	4.39
	Female	21	7.85
		25	7.87
		26	6.65
	S.E.D[1]		0.584
P-value			
Treatment			<0.001
Day			0.001
Gender			0.973
Treatment x day			<0.001
Treatment x gender			0.013
Day x gender			0.246
Treatment x day x gender			0.242

Means within a row with different letter superscripts indicate a significant (P<0.05) difference

[1]SED: Standard error of differences of means

Data are means of eight pens, and one male and one female per pen per time point.

Figure 7.8 shows the effect of challenge on *C. perfringens* specific α-toxin in the ileal digesta of control and challenged birds. *C. perfringens* specific α-toxin was detected in the ileal digesta of birds in both the control and challenged treatment groups. A significantly higher level of *C. perfringens* α-toxin (P=0.022) was found in the ileal digesta of birds with *C. perfringens* challenge treatment compared to the control group (Figure 7.8).

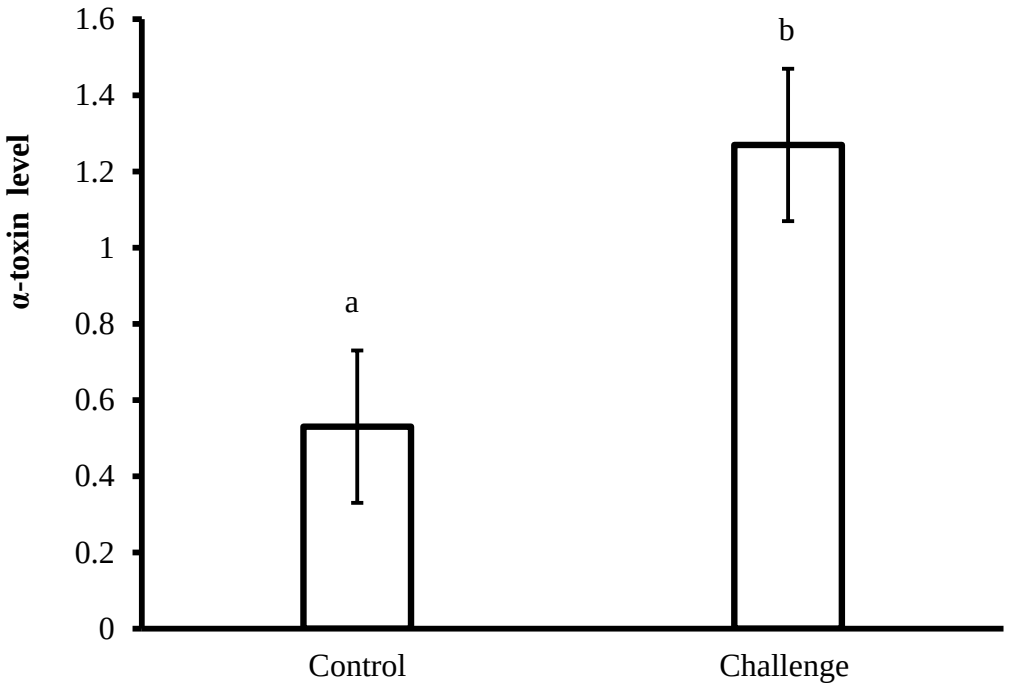


Figure 7.8: Alpha toxin level (optical density unit, OD450) in the ileal digesta of broilers chickens in control and *C. perfringens* challenged treatment groups. a-b values within treatment groups with different superscripts are significantly different (P<0.001).
The error bars are standard error of the means (SEM).

7.5.5 Growth performance

Table 7.5 shows the effect of challenge on weight gain, feed intakes and FCR during days 7-16, 16-21 and 21-25. Weight gain was not affected by challenge treatment during days 7-16 and 21-25 (P>0.05). However during days 16-21, there was a trend for the control birds to be heavier than challenged birds. Challenge did not significantly affect feed intake on any of the days. During days 7-16 and 21-25, FCR was not affected by *C. perfringens* challenge but during days 16-21, challenged birds had significantly higher FCR than control birds.

Table 7.5: The growth performance of broiler chickens in challenge and control treatment group

	Days	Control	Challenge	SEM[1]	Probability of difference
Weight gain (g/bird)	7-16	245	256	6.71	0.272
	16-21	271	237	12.58	0.080
	21-25	257	239	7.60	0.114
Feed intake(g/bird)	7-16	376	387	6.35	0.236
	16-21	266	263	7.02	0.706
	21-25	361	370	16.68	0.715
Feed conversion ratio (FCR)	7-16	1.54	1.51	0.03	0.572
	16-21	0.99[a]	1.11[b]	0.03	0.020
	21-25	1.41	1.56	0.08	0.247

Means within a row with different letter superscripts indicate a significant ($P<0.05$) difference.

[1]SEM: Standard error of the means.

Data are means of eight pens.

7.6 Discussion

With an absence of clinical signs to indicate NE, the presence of gross lesions in the intestine with related histopathological changes, suggests that the type of disease induced in the present study met the recognised criteria to warrant its classification as sub-clinical NE (Lovland & Kaldhusdal, 2001; Skinner *et al.*, 2010).

The birds in the challenged treatment group that had been repeatedly gavaged with *C. perfringens* showed an increase in their intestinal lesion score, compared with the non-challenged control group birds (Table 7.2). Typical gross lesions consisted of necrotic areas surrounded by hyperaemic zones on the mucosal surface of the small intestine. Some cases also showed the formation of a yellow to brown, diphtheric pseudo-membrane covering the intestinal mucosa (Kaldhusdal & Hofshagen, 1992). In most field cases of NE gross lesions are most commonly found in the jejunum, followed by the ileum and duodenum (Long *et al.*, 1974; Kwatra & Chaudhury, 1976; Alsheikhly & Alsaieg, 1980). It is difficult to predict the time when gut lesions peak (Lovland *et al.*, 2003). In the present study similar gross lesions were observed in experimentally-infected birds. Birds were sampled on 3 days (21, 25 and 26) and most of lesions were observed on days 21 and 25 (31.2% and 35.9% respectively). However, on day 26 no lesions were detected in any of the birds. This experiment therefore appears to suggest the optimal time point for lesion detection is between 21 and 25 days of age. These findings agree with those of (Gholamiandekhordi *et al.*, 2006) who also detected lesions only for 4 days after the final *C. perfringens* inoculation although further lesions were not detected after an additional 2 days. Riddell & Kong (1992) found that mortality began in the birds just one day after commencement of experimental challenge with most mortality occurring during the following 3 days. The exact reason(s) for a higher number of birds with lesions on day 25, yet not a single bird with lesions on day 26 in the *C. perfringens* challenged treatment group remains unclear, although it can not be excluded that this is may be to some extent the result of random sampling of the remaining birds.

The finding that three chickens (two on day 25 and one on day 26) in the control group, which had not been challenged with *C. perfringens* also developed NE lesions is not unexpected. Since the broiler chickens in the control group had been subjected to the same predisposing factors underlying our NE model, it is likely that their intestines

were primed for the development of NE arising from proliferation of the low level of *C. perfringens* present in their microflora. In addition, and despite the precautions described in section 3.2.1. it is impossible to totally exclude the possibility that *C. perfringens* may have spread from the challenged pens to one of the unchallenged pens, since the available pen space dictated that both challenged and unchallenged pens were in the same experimental building. Floor litter has been recognised as being a continuous source of *C. perfringens* due to the coprophagic activity of the birds (Line *et al.*, 1998). Another possibility is the ubiquitous nature of *C. perfringens*: the bacteria is widely present throughout the environment, having been isolated from wall swabs, litter, feeders, drinking water, transport coops and even from hatcheries in egg shell material and paper pads (Craven *et al.*, 2001a; Craven *et al.*, 2001b). Experimental conditions in the present experiment were optimum for the growth of *C. perfringens* leading to a subsequent release of α-toxin and sub-clinical disease induction. It is therefore possible that *C. perfringens* from either an endogenous or environmental source was responsible for causing the sub-clinical NE that arose in a small number of unchallenged birds. Kaldhusdal *et al.* (1999) found the frequency of NE lesions in control birds was actually higher than in challenged birds on 19 days post-challenge.

C. perfringens proliferation and sub-clinical NE are also associated with cholangohepatitis together with multifocal to massive necrotic hepatitis, collectively referred to as *C. perfringens* associated hepatitis (CPH). Affected livers are usually enlarged, firm, pale and mottled in appearance with small focal lesions (Kaldhusdal *et al.*, 1999; Lovland & Kaldhusdal, 2001). In the present study none of the birds had any form of liver lesions. Liver lesions are usually found in broiler carcasses at processing, both with and without clinical NE being recognized in the flocks. Results from Lovland & Kaldhusdal (1999) showed that 24 out of 45 (55%) livers showing lesions were positive for *C. perfringens*, which suggests that liver lesions may not always be seen in birds with sub-clinical NE.

The histopathological changes typical of sub-clinical NE were observed in the intestine of all the birds with lesions. There was a marked inflammatory response to the *C. perfringens* with focal areas of diffuse necrosis. These changes were consistent with those described in the literature (Parish, 1961; Alsheikhly & Truscott, 1977b; Kaldhusdal & Hofshagen, 1992; Kaldhusdal *et al.*, 1995). Kaldhusdal & Hofshagen (1992) explained that microscopic changes of sub-clinical NE start with slight oedema

in the lamina propria, together with dilation of vessels and a desquamation of epithelial cells, later resulting in oedema followed by detachment of the epithelial cells from the underlying lamina propria. Tips of the villi have coagulated necrosis and sloughed epithelium. The affected area has large amounts of fibrinous exudates with mononuclear cell infiltration but limited heterophil infiltration. As time passes the necrotic lesions involve more than half the length of the villi (Alsheikhly & Truscott, 1977b). Gram positive bacilli colonizing the necrotic areas in birds with sub-clinical NE, as in the present study, have also been observed by many researchers (Parish, 1961; Arbuckle, 1972; Helmbold & Bryant, 1971; Long, 1973). On day 26, *C. perfringens* appeared to have been trapped within the necrotic area and remained away from regenerated villi. Similar changes have been seen by Alsheikhly & Truscott (1977b) who reported them as a regeneration process; they observed that lesions of NE developed after 3hrs of infusion of crude toxin subsided (regeneration of villi) after 12-20hr (Alsheikhly & Truscott, 1977b).

Baseline *C. perfringens* concentrations determined on day 16 (pre-challenge), showed no difference between the counts of *C. perfringens* in the ileal digesta of subsequently challenged birds and the unchallenged birds in the control group. This suggested that all chicks in the experiment probably had a similar concentration of intestinal *C. perfringens*, prior to commencing the *C. perfringens* challenge. On day 21 (post challenge), the *C. perfringens* challenged birds had a significant increase in concentrations of *C. perfringens* compared to the unchallenged/uninfected control group (Table 7.4). This demonstrates a successful challenge. The highest *C. perfringens* counts were found on day 25, followed by 21 whilst the lowest counts were found on day 26, which agrees with an absence of lesions on that day. In further agreement, Pedersen *et al.* (2003) found that *C. perfringens* was not able to persist in the intestine of broilers for more than 6 days after cessation of experimental inoculation.

The main virulent factor of sub-clinical NE is believed to be α-toxin produced by *C. perfringens* type A (Alsheikhly & Truscott, 1977a). There was a significant increase in the antibodies to α-toxin in the ileal digesta of birds challenged with *C. perfringens*. However, there is on going debate regarding the involvement of α-toxin in producing NE. Recent studies have shown that an α-toxin mutant strain can induce severe necrotic lesions (Keyburn *et al.*, 2006), whilst involvement of α-toxin in disease

production has been confirmed by successfully producing NE using bacteria free crude toxin (Alsheikhly & Truscott, 1977b).

Intestinal coccidial lesions, consistent with typical coccidial infection, were observed in most of the birds in both treatments (Table 7.2). These findings are in line with data from the literature which reports finding coccidial lesions whilst developing a disease model for NE (McReynolds *et al.*, 2004b; Pedersen *et al.*, 2008). Overdose of live coccidial vaccine (10 times above that prescribed by the manufacturer) induces gut damage. So it can reasonably be hypothesized that mucosal damage caused by a live overdose of coccidial vaccine will allow a portal of entry for *C. perfringens,* releasing toxin(s) that may further affect many cells including enterocytes. Both *C. perfringens* and coccidia act synergistically in inducing gross, visible lesions. Intestinal coccidiosis may slow intestinal motility resulting in transient stasis of digesta and subsequent anoxia since *C. perfringens* is a facultative anaerobic that will lead to a proliferation of *C. perfringens* and sub-clinical NE (Helmbold & Bryant, 1971; Hong *et al.*, 2012)

Another possible explanation of the association between coccidiosis and NE is intestinal damage by coccidian sporozoites causing release of proteins into the gut lumen. Proteolytic enzymes may degrade these proteins into amino acids that increase the acidity of digesta. Lower pH (5.6 or below) affects the permeability of the smooth muscles of the intestine, causing stasis. Some species of coccidia not only cause a decrease in the pH of the small intestine but also cause an increase in caecal pH, where *C. perfringens* normally exists. A decrease in the pH of the small intestine, together with increase in pH of the caeca and intestinal stasis all are factors that promote the growth of *C. perfringens* (Shane *et al.*, 1985; Ruff & Reid, 1975). Since *C. perfringens* lacks the ability to form amino acids it requires a medium containing amino acids. This means that a diet rich in fish meal provides an excellent nutritional medium, further enhancing the growth of *C. perfringens* so may help in the induction of sub-clinical NE (McDevitt *et al.*, 2006b). Collier *et al.* (2008) proposed that coccidial infection induces mucogenesis as a result of the host's inflammatory response, leading to an increase in mucin that subsequently enhances *C. perfringens* growth, due to its ability to utilize the mucus as a substrate (Deplancke *et al.*, 2002). Lesions of NE have been found associated with the sexual forms of coccidia in field studies (Long *et al.*, 1974). The same results also confirmed that co-infection with *Eimeria* species is an important predisposing factor in the development of sub-clinical NE.

It was particularly noted during examination of the gross coccidial lesions that most were superficial and did not pierce deeply into the intestinal wall. This was later confirmed to be consistent with earlier findings (Alsheikhly & Alsaieg, 1980), showing that superficially developing coccidial species were more significant in providing optimum conditions for *C. perfringens* to establish and multiply in the gut, so causing NE.

Several researchers have reported that coccidiosis does not always result in NE (Long, 1973; Broussard *et al.*, 1986; Dhillon *et al.*, 2004; Hermans & Morgan, 2007). However Long (1973) reported that coccidiosis is a factor associated with the occurrence of NE under practical field conditions. Coccidiosis has been described as a contributory factor for NE by many researchers as it has usually been observed in conjunction with NE (Alsheikhly & Alsaieg, 1980; Shane *et al.*, 1985; Wu *et al.*, 2010). In the present study, no relation was found between the number of *C. perfringens* in the ileal digesta and coccidia lesions (data not shown). This appears to confirm that coccidia only provide a gateway for the entry of *C. perfringens* into the intestinal wall, but do not have any direct effect on the growth of *C. perfringens.*

No mortality was observed in any of the treatment pens, and birds showed no clear symptoms. There was also no anorexic response to challenge; *C. perfringens* challenged birds had similar intake to their unchallenged counterparts. However there was a trend in the challenged group to show a lower weight gain compared to the control birds during days 16-21. This was reflected in a significantly increased FCR in the *C. perfringens* challenged birds compared to the unchallenged controls. The results are in accordance to the findings of other researchers, showing that inoculation with *C. perfringens* and the subsequent production of α-toxin results in reduced growth and increased FCR (Kaldhusdal & Lovland, 2000; Lovland & Kaldhusdal, 2001; 2010 Liu *et al.*, 2010; Miller *et al.*,). Damage to the mucosal layer of the intestine of birds, as indicated by necrotic lesions, may result in decreased digestion as well as reduced absorption of nutrients, which together lead to both reduced weight gain and an increased FCR. The overall performance of birds in the challenged treatment group was consistently poor on all days (Table 7.5). This might have been due to the fact that the birds were under stress of *C. perfringens* challenge with higher levels of *C. perfringens* in their intestinal tract. Broilers having higher number of *C. perfringens* in their gut

have decreased weight gain and poor feed conversion efficiencies (Kaldhusdal *et al.*, 1999; Dahiya *et al.*, 2005b).

7.7 Conclusion

It was concluded that multiple *C. perfringens* inoculations through gavage, in the presence of high levels of live coccidial vaccine, together with other predisposing factors such as feed withdrawal, and higher levels of fish meal (30%) in the grower diet with CM do provide a suitable model for induction of sub-clinical NE. In the present experiment a statistically significant proportion (>40%) of the challenged birds developed necrosis of their intestinal mucosa without inducing mortality. Therefore what is successfully developed here is a sub-clinical NE model, though subsequent studies are required to confirm its repeatability. Molecular studies conducted by various researchers indicated that NE usually occurs following multiplication of one or two specific strains of *C. perfringens* in the gut (Engstrom *et al.*, 2003; Nauerby *et al.*, 2003). Therefore it may be reasonable to assume that the presence of numerous predisposing factors that help to create optimum growth for multiplication of a specific strain of *C. perfringens* is necessary for induction of sub-clinical NE. Gholamiandehkordi *et al*. (2007) did not find any visible NE lesions when using a single *C. perfringens* infection along with an high dose of coccidial vaccine. However the same researcher found that multiple oral doses of *C. perfringens* in combination with an high dose of live coccidial vaccine did give a suitable model for subclinical NE (Gholamiandehkordi *et al*., 2007). According to Wu *et al.* (2010) diets containing high levels of fish meal (250- 500g/kg) and *Eimeria* infection is the most effective combination to produce effective NE lesions. Sub-clinical NE is a complex multi-factorial disease so successful reproduction needs careful incorporation of all these factors that, acting synergistically, induce gross, visible gut damage.

The ideal model to study the pathogenesis of sub-clinical NE is one in which a statistically significant proportion of challenged birds develop grossly visible lesions, but without clinical signs or associated mortality. The disease model used in the present study incorporates the different predisposing factors that commercial poultry operations are likely to face on a day to day basis. Most of the challenged models used a mixture of *C. perfringens* strains to induce NE (McReynolds *et al.*, 2005; McReynolds *et al.*, 2008), although only one strain was used in the present study. Ficken & Wages, (1997)

suggested that factors such as coccidiosis, IBD and dietary stress along with high doses of *C. perfringens* would favour experimental induction of NE in chickens. The present study confirms this finding. Above all, this model will be more acceptable to animal welfare activists since it presents minimal ethical issues for consideration due to the lack of mortality associated with this *C. perfringens* challenge.

8 SIX:

IDENTIFICATION OF BIOCHEMICAL MARKERS FOR SUB-CLINICAL NECROTIC ENTERITIS IN BROILER CHICKENS

8.1 Introduction

Sub-clinical NE is a commonly prevalent condition that causes a serious threat to the global broiler poultry industry, as under field conditions the sub-clinical form of disease is difficult to detect, making it one of the major causes of economics losses to today's world wide broiler industry (Kaldhusdal & Lovland, 2000; Shane *et al.*, 1984). Although the presence of intestinal necrotic lesions is considered as a characteristic feature of the sub-clinical disease, the timing of the peak in numbers of gut lesions is largely unpredictable, so using measurement of gut lesions alone to quantify the sub-clinical NE severity would need repeated examinations of the flock (Lovland *et al.*, 2003).

Currently the only way to assess the degree of host response is by scoring gross pathological lesions within the intestine and most of the cases of sub-clinical NE are only identified at the time of carcass rejection (Kaldhusdal & Lovland, 2000). To date there is no validated lesion scoring system that characterizes the disease condition in poultry. The different lesion scoring systems that have been used makes identification even more complex (Shane *et al.*, 1985; Prescott, 1979; Lovland *et al.*, 2004). Olkowski *et al.* (2006a) also emphasised that diagnosis of sub-clinical NE can not be made merely on gross observations. It is recognized that there is an urgent need to develop another test that can be reliably used to identify sub-clinical NE presence, and possibly to quantify its severity. An alternative, objective approach could be to measure the levels of acute phase proteins (APP) and the expression of different genes in response to *C. perfringens* challenge in the intestinal tract of broiler chickens.

The acute phase proteins are a group of blood proteins released as a result of an acute phase response, a natural systemic reaction to neutralize the effect of pathogens, trauma and immune disorders. In response to inflammation, inflammatory cells (macrophages) secrete a number of cytokines into the blood stream, most noticeably IL1, IL6, IL8 and TNF-α. At the same time, the liver responds by producing a large number of acute phase proteins/reactants (Murata *et al.*, 2004). Similarly, during infection, inflammation of the gut mucosa can cause a significant change in the expression pattern that has a harmful effect on the underlying epithelium which can also trigger the release of different APPs. Acute phase response appears earlier than specific antibodies, and decreases as the inflammatory response subsides. In cases of chronic

infection, levels of APPs may remain elevated if stimulatory cytokines are still active (Chamanza *et al.*, 1999;Gruys *et al.*, 1994). APPs can be considered as positive or negative, depending on whether their level increase or decrease in response to challenge. The positive APPs include haptoglobin, C- reactive protein, ceruloplasmin, fibrinogen and alpha 1 acid glycoprotein, although there are differences between species. Negative APPs include albumin and transferrin (Yoshioka *et al.*, 2002; Murata *et al.*, 2004). Mammalian acute phase protein responses are well studied for many infections, however information regarding avian APPs are much less available. Much of the research on APP in fowls has been published in recent years (Chamanza *et al.*, 1999), though little is still known of the dynamics of APP in chickens.

Measurement of levels of various APPs has been used for detecting various pathological conditions. Ceruloplasmin, an important APP with well known beneficial functions, is an extracellular ferroxidase, which plays a role in regulating iron homeostasis by oxidizing toxic ferrous iron to its non-toxic ferric form, so protecting tissues from oxidative action (Chamanza *et al.*, 1999; Patel *et al.*, 2002; Olivieri *et al.*, 2011). Various studies have confirmed that ceruloplasmin, can be used as an indicator of infection in cattle (Conner *et al.*, 1986), dogs (Conner *et al.*, 1988) and chickens (Piercy, 1979). The application of another important APP, haptoglobin has been investigated in various species of infected animals i.e: cattle with acute mastitis and dogs with polyarthritis. However in avian species it is present in the form of an analogue, known as PIT 54 (Eckersall *et al.*, 1999; Georgieva, 2010).

Functional haptoglobin has two chains (alpha and beta), PIT 54 is a single chain polypeptide belonging to blood plasma α 2-glycoprotein and form a complex after binding with the globin portion of free blood haemoglobin. This large complex can not pass through the renal glomeruli. Therefore the binding of haptoglobin with haemoglobin prevents the systemic loss of iron following systemic haemolysis. Production of haptoglobin is enhanced by growth hormone, insulin, bacterial endotoxins, prostaglandins and different cytokines (IL-1, IL-6, and TNF) (Raynes *et al.*, 1991; Georgieva, 2010).

Ovotransferrin (OTF) or conalbumin is an iron binding protein in chickens, the concentration of which is significantly increased in response to chemical, bacterial and viral inflammation. The exact function of OTF in avian species is not known, however

in laying hens OTF is synthesized under the control of oestrogen (Xie *et al.*, 2002). Studies have shown that OTF may be used as a marker of inflammation associated with various infectious and non-infectious conditions (Xie *et al.*, 2002).

Ceruloplasmin, PIT 54 and OTF have been reported to increase in response to certain bacterial, viral and parasitic infections (Rath *et al.*, 2009). Various APPs have been used as diagnostic and prognostic markers of inflammation in both humans and animals (Chamanza *et al.*, 1999; Olivieri *et al.*, 2011). Economically important diseases, especially sub-clinical forms which are not detectable by clinical signs or even by post-mortem examination, may be diagnosed by levels of APPs, since APR in chickens is linked to growth depression and decreased production (Klasing & Korver, 1997). Its is known that changes in APP concentrations remain detectable until the inflammation or infection subsides in response to treatment or self recovery (Xie *et al.*, 2002). The potential use of APPs for examining the existence of infectious diseases during ante-mortem and post-mortem meat inspection has also been suggested (Saini & Webert, 1991). Thus, whilst APP response in chickens is different to that of mammals and humans (Georgieva, 2010), changes in levels of APPs have in addition mostly been assessed for aseptic inflammation, induced by intramuscular injection of turpentine (Chamanza *et al.*, 1999) or by lipopolysaccharide administration (Hallquist & Klasing, 1994).

There has been no previous attempt to characterise the APP changes that occur as a result of sub-clinical NE in broilers. Therefore the objective here was to assess the response of three main APPs, i.e. ceruloplasmin, PIT 54 and OTF in response to sub-clinical NE so to investigate whether (some of) these APPs could be used as biochemical diagnostic markers for sub-clinical NE in poultry.

During infection, inflammation of the gut mucosa can cause a significant change in the gene expression patterns having a harmful effect on the underlying epithelium. Earlier our laboratory (Athanasiadou *et al.*, 2011) utilised a novel *in situ* broiler model for genomic wide transcriptomic analysis to characterise the consequences of α-toxin infusion in the duodenum of broilers and early host responses through microarray. More than 30 genes were differentially expressed between toxin-infused and control birds. The expression level of 9 out of 11 genes expressed in microarray was validated by qPCR analysis.

Here, 6 gene transcripts that were differentially expressed in the aforementioned study were studied in challenged and sham-challenged birds. Different species of broilers can differ in their susceptibility to different infectious diseases. Resistance to diseases could be due to genetic differences between the species (Zekarias *et al.*, 2002; Lamont, 1998). Therefore an additional objective was to observe the pattern of up / down regulation of the same genes between two different broiler species (Ross 308 vs Hubbard yield). It is hoped that host responses in terms of APPs and gene expression will provide greater insight into the pathogenesis of NE.

8a: Acute Phase Protein

8.2 Materials and Methods

The chickens used in the APP studies were the same as used in the experiment of Chapter 7. Briefly a total of 112, one-day-old mixed sex Ross 308 birds were obtained from a commercial hatchery and reared in a single solid-floored pen from 0 to 7 days of age in an environmentally controlled house. All the birds were fed the starter diet from day 0 to day 7, followed by a canola-rich grower diet from day 8 until day 15. Thereafter all birds were fed the grower diet, mixed 3:1 with fish meal until the end of the study. Throughout the experimental period birds were fed and watered *ad libitum* except for a 20 hour feed withdrawal on day 16 prior to first gavage.

All the birds on the challenge treatment were orally gavaged with 1.5ml of inoculum (BHI broth containing an actively growing culture of *C. perfringens*) using a 10ml bottle equipped with vinyl tubing about 3-4cm long on days 17,18, 19 and 20. *C. perfringens* inoculum was gavaged twice a day, using freshly prepared inoculum each time as detailed in (Appendix- C). The chickens in the control treatment were orally gavaged at the same time with 1.5ml of freshly prepared sterile BHI only. Bacterial counts were performed on the culture every time prior to gavaging. On day 16, all birds were vaccinated with infectious bursal disease (IBD) vaccine (Poulvac® Bursine2, Pfizer Animal Health) in the drinking water. On day 18, all the birds received anticoccidial vaccine “Paracox- VIII (Schering-Plough Animal Health, Brussels, Belgium).

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8.2.1 Determination of ceruloplasmin in blood serum

Serum ceruloplasmin (Cp) was measured indirectly using p-phenylenediamine (PPD) oxidase activity. Ceruloplasmin catalyses the oxidation of PPD to yield a purple coloured product whose rate of formation can be determined by spectrophotometry. The rate of formation of the coloured oxidation product is proportional to the concentration of serum Cp (Sunderman and Nomoto, 1970). Before applying the PPD assay to chicken serum the optimum pH was established according to Martinez *et al.* (2007) and found to be pH 6.2. The PPD reagent was made by adding 61.5mg of p-phenylene di amine dihydrochloride (Sigma P1519) to 25ml of 0.598M sodium acetate buffer pH 6.2. Porcine serum of a known cerloplasmin concentration was used as standard and double diluted 4 times to achieve a standard curve. 50µl of PPD and 15µl of either serum sample or standard solution was added to each well of a 96 well plate. This was left in the dark for 20 minutes and then read on a Flurostar optical density reader at 550nm. The same method was subsequently used with a biochemical auto analyser (Figure 8.1; ABX Pentra 400, Horiba medical) to measure the experimental samples all in one batch.

8.2.2 Determination of PIT54 in blood serum

Commercial methods for measuring haptoglobin, which is the haemoglobin binding protein in mammals, has found to be effective with its chicken equivalent PIT54 (Eckersall *et al.*, 1999). The peroxidise activity of a haemoglobin haptoglobin complex at low pH (or PIT54 when measured in chickens) is directly proportional to the

concentration of the haptoglobin in a sample. A biochemical auto analyser (Figure 8.1; ABX Pentra 400, Horiba medical) was used to measure this activity and calculate the concentration of PIT54.

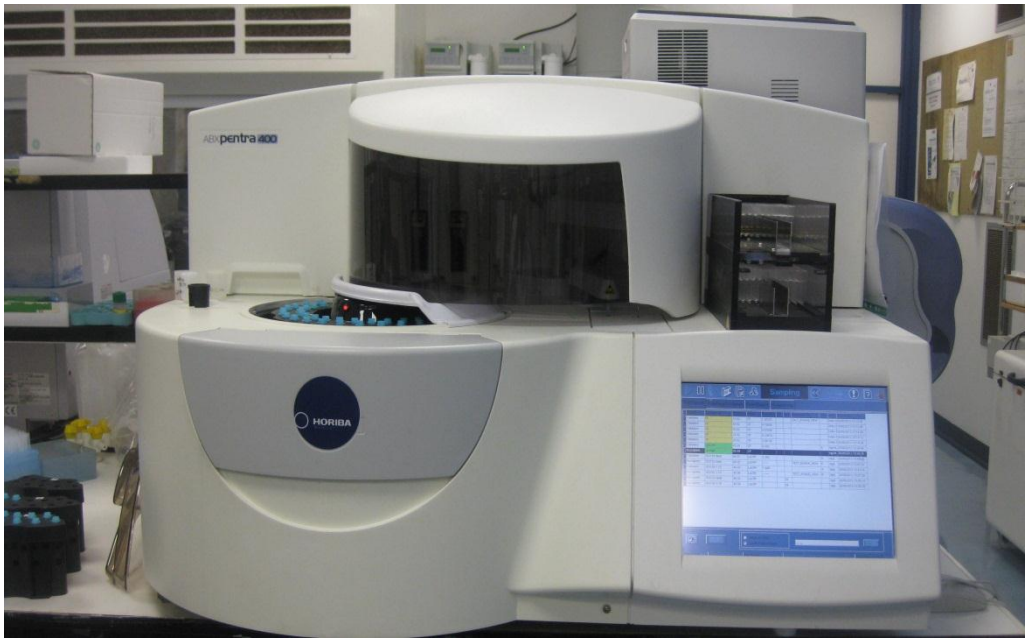

Figure 8.1: Biochemical auto analyser used to determine ceruloplasmin and PIT 54 concentrations in the serum of broiler chickens.

8.2.3 Determination of ovotransferrin in blood serum

An ovotransferrin (OVT) assay detailed by Rath *et al.*, (2009) was modified and used in the investigation. A 96 well plate was coated with chicken OVT (Conalbumin Sigma C0755) at a concentration of 1µg/ml in 0.2M Sodium hydrogen carbonate and stored at 4°C overnight. Wells were washed with TBS and blocked with 0.5% fish gelatin (Sigma G7765) for 1 – 2hours, although this was removed before the addition of the test solutions. Ovotransferrin standards were made by serially diluting 32µg of OVT in 1 ml TBS to 0.125µg/ml. Rabbit anti-chicken transferrin (OVT) antibody (Accurate Chemical AI-AG 8240) was diluted to a concentration of 0.2µg/ml in TBS. Serum was diluted 1:20 (5 µl in 1ml) in TBS. 50 µl of diluted standards or samples were added in duplicate to each well before the addition and mixing of 50 µl anti-chicken transferrin antibody with this final mixture being incubated by rocking for a further 2 hours. The wells were washed successively 4-5 times using TBS containing 0.05% tween-20 (TBS-T). 100µl of 0.02% goat anti-rabbit IgG-HRP was added to each well. After 1 hour

incubation with constant shaking, each well was aspirated and further washed 3-4 times with TBS-T. 100µl of IMB peroxidase substrate (KPL 50-76-00) was then added to each well followed by incubation for 4 – 5 minutes at room temperature as the wells turned blue. The reaction was halted with the addition of 100µl 1M NaOH. Absorbance was measured at 450nm on a Flurostar optical density reader.

8.3 APP: Statistical analysis

The effect of challenge on serum concentration of ceruloplasmin, PIT54 and OTF were compared using a split plot design (Genstat 14 for Windows, IACR Rothamstead, England). The partitioned sources of variation included challenge, gender, post-challenge days and their interactions.

Because of the skewed nature of the concentrations of ceruloplasmin, data was transformed according to log (n + 1) to normalize the data before statistical analysis. Means were compared by Duncan's multiple range test. Effects were reported as significant at $P<0.05$.

8.3 APP: Results

8.3.1 Serum Ceruloplasmin concentration

Figure 8.2 shows serum ceruloplasmin concentrations on day 16, and the effect of challenge (infected and sham infected), gender (male and female) and post challenge days (21, 25, 26 days). Serum ceruloplasmin concentrations were significantly affected by challenge treatment ($P= 0.023$) but not by its interactions with gender ($P= 0.739$) and post challenge days ($P= 0.346$). Challenged birds showed a significantly larger increase in concentrations of serum ceruloplasmin compared to the unchallenged control birds. There was a significant difference in ceruloplasmin concentrations on different experimental days ($P< 0.001$). Concentrations of serum ceruloplasmin increased from day 21 (one day post-challenge), to a maximum at day 25 followed by a sharp decline on day 26 (5 days post-challenge). Serum ceruloplasmin concentrations were not affected by gender ($P= 0.881$) or the interaction between post challenge days and gender ($P= 0.092$).

8.3.2 Serum PIT54 concentration

Figure 8.3 shows serum PIT 54 concentrations on day 16, and the effect of challenge (infected and sham infected), gender (male and female) and post challenge days (21, 25, 26 days). Serum PIT 54 concentrations were not affected by treatment (P= 0.872) post challenge days (P= 0.118) but it was affected by the interaction between challenge treatment and post challenged days; challenged birds had higher concentrations of serum PIT 54 on day 25 whereas on day 26 control birds had higher concentrations of serum PIT 54 (P= 0.008). Serum PIT 54 concentrations were not affected by gender (P= 0.959) or its interaction with treatment (P= 0.884) or post challenge days (P= 0.172).

8.3.3 Serum ovotransferrin concentration

Figure 8.4 shows serum OTF concentrations on day 16 and the effect of challenge (infected and sham infected), gender (male and female) and post challenge days (21, 25, 26 days). Concentrations of serum OTF was not affected by challenge treatment (P= 0.303), post challenge days, (P= 0.795) or their interaction (P= 0.972). Serum OTF concentrations was significantly affected by gender (P= 0.001) but not by its interactions with challenge (P= 0.256) and post challenge days (P= 0.539). Concentrations of serum OVT was significantly higher in females compared to male birds (P < 001).

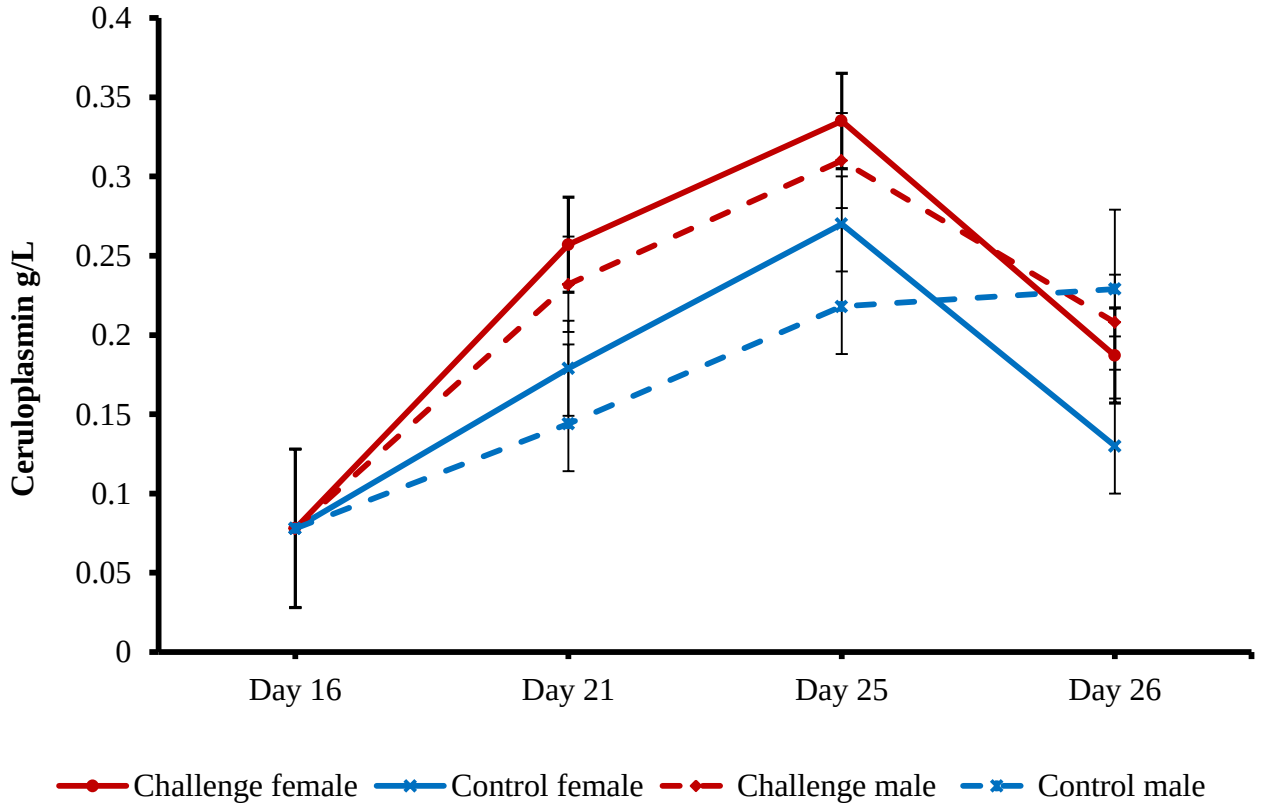

Figure 8.2: Serum ceruloplasmin concentration of male and female in challenged and unchallenged control treatment groups on days 16 (pre-challenge), 21,25, and 26 (post challenge).

The error bars are standard error of means (SEM).

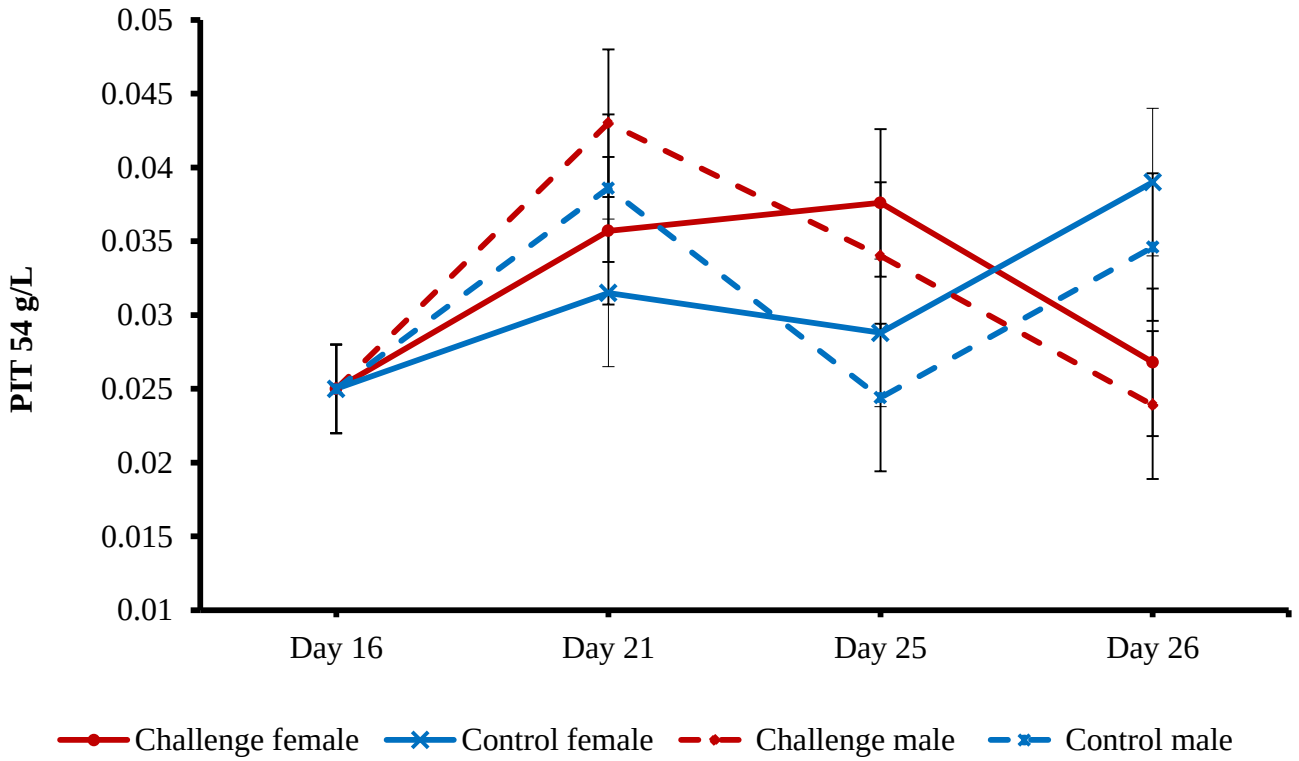

Figure 8.3 : Serum PIT54 concentration of male and female in challenged and unchallenged control treatment groups on days 16 (pre-challenge), 21,25, and 26 (post challenge).

The error bars are standard error of means (SEM).

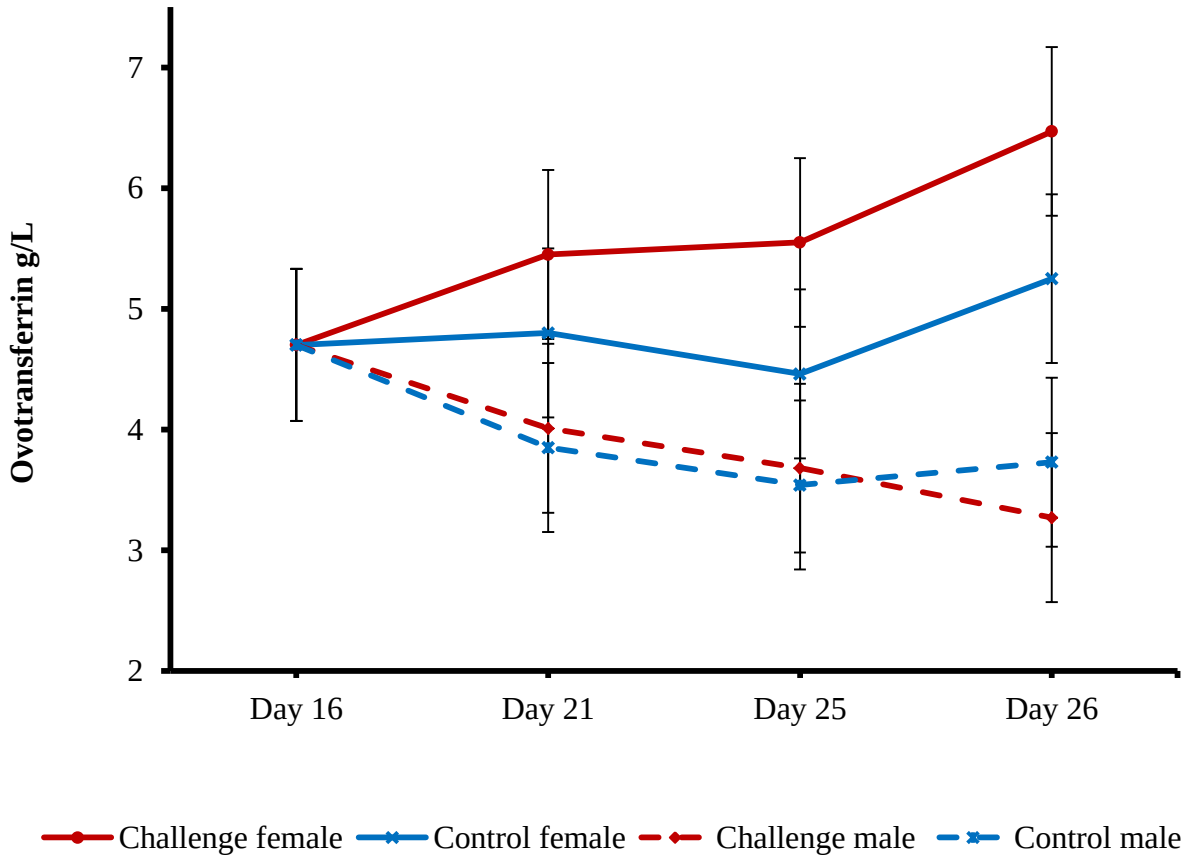

Figure 8.4: Serum Ovotransferrin concentration of male and female in challenged and unchallenged control treatment groups on days 16 (pre-challenge), 21,25, and 26 (post challenge).

The error bars are standard error of means (SEM).

8.5 APP: Discussion

Experimental models of sub-clinical NE are increasingly required for studies of disease progress and the effect of novel interventions products. However reliable, easily quantifiable indicators of sub-clinical NE are not readily available. Even scoring system for the disease characterization varies greatly (Prescott, 1979; George *et al.*, 1982; Lovland *et al.*, 2004) Acute phase proteins as a result of early inflammation can represent a potential useful marker of bacterial infection (Chamanza *et al.*, 1999). Economically important diseases especially sub-clinical forms which are difficult to detect by clinical signs or even by post-mortem examination may be diagnosed by levels of APPs, as the acute phase response in chickens is linked to growth depression and decreased production (Klasing & Korver, 1997). The effect of sub-clinical NE on the concentration of various APPs has not been assessed in broiler chickens. The present study analysed the effect of experimental challenge on three important APPs and their relation to attempted induction of sub-clinical NE.

The disease was successfully produced in the present study. The appearance of typical lesions of sub-clinical NE following *C. perfringens* challenge confirmed the presence of infection in the gut. During the initial phase of inflammatory process induced by infection involves the release of acute phase proteins (Gruys *et al.*, 1994). The present study indicates that serum levels of the positive acute phase protein ceruloplasmin increased in challenged birds compared to unchallenged controls probably due to gut lesions mediated through gut microbiota. These findings are in agreement with previous studies which reported significant elevations of ceruloplasmin concentrations during experimental infection with *E. coli* and *E. tenella* (Georgieva, 2010). Mazur-Gonkowska *et al.* (2004) found a significant increase in concentrations of ceruloplasmin in turkeys infected with *E. coli,* and levels remained elevated until day 10 post infection. This indicates that ceruloplasmin is an important APP in chickens responding to bacterial infection.

The present study showed that serum ceruloplasmin levels increased during the course of infection, reaching a peak at day 25 (4 days post challenge) when the maximum number of birds were found to be positive for sub-clinical NE lesions (Chapter 7). This was followed by sharp a decline on day 26, although levels remained elevated compared to pre-challenge (day 16). This pattern of response is similar to the

previous study in chickens experimentally infected with collibacillosis (Piercy, 1979). Piercy (1979) found peak ceruloplamin concentrations on day 4 post-challenge after which the concentration decreased. Piercy (1979) suggested that an increase in concentration of serum ceruloplasmin may be a change mediated by pathogenic bacteria to overcome the host hypoferraemic response. Similarly serum levels of ceruloplasmin found to be increased following experimental infection with salmonella in commercial layers (Garcia *et al.*, 2009).

Ceruloplasmin was the only APP to be affected by sub-clinical NE challenge. Increased levels of pro-inflammatory cytokines such as IL-1 and IL-6 and TNF-α following infection stimulate liver production of APPs resulting in an increased concentration in the blood (Tosi, 2005). A similar mechanism may apply to the elevation of ceruloplasmin in birds challenged with *C. perfringens.*

In the present study no significant effect of challenge was observed on PIT 54 in the serum of male and female broilers. Literature regarding haptoglobin in poultry is very scarce. In cattle haptoglobin has been reported as useful indicator of bovine bacterial infection (Eckersall & Conner, 1988). Deignan *et al.* (2000) after experimental infection with salmonella in calves found a significant increase in serum haptoglobin with in 3 days of post-challenge, although after 5 further days the levels of haptogobin returned to normal. Conversely Georgieva (2010) found no difference in PIT 54 concentrations after experimental infection with *Eimeria tenella*. This may be the case in the present experiment as some birds did develop sub-clinical lesions which may offset the response from birds in the challenge groups. However increase in levels of haptoglobin has not been studied with common chicken diseases as has been done with ruminants (Chamanza *et al.*, 1999).

Transferrin is a negative APP in cattle as its levels are known to decrease with acute infection (Moser *et al.*, 1994). Conversely in chicken transferrin act is a positive APP, as the levels of OTF increase with inflammatory and infectious diseases (Xie *et al.*, 2002). In mammals OTF is down-regulated during inflammation (Murata *et al.*, 2004). Conversely OTF is up-regulated in fibroblasts and chondrocytes in response to viral infection (Morgan *et al.*, 2001) and inflammation (Xie *et al.*, 2002). In the current study levels of OTF neither increased nor decreased in the serum of challenged and unchallenged treatment groups. Xie *et al*. (2002) reported that ovotransferrin increased

in SPF chickens upon challenge with different viral diseases, like fowl pox virus, infectious bronchitis virus or infectious laryngotracheitis virus. However, there are studies where levels of transferrin remained unchanged after administration of endotoxin (Moser *et al.*, 1994). In agreement with the present study, Rath *et al.* (2009) did not find any significant change in chickens infected with pulmonary hypertension syndrome and tibial dyschondroplasia compared to their controls. However, phenomenon of changes in concentration of serum OTF remains unexplained. Murata *et al.*, (2004) suggested an increase in concentration in many microbial challenges may help the host's non-specific defence. In the current study female broiler chicks had significantly higher concentrations of OTF compared to male birds possibly due to the fact that OTF synthesis is under the control of oestrogen (Palmiter *et al.*, 1981).

The expression of these APPs has yet to be fully determined through further investigation in experimental models. It is noteworthy that a proportion of birds in the control unchallenged groups developed lesions of sub-clinical NE. That could explain the difference in the levels of PIT 54 and OTF compared to ceruloplasmin (O'Reilly, Personnel communication). However this is the first study to measure/analyse the response of APPs in broiler chickens challenged with *C. perfringens.*

8.6 APP: Conclusion

In conclusion, our results showed that ceruloplasmin may be considered a moderate APP in sub-clinical NE in chickens as it increased around 3 fold, suggesting that monitoring serum ceruloplasmin concentration could indicate the presence of *C. perfringens* infection in poultry. However further research confirming its role and concentrations in sub-clinical NE are needed to provide a diagnostic and prognostic marker for flock health and welfare to ultimately help in better understanding of the pathophysiology of sub-clinical NE. The potential for PIT54 and OTF as APP has not been confirmed in this study, perhaps the subclinical infection of the experimental model was not sufficient to cause substantial increases in the APP of chicken.

8b: Gene expression

8.7 Materials and Methods

The chickens used in the gene expression studies were the same birds referred to in chapter 6 (b) (previously published paper Saleem *et al*., 2012). Briefly a total of 45 day-old male (30 Ross 308 and 15 Hubbard) were obtained from a commercial hatchery, reared in solid-floored pens in an environmentally controlled house. Three groups of birds (2 Ross and 1 Hubbard) were subjected to one of three different treatments (Table 6.1). All three treatments were randomly allocated to pens in three positional blocks. A total of nine floor pens, with 5 birds per pen were used in an environmentally controlled room with the birds reared as a single flock from day 0 to day 7.

All the groups were fed the starter diet from day 0 to day 7, followed by the grower diet from day 8 until day 15. Thereafter all birds were fed the grower diet, mixed 3:1 with fish meal until day 21. Challenged groups were given a *C. perfringens* culture in their diet on days 17, 18 and 19. In contrast, the birds of the control group were given feed mixed with sterile brain heart infusion broth (BHI) only, as described in Chapter 3.2.2. On day 18, all birds received anticoccidial vaccine "Paracox-8™" (Schering-Plough Animal Health, Welwyn Garden City, UK) by oral gavage at 10 times the dosage prescribed by the manufacturer.

8.7.1 Tissue collection

On day 21 all the birds were humanely killed by intravenous administration of an overdose of barbiturate. Gut samples was taken (from lesions and jejunum) from all the birds (n= 48), briefly washed with PBS and stored in RNA Later for possible further gene expression analysis. All samples were kept and analysed on a per bird basis. RNA in animal tissue is not protected that is why each sample was treated with RNA later, after which samples were immediately stored at -80°C until further use.

8.7.2 Tissue disruption and RNA Isolation

Efficient disruption and homogenization of the tissue is an absolute requirement for all total RNA purification procedures. Tissue disruption is required to break cell membrane and organelles to release all RNA contained in the sample. A maximum amount of 30µg of duodenal sample was taken using a sterilized forceps with the tissue sample being cleaned and placed in ceramic beaded tube (CK28 tubes; Stretton Scientific, Stretton, UK) with 600µl of RLT buffer (Appendix -D). The beaded tube was placed in the cell disrupter machine (FastPrep®, model FP100, Qbiogene, Inc, Cedex, France) at 6.5 for 40 seconds. The mixture was then centrifuged for 3 minutes at 13,000rpm, and 500ml of the supernatant was pipetted and transferred to new 2ml centrifuge tube.

RNA isolation was done using RNeasy Mini Kit (Qiagen, according to instructions of the manufacturer with an additional step. One volume of the 70% ethanol (Appendix -D) was added to the cleared lysate mixed immediately by pipetting. Up to 700µl of the sample with any precipitate that may have formed was transferred to an RNeasy spin column placed in a 2ml collection tube. This mixture was centrifuged for 15 seconds at 10,000 rpm and flow throw was discarded. 700µl of buffer RW1 was added to the RNeasy spin column, before gentle lid closure and 15 seconds of centrifuging at 10,000rpm followed by discarding of the flow throw. 500µl of buffer RPE was added to the RNeasy spin column, centrifuged for 2minutes at 10,000rpm before discarding both flow throw and collection tube. The RNeasy spin column was placed in a new 2ml of collection tube, centrifuged at full speed for 1minute. The RNeasy spin column was placed in a new 1.5ml collection tube 30-50µl of RNase-free water was added directly into the spin column membrane and centrifuged at full speed for 1minute to elute RNA.

After extraction of RNA, the quantity of RNA in each sample was determined spectro-phometrically at 260/280nm (Nano Drop®, ND-1000, Nanodrop Technologies Inc).

8.7.3 Conversion of RNA into cDNA

As the extracted RNA was unstable, RNA was converted into complementary DNA (cDNA) using Verso™ cDNA synthesis kit (Thermo Scientific, UK). Different quantities of samples were used for conversion of RNA to cDNA:

- Samples having more than 2000ng/µl of RNA used 0.5µl of sample
- Samples having less than 800ng/µl of RNA used 2 µl of sample
- Samples having RNA from 800 to 2000ng/µl used 1µl of sample.

Following the manufacturer's protocol for reverse transcription in RNA samples the following were added: 4µl 4x cDNA synthesis buffer, 2 µl 2 µM dNTP mix, 1 µl RNA primer, 1 µl of RT enhancer, 1 µl of vero enzyme mix, water (PCR grade) to a final volume of 20µl. The reaction was incubated for 30 minutes at 42°C. The reaction was inactivated by heating at 95°C for 2 minutes. Generated cDNA was stored at -20°C until further use.

8.7.4 Quantitative reverse transcriptase polymerase chain reaction (qPCR)

PCR amplification and analysis were achieved using a MX 3000P™ thermocylcer (Startegene) using SYBR green (Sigma, UK). cDNA was diluted 10 fold in RNase free PCR grade water and 1µl added to a 20µl reaction. The reaction mixture consisted of 1µl of cDNA (1:10 diluted). 0.5 µl of each primer, 10 µl of Brilliant III ultra fast SYBR® qPCR Master Mix and 7.75µl of RNase free water. The primers for each gene analysed are shown in the Table 8.1. Since the PCR products were sequenced, this confirmed that analysed genes were correct. All templates were amplified using the following protocol: a pre-incubation for 2 minutes at 50°C, 10 minutes at 95°C, amplification for 40 cycles, 30sec at 95°C, primer specific annealing temperature for 1 min (Table 8.1) and 30sec at 72°C. Fluorescent data was acquired during each extension phase. After 40 cycles a melting curve was generated by heating the samples to 95°C for 1 min followed by cooling down to 55°C for 30sec and heating the sample to 95°C for 30sec. with continuous measurement of the fluorescence. All

RT-PCRs were amplified as a single product as determined by melting curve analysis. In each run 6 standards of the gene of interest in duplicate were included with appropriate dilutions to determine the cDNA concentration in the sample. Relative mRNA levels were calculated for genes using an included standard curve for each individual gene and values were normalised to the housekeeping gene β-actin that was used as an internal control, the expression of which was measured in the same way. Expression of β-actin did not significantly affected by different treatment groups (P= 0.896).

Table 8.1: Sequence of oligonucleotide primers used in quantitative real-time PCR

Gene name	Primer sequence (5′- 3′)		Size for PCR product (bp)	Annealing temperature
	Forward	Reverse		
ßAct	GAGAAATTGTGCGTGACATCA	CCTGAACCTCTCATTGCCA	180	61
NBL1	CGGCTGCGAGTCCAAGTCCATC	TCCACCAGCTTGTCAACCCTGG	200	60
BL-A	ACGTCCTCATCTGCTACGCCGA	TTCCGGCTCCCACATCCTCTGG	236	59
FAS	CCTGACCCACCACGTCCCTGA	GGTTTCGTAGGCTCCTCCCATTCCA	196	61
GIMAP8	TCGTGGGCAAGACGGGGAGT	CCGCAGAAGCGGCCTTTAGC	130	60

Primer sequences are proprietary; assays were designed and provided by Qiagen (Quantitect Primer Assay) and conditions used were as described in the Qiagen Quantitect Primer Assay handbook.

8.8 Gene expression: Statistical analysis

cDNA levels of genes of interest and the housekeeping gene were then subjected to a comparative quantitation analysis, using the MxPro software (Stratagene). Statistical differences between groups were compared using generalized analysis of variance (ANOVA). Effects were reported significant at $P< 0.05$. All procedures were performed using Genstat 14 for Windows (IACR Rothamstead, UK).

8.9 Gene expression: Results

8.9.1 Expression of fas

Figure 8.5 shows the effect of *C. perfringens* challenge (infected and sham infected), on the expression of the *fas* gene in intestinal tract of broilers. Challenge did not significantly affect the expression of *fas* in different treatment groups ($P= 0.249$). Moreover the expression of *fas* did not differ between the challenged Ross and Hubbard birds (5.36×10^4 vs. 7.69×10^4).

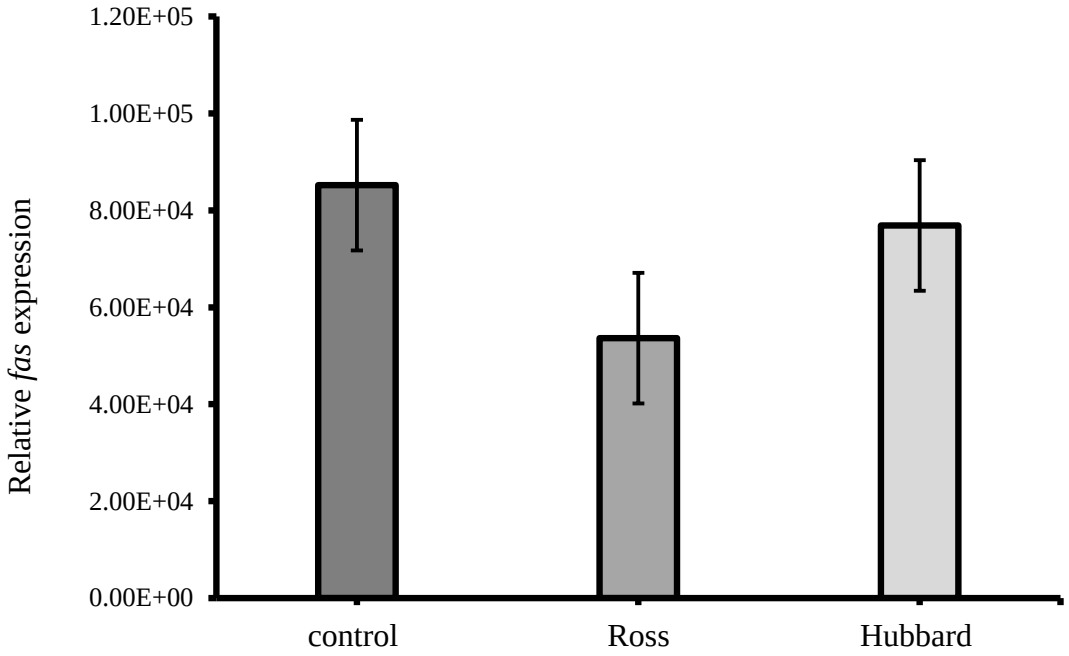

Figure 8.5: Effect of experimental challenge with *C. perfringens* on relative expression of *fas* in intestine of broiler chickens.
The Error bars are the standard error of means (SEM).

8.9.2 Expression of BL-A

Figure 8.6 shows the effect of *C. perfringens* challenge (infected and sham infected), on the expression of the *BL-A* gene in the intestinal tract of broilers. Challenge did not significantly affect the expression of *BL-A* in different treatment groups (P= 0.592). Moreover the expression of BL-A did not differ between the challenged Ross and Hubbard birds (3.52×10^2 vs. 2.98×10^2).

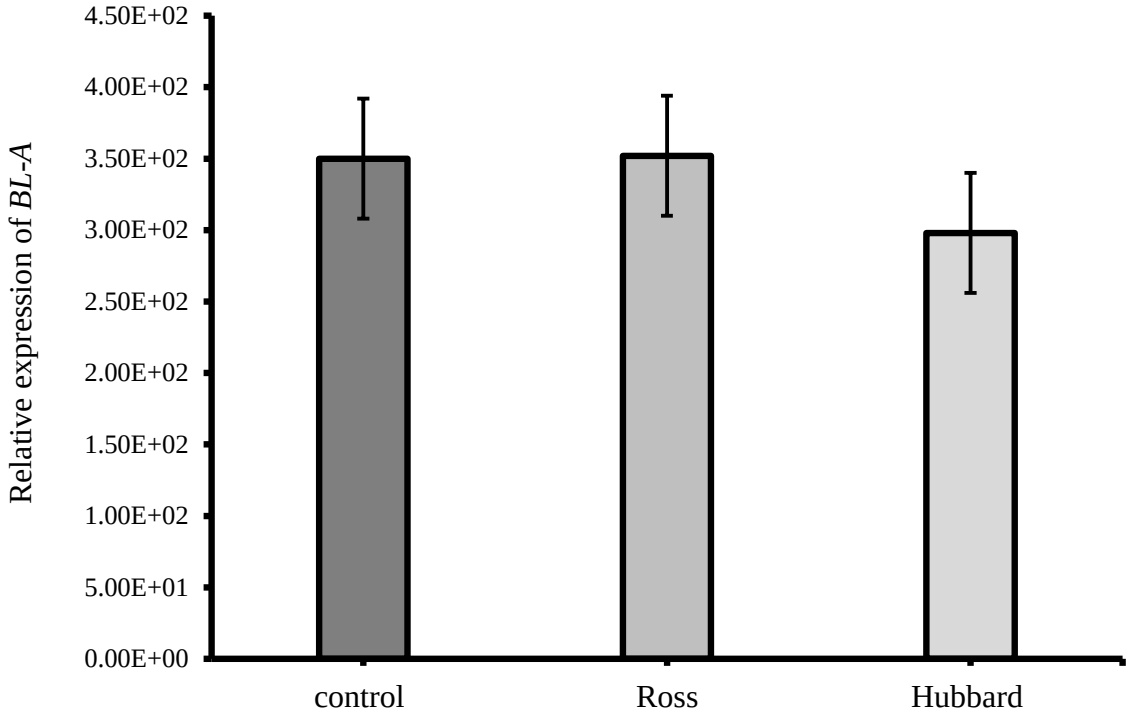

Figure 8.6: Effect of experimental challenge with *C. perfringens* on relative expression of BL-A in intestine of broiler chickens.
The Error bars are the standard error of means (SEM).

8.9.3 Expression of NBL1

Figure 8.7 shows the effect of *C. perfringens* challenge (infected and sham infected), on the expression of the NBL1 gene in the intestinal tract of broilers. Infection tended to reduce expression of NBL1 in Ross birds (P= 0.072). Moreover the expression of NBL1 did not differ between the challenged Ross and Hubbard birds (3.13×10^3 vs. 6.07×10^3).

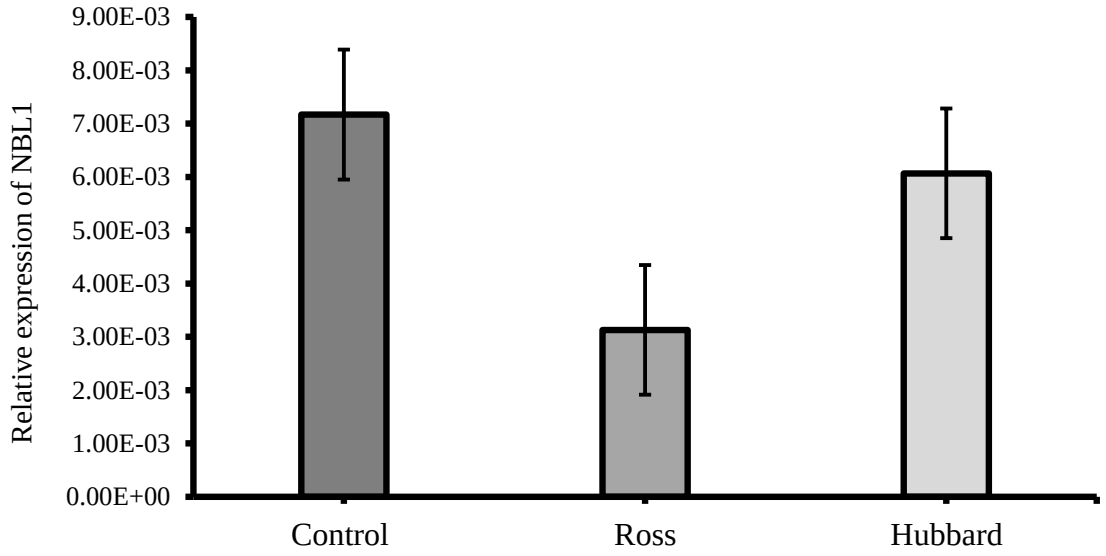

Figure 8.7: Effect of experimental challenge with *C. perfringens* on relative expression of NBL1 in intestine of broiler chickens.
The Error bars are the standard error of means (SEM).

8.9.4 Expression of GIMAP8

Figure 8.8 shows the effect of *C. perfringens* challenge (infected and sham infected), on the expression of the *GIMAP8* gene in the intestinal tract of broilers. Challenge did not significantly affect the expression of *GIMAP8* in different treatment groups (P= 0.377). Moreover expression of *GIMAP8* did not differ between the challenged Ross and Hubbard birds (1.92×10^3 vs. 5×10^7).

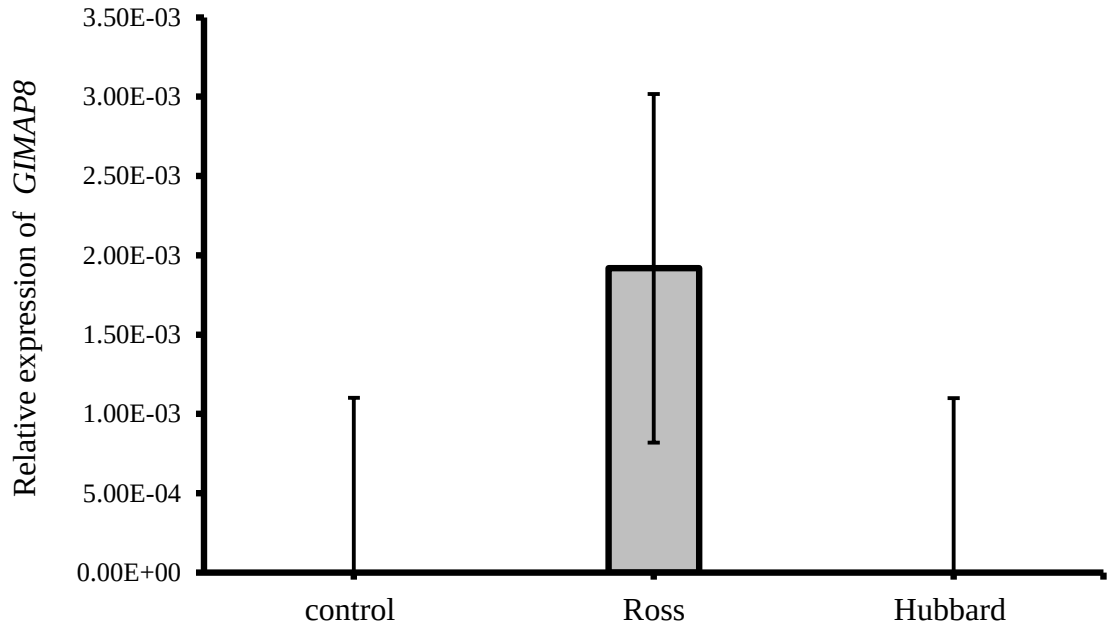

Figure 8.8: Effect of experimental challenge with *C*. *perfringens* on relative expression of *GIMAP8* in intestine of broiler chickens.
The Error bars are the standard error of means (SEM).

8.10 Gene expression: Discussion

At both cellular and molecular level a pathogen can modulate and interfere with different programmes within an organism. In terms of primary host response, it is necessary to recognise that a pathogen can cause a number of changes in gene expression levels in particular host cells. Gene changes that occur can vary between susceptible and resistant chickens as well as between infectious and control birds (Hemert, 2007). The literature reports differences in gene expression levels between control and challenged birds following experimental induction of diseases (Liu *et al.*, 2001; Hong *et al.* 2012; Sumners *et al.*, 2012). Following experimental induction of malabsorption syndrome (MAS), chickens susceptible to MAS had much lower expressions of IL-2, IL-6, IL-18 and IFN-gamma in their small intestine when compared with birds with a high resistance to MAS with higher expressions of the same genes. Following induction of Marek's disease, similar differences in gene expression were found between the lymphocytes of birds resistant or susceptible to the disease (Liu *et al.*, 2001).

By using a genome-wide transcriptomic analysis and an *in situ* broiler gut loop model from earlier work, Athanasiadou *et al.*(2011) identified the pathways that regulate early host responses to *C. perfringens* toxin in the duodenum of broilers. Genome-wide transcriptomic analysis of duodenum in broiler chickens infused with crude *C. perfringens* α-toxin showed a wide range of genes associated with an innate immune response, which were up and down-regulated in toxin-infused birds compared to controls. 11 genes were confirmed by qPCR (Athanasiadou *et al.*, 2011). Out of 11 genes that were confirmed by qPCR, six genes were selected to be analysed. The expressions of 6 gene transcripts were analysed in broilers experimentally challenged with *C. perfringens* (1.54×10^9 cfu/g of feed) in two genetically different commercial broiler chicken lines (Ross, Hubbard) with the objective of obtaining a better understanding of the immune competence possessed by these two commercial lines.

Fas is a cell surface protein, belonging to the tumour necrosis factor family that plays a key role in cell death. It acts as a receptor for Fas ligand, a cell surface molecule. When *Fas* ligand binds to *Fas* it induces apoptosis of *Fas* bearing cells (Nagata & Golstein, 1995). In the present study *C. perfringens* challenge did not have a significant effect on expression of the *Fas* gene. The mechanism of cell death in *C. perfringens*

infection is attributed to both necrosis and apoptosis. One hour post infusion with *C. perfringens* crude toxin, cell death is attributed to necrosis as Pro-apoptotic genes, such as *Fas*, CASP8 and TNFRSF1B, that have been shown to be down-regulated in toxin-infused birds compared to controls (Athanasiadou *et al.*, 2011) Whereas, 24 hrs post *C. perfringens* inoculation, cell death in the spleen of infected broilers is said to be controlled via apoptosis (Zhou *et al.*, 2009).

GTPase of the immunity-associated protein family member 8, *GIIMAP8*, belongs to an immunity associated protein family also called immune-associated nucleotide binding proteins (IANs). They are functionally uncharacterized GTP-binding proteins expressed in vertebrate immune cells as well as in plant cells during antibacterial responses and during T-cell differentiation so are associated with immunological function (Filen & Lahesmaa, 2010; Nitta *et al.*, 2006). *GIMAP8* was one of the top gene that was down-regulated gene in toxin infused birds. In the present study *C. perfringens* challenge did not have a significant effect on expression of the *GIMAP* gene.

Major histocompatibility complex (MHC) is known to encode class I and class II molecules. MHC class II molecules are highly polymorphic, consisting of two structurally related trans-membrane glycoproteins, α and ß chains that play a central role in immune response by binding peptides for presentation as an antigen to T-lymphocytes of the immune system. *BL-A* is one of the MHC class II alpha chain paralogues and lacks polymorphism (Salomonsen *et al.*, 2003; Kaiser, 2010). There were no significant differences detected for expression of the *BL-A* gene in this study. *BL-A* has been found to be down-regulated in spleen lymphocytes of broilers experimentally infected with *Salmonella gallinarum* (Lim *et al.*, 2009).

Another gene *NBL1,* neuroblastoma, suppression of tumourigenicity 1: also known as DAN, DANDI. This, was selected as it was one of the top up-regulated genes in the gut loop model (Olakowski *et al.*, 2009). *NBL1* has been identified as a tumour supressing gene and also has roles in the inflammatory response. In the present study *C. perfringens* challenge did not have a significant effect on expression of the *NBL1*gene. In addition to these gene transcripts two more gene transcripts (IRAK-4 and VTN) were also analysed but unfortunately problems with these genes, failed to generate a qPCR standard curve so were excluded from the analysis.

The difference in results of the expression level of genes between the gut loop model and the present study could be due to several reasons (Athanasiadou et al., 2011). It may be due to the fact that toxin infused birds were investigated 1hr after toxin infusion, compared with the present experiment which investigated gene expression after 2 days *C. perfringens* challenge per se (so enough time is given to *C. perfringens* to grow and release its α-toxin into the gut). This time difference may account for the difference in gene expression patterns. Sumners *et al.* (2012) found higher expression of IL-13 gene after 2hrs of exposure to *C. perfringens* toxin but not after 4hrs. The expression pattern of one gene can be varied between different diseases (Hong *et al*., 2012). Different genes respond differentially to various infections: production of several pro-inflammatory cytokines (IL-1ß, TNF-α, IL-6) are increased following infection with *C. perfringens* (Hong *et al*., 2006a; Hong *et al*., 2006b; Park *et al*., 2008). However Hong *et al*. (2012) could not find any differences in the expression levels of IL-1ß and IL-6 between NE infected and control birds. Similarly Park *et al.* (2008) did not find any altered response of some pro-inflammatory cytokines genes (IL-6, IL-16) in birds experimentally infected with *C. perfringens*.

Moreover one cannot exclude the possibility of differential expression in genes response to crude toxin directly infused into the duodenum and *C. perfringens per se*. Challenging birds through in feed C. *perfringens* may not be able to produce sufficiently high amounts of α-toxin to produce a significant effect on gene expression, or vice versa, infusion studies may result in higher local toxin concentrations than occurring in (natural) infections. However the low numbers of birds exhibiting lesions in the present experiment is not enough to warrant ignoring the inability to find a difference between the different treatments groups.

The two different commercial lines did not show any differential gene expression pattern for the different genes analysed in the present experiment. Hong *et al.* (2012) analysed the expression levels of different avian ß-defensins in two lines of commercial broiler chickens: Ross and Cobb following *E. maxima* and *C. perfringens* co-infection and found differential gene expression patterns. However not all of the genes investigated were expressed differentially as out of 14 ß-defensins only 8 showed differential gene expression in the jejunum across two species of chickens. Sumners *et al.* (2012) documented that resistance to *C. perfringens* infection is one instance where genetically divergent species do not follow their established trend of pathogen

susceptibility. High antibody response chickens have also been observed to be more resistant to *C. perfringens* infection compared to those with a low antibody response. Highly up-regulated expression of various cytokines, IFN-γ, IL-8, IL-15 has been observed in chicken species with a high antibody response during exposure to *C. perfringens* α-toxin compared to a species with a low antibody response (Sumners *et al.*, 2012).

The comparatively low NE lesion score in the present study, together with insignificantly different counts of *C. perfringens* and α-toxin between the different treatment groups might have caused such subtle changes in mRNA transcripts of the genes analysed that detection of significant changes was not possible in qRT-PCR analysis. Si *et al.*, (2007) found higher numbers of differentially expressed genes following *C. perfringens* challenge on the day when NE lesions were most serious.

8.11 Gene expression: Conclusion

In conclusion, our results showed that challenging birds with higher in-feed doses of *C. perfringens* did not alter the expression level of the gene transcripts (*fas*, *BL-A*, *GIMAP8* and *NBL*) that were up and down regulated following infusion of crude toxin. Results of present study also indicate the difference of host response in term of gene expression, to toxin and *C. perfringens* per se, and/ or different stages in pathogenesis of *C. perfringens* infection. However, this preliminary study has paved the way for more extensive studies into changes in gene expression analysis, as in agreement with lack of gene expression variation, very few birds were observed to have sub-clinical NE. Further studies on gene expression in an experimental disease model will be necessary for better understanding of host pathogen interactions.

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9 GENERAL DISCUSSION

The objective of this section is to discuss the overall findings of the project rather than provide a comprehensive discussion of individual experiments' results.

9.1 Introduction

Although NE is not a new disease it has, until now, been controlled by in-feed microbials, and ionophore anti-coccidials (Collier *et al.*, 2003). Following an EU wide ban on in-feed growth promoters, it has re-emerged as a significant problem that ultimately results in reduced growth performance as well as increasing feed costs. Epidemiological data suggests that sub-clinical NE results in a 20% reduction in bird weight and a 10.9% increase in FCR compared to healthy birds – thus having the potential to cause a significant effect on both the growth and feed efficiency of broiler flocks. (Kaldhusdal *et al.*, 2001; Hofacre *et al.*, 2003; Skinner *et al.*, 2010; Skinner *et al.*, 2010; Timbermont *et al.*, 2009a). However, the nature of sub-clinical NE makes it difficult to determine accurately the total impact of fully developed NE on chicken production. The primary etiological agent of sub-clinical NE is a gram positive, anaerobic, spore forming bacterium, *Clostridium perfringens* (*C. perfringen*s), a commensal in the GIT of poultry (Ficken & Wages, 1997) that has been isolated from feed, litter, dust, and faeces (Wages & Opengart, 2003).

The economic impact of sub-clinical NE has not been formally investigated although it is becoming progressively more apparent (Skinner *et al.*, 2010). The sub-clinical form of the disease causes damage to intestinal mucosa leading to decreased digestion and absorption, reduced weight gain and increased feed conversion ratio (Kaldhusdal *et al.*, 2001). Although NE currently is a sporadic disease in developing countries it is still causing large scale outbreaks in chicken production units, so poultry farms cannot afford to ignore the economic losses caused by this disease. The exact conditions that precipitate the out breaks of NE under field conditions are still ambiguous, despite identification of numerous factors that promote the development of sub-clinical NE. These predisposing factors are mainly dietary in nature together with a degree of co infection with *Eimeria*. Despite the present understanding of the disease's progression and identification of *C. perfringens* as the disease's primary etiological agent, it is still unclear as to the predisposing factor(s) that lead to the overgrowth of *C. perfringens* in the GIT and the subsequent induction of NE. It has proved difficult to reproduce sub-clinical NE under experimental conditions. There are numerous

predisposing factors, but they are ill defined with contradictory results from experiments (Kaldhusdal *et al.*, 1999). In order to control strategies and explore new methods for controlling sub-clinical NE it is essential to develop a reproducible experimental model for induction of sub-clinical NE that allows testing of a variety of factors, such as feed additives, vaccines and a range of new approaches, in order to achieve more effective control of this billion-dollar-costly disease.

The focus of this thesis was:

1. To develop a working infection model that enables experimental induction of sub-clinical NE in broiler chickens with special reference to prioritize nutrition related risk factors that purportedly predispose birds to sub-clinical NE.

2. To test breed sensitivity to disease induction, two chicken breeds, Ross and Hubbard, with varying degrees of susceptibility to the induced infection.

3. To identify the novel biochemical markers for sub-clinical NE.

The major focus of the work described in this thesis is to gain a better understanding of the involvement and relevance of various anecdotal and potential risk factors for NE in isolation and/or in combination in order to develop a model of sub-clinical NE, that is able to contribute to advancing new preventative strategies enabling control of this billion dollar disease in a post antibiotic era.

9.2 Responses of broiler chickens to various predisposing factors used in various experiments of this PhD project

9.2.1 Feed withdrawal /Fasting

The first study, detailed in Chapter 3, was done to determine whether feed withdrawal assists the induction of sub-clinical NE in broiler chickens inoculated with *C. perfringens*. To our knowledge, this experiment is the first to have assessed the effect, in isolation, of feed withdrawal on sub-clinical NE in young broiler chickens. No specific lesions with low digesta counts of *C. perfringens* demonstrated that feed

withdrawal at the durations used, did not predispose the birds to NE. It can therefore be concluded that feed withdrawal alone does not predispose the birds to sub-clinical NE. This lack of expected results suggests that, if fasting is indeed predisposing to sub-clinical NE, there must be other critical variables involved.

Many earlier experiments applied feed withdrawal together with very high levels of *C. perfringens*, yet failed to induce NE (Jia *et al.*, 2009; Pedersen *et al.*, 2003). Digestive enzymes such as Trypsin, recognised as an effective in-activator of the α-toxin of *C. perfringens*, increase in quantity during short hours of starvation (Baba *et al.*, 1992). Although mucin quantity was not measured in this study it is possible that paracox-5®, used in the present experiment, was also unable to produce sufficient mucin to encourage proliferation of *C. perfringens*.

9.2.2 Vegetable protein source

A wide number of scientific publications have identified, dietary ingredients, such as wheat, barley and fishmeal, which affect the proliferation of *C. perfringens*, so also affect the incidence of NE (Kaldhusdal & Hofshagen, 1992; Kaldhusdal & Skjerve, 1996; Drew *et al.*, 2004). However, the experimental induction of sub-clinical NE is hampered due to poor definition of the relationship between these factors.

The second study, detailed in chapter-4, was done to determine the effect, on the incidence of NE, of three alternative sources of vegetable protein, (SBM, PPC and CM), in nutritionally complete diets with similar crude protein content (212 g/kg). It has long been suggested that a relationship exists between the type of litter and the incidence of NE under field conditions since *C. perfringens* has been isolated from poultry litter (Nairn & Bamford, 1967). An important component in the development of NE is the recycling of *C. perfringens* through both litter ingestion between birds and transmission to subsequent flocks placed on old litter (Nairn & Bamford, 1967; Craven *et al.*, 2001a; Craven *et al.*, 2001b; Alexander *et al.*, 1968; Craven *et al.*, 2001b). Therefore this experiment challenged the birds by exposing them to reused litter material providing an innate infection, without the need to dose them with actively pathogenic *C. perfringens* to reproduce the disease. Reused litter material was obtained from a commercial farm in order to simulate the natural conditions for the development of sub-clinical NE.

9.2.3 Trypsin inhibitor

Trypsin inhibitor is a recent addition to the list of predisposing factors for NE (Palliyeguru *et al.*, 2010). It was therefore decided to include a fourth treatment, in a wheat-soya diet with added synthetic TI (6µg/ml) to study its impact on the onset of sub-clinical NE. To our knowledge this experiment is, again, the first to assess the effect of synthetic TI on the induction of sub-clinical NE in poultry. However, *post hoc* dietary analysis in this study showed that all four diets (SBM, SBM+TI, PPC and CM) had almost the same levels of TI activity (0.8mg/g of feed) so no comparison of dietary treatments can be made solely on the basis of the TIA content of the diet. This could indicate the degradation of synthetic TI after mixing with the experimental diet. It might also be due to too small a concentration of the synthetic TI or sufficiently significant differences between natural and synthetic TI that may have required different analytical techniques.

Sub-clinical NE was not induced by the use of SBM, PPC or CM. However, a CM diet did predispose the birds to coccidiosis to such an extent that it was necessary to cull them all on day 21, although the other dietary treatment groups were continued to the planned completion of the experiment at day 30. It is postulated that this increased susceptibility to coccidiosis could have been the result of damage to the intestinal mucosal layer by anti nutrients present in CM such as phytate. Although not shown in this study, coccidia are recognised as a co-factor in NE so it is reasonable to assume that, under commercial conditions, CM would also predispose birds to sub-clinical NE.

9.2.4 Fish meal

The third study, detailed in Chapter 5, was done to determine the effect of fishmeal addition on *C. perfringens* proliferation on *in vitro* digested grower diets. There is a close relationship between fish meal and the incidence of NE in poultry as dietary levels of animal protein like fish meal are known to contribute to the growth of *C. perfringens* (Truscott & Alsheikhly, 1977; McDevitt *et al.*, 2006b). This is reflected in the number of experimental models relying on fish meal addition to diets (Pedersen *et al.*, 2008; Timbermont *et al.*, 2010; Gholamiandehkordi *et al.*, 2007). However, it is worth noting that not all experimental models using fish meal were able to reproduce NE under controlled environmental conditions (Pedersen *et al.*, 2003). The overall

findings of the present study support the view that high levels of dietary fish meal may assist survival of *C. perfringens*, suggesting that the role of fish meal cannot be excluded as a predisposing factor for (subclinical) NE. Therefore it was decided to use fish meal (30% added on top of a grower diet) in all subsequent experiments attempting to induce sub-clinical NE.

9.2.5 Effect of combination of predisposing factors on experimental induction of sub-clinical NE

The fifth experiment, detailed in Chapter 7, was done to induce sub-clinical NE in broiler chickens by combining all the predisposing factors previously considered individually (i.e. diet, coccidial vaccination, IBD vaccine and added fishmeal) following challenge through oral gavage with high numbers of *C. perfringens* (10^8 cfu/ml). This experiment successfully produced the disease with a statistically significant percentage (40.6%) of birds developing NE lesions, but with no mortality. Ante-mortem examination of the birds had shown no clear symptoms. Post-mortem examination revealed gross lesions consisting of necrotic areas, surrounded by hyperaemic zones. Some cases also showed the intestinal mucosa covered by a yellow to brown, diphtheric pseudo-membrane. The overall performance of birds in the challenged treatment group was consistently poor on all days confirming the economic importance of the sub-clinical form of the disease.

The results of this experiment provides a suitable model for induction of sub-clinical NE under controlled environmental conditions: multiple *C. perfringens* inoculations through gavage; together with a combination of different predisposing factors such as in the presence of high doses of live coccidial vaccine, feed withdrawal, and higher levels of fish meal (30%) in the grower diet with CM.

It was possible to produce the disease by an appropriate combination of different predisposing factors. Various factors such as environment, diet and co infection with other pathogens can provide endogenous strains of *C. perfringens* with optimal conditions for proliferation of *C. perfringens* and subsequent production of α-toxin. Minor intestinal damage and sufficient numbers of *C. perfringens* in the intestine are pre-requisites to producing the disease as this research failed to induce NE lesions even after infusion of large number of *C. perfringens* alone (Alsheikhly & Truscott, 1977a).

Therefore it may be reasonable to assume that the presence of numerous predisposing factors applied in the experiment helped to create optimum conditions for multiplication of *C. perfringens* leading to induction of sub-clinical NE, confirming the findings that multiple oral doses of *C. perfringens* in combination with an high dose of live coccidial vaccine did give a suitable model for subclinical NE (Gholamiandehkordi *et al*., 2007). Much of the literature on induction of NE has shown that the most successful models, in addition to high and repeated doses of *C. perfringens*, also included a combination of different predisposing factors such as higher doses of coccidial vaccine, diets containing high levels of fish meal and IBD vaccination (Wu *et al.*, 2010).

Under controlled environmental conditions, earlier experiments appeared to show that it was easy to successfully produce NE (Alsheikhly & Truscott, 1977b; Cowen *et al.*, 1987; Alsheikhly & Truscott, 1977a; Hamdy *et al.*, 1983b; Prescott *et al.*, 1978a). However more recent attempts have failed to replicate their results with many researchers unable to induce the disease even in the presence of recognised predisposing factors (Pedersen *et al.*, 2003; Olkowski *et al.*, 2006b). Pedersen *et al*, (2003) failed to induce NE in broiler chickens in three of their experiments even after inoculation of very high numbers of *C. perfringens*. Challenge trials conducted by Olkowski *et al.* (2006a) also failed to produce clinical and/or pathological features characteristics of typical NE.

The results of experiments 4b and 5 show that the response of challenge may differ from experiment to experiment depending upon the route of challenge, challenge dose and the number of days of challenge. The response in experiment 4b was weaker than that in experiment 5, although there were a number of differences between the other experimental conditions. A combination of all these factors may responsible for the onset of sub-clinical NE in experiment 5 (Chapter 7).

The experiments detailed in this thesis measured the disease frequency per treatment on the basis of lesions of sub-clinical NE found in randomly euthanized birds, subsequently confirmed by histopathology. The use of lesion-like thin walled, friable intestines for diagnosis creates problems due to the difficulty of achieving objectivity. However, restriction of diagnostic criteria to just necrotic lesions can appear to reduce the incidence of the disease by not including the evidence of lesion-like thin walled, friable intestines.

9.2.6 Coccidiosis

The most frequently described risk factor for the onset of NE is concurrent intestinal disease, such as coccidial infection. The *Eimeria* species particularly that colonize the small intestine, such as *Eimeria maxima* and *Eimeria acervulina,* are known to predispose to NE (Alsheikhly & Alsaieg, 1980). Bradley and Radhkrishnan, (1972) observed that growth of *C. perfringens* in the caecum increased during infection with E. *tenella* this increased growth also resulted in reduction in the number of *Lactobacilli*. Whereas Baba *et al.* (1997) suggested that concurrent infection with E. *nacatrix* and *C. perfringens* has a synergistic effect and increases clostridial population in the intestine of the chickens.

Coccidiosis and NE are usually linked as they both have similar symptoms (Williams, 2005). Alsheikhly & Alsaieg, (1980) and Shane *et al.* (1985) suggested that supplementation with anti-coccidial preparations may reduce the incidence of NE. The precise mechanism of coccidial infection in the pathogenesis of NE is not clear but Alsheikhly & Truscott, (1977b) hypothesized that intestinal damage due to coccidiosis along with sufficient numbers of *C. perfringens* are prerequisites for NE to occur, although some of the experimental evidence lacks reliable results to support this hypothesis (Pedersen *et al.*, 2003). The present project has demonstrated (Chapters 3, 4, 6) that coccidial vaccination alone seemed insufficient to induce sub-clinical NE.

9.2.7 Dietary amino acids

Unfortunately there is scarce information in the literature on the affect of dietary amino acids on lesions of sub-clinical NE, although these have been recognized as a factor that affects the *C. perfringens* population in the gut so have been suggested as a risk factor for sub-clinical NE (Wilkie *et al.*, 2005; Dahiya *et al.*, 2007b; Dahiya *et al.*, 2007a; Drew *et al.*, 2004). Drew *et al.* (2004) found significant increases in the ileal and caecal population of *C. perfringens* in chickens fed a fish meal diet (400g/kg) compared to a soy protein concentrated diet. This increase was attributed to the higher glycine content of fish meal in the diet. In addition to glycine, the dietary methionine level was also reported to stimulate the growth of *C. perfringens* (Muhammed *et al.*, 1975; Drew *et al.*, 2004).

Analysis of different grower diets (SBM, PPC, and CM), used in experiment two (Chapter 4) showed no major differences between the amino acids levels. Glycine levels in the SBM (7.5%), PPC (8.2%) and CM (8.0%:Figure 9.1; Figure 9.2). Similarly there were no major differences in levels of methionine between CM (4.4%), SBM (4.7%) and PPC (3.8%) diets. Whereas *C. perfringens* counts in digesta of broilers fed SBM, PPC, and CM grower diets were 2.53 2.91 and 3.15 $\log_{10}$ cfu/g respectively. Moreover no lesions of sub-clinical NE were observed in any of the treatment groups.

Analysis of different grower diets, used in experiments 4a 4b and 5 (Chapters 6 and 7) showed no major differences between the amino acids levels. There were no major differences between glycine levels in the grower diets used in experiment 4 and 5 (15 vs. 14 % respectively Figure 9.3: Figure 9.4). Similarly no major differences were found in methionine levels in the grower diet fed to birds in experiments 4a and 4b (7 % vs 6 % respectively). Counts of *C. perfringens* ranged from 1.3 -3.35 $\log_{10}$ cfu/g of digesta in experiments 4a and 4b whereas in experiment 5 counts ranged between 3.67 – 8.27 $\log_{10}$ cfu/g of digesta over different dissection days. The total percentage of birds with lesions of sub-clinical NE in the Ross and Hubbard birds of experiments 4b (Chapter 7) was 13.3% and 6.66% respectively whereas in experiment 5 lesions of sub-clinical NE were seen in 31.2% and 35.9% birds on days 21 and 25 respectively.

Palliyeguru *et al.* (2010) found a higher incidence of sub-clinical NE in birds fed a potato protein diet in comparison to a soya diet although dietary analysis showed similar glycine levels in potato (9.4g/kg) and soya (9.0g/kg) diets. Dahiya *et al.* (2007b) found decrease in ileal and caecal populations of *C. perfringens* fed diets with high methionine concentrations. In agreement with studies (Palliyeguru *et al.*, 2010; Dahiya *et al.*, 2007b), the present Phd project provide further confirmation of probable involvement of other factors as the results clearly demonstrate that the changes in *C. perfringens* levels are not adequately explained by the level of dietary amino acids which also do not appear to significantly affect the incidence of sub-clinical NE.

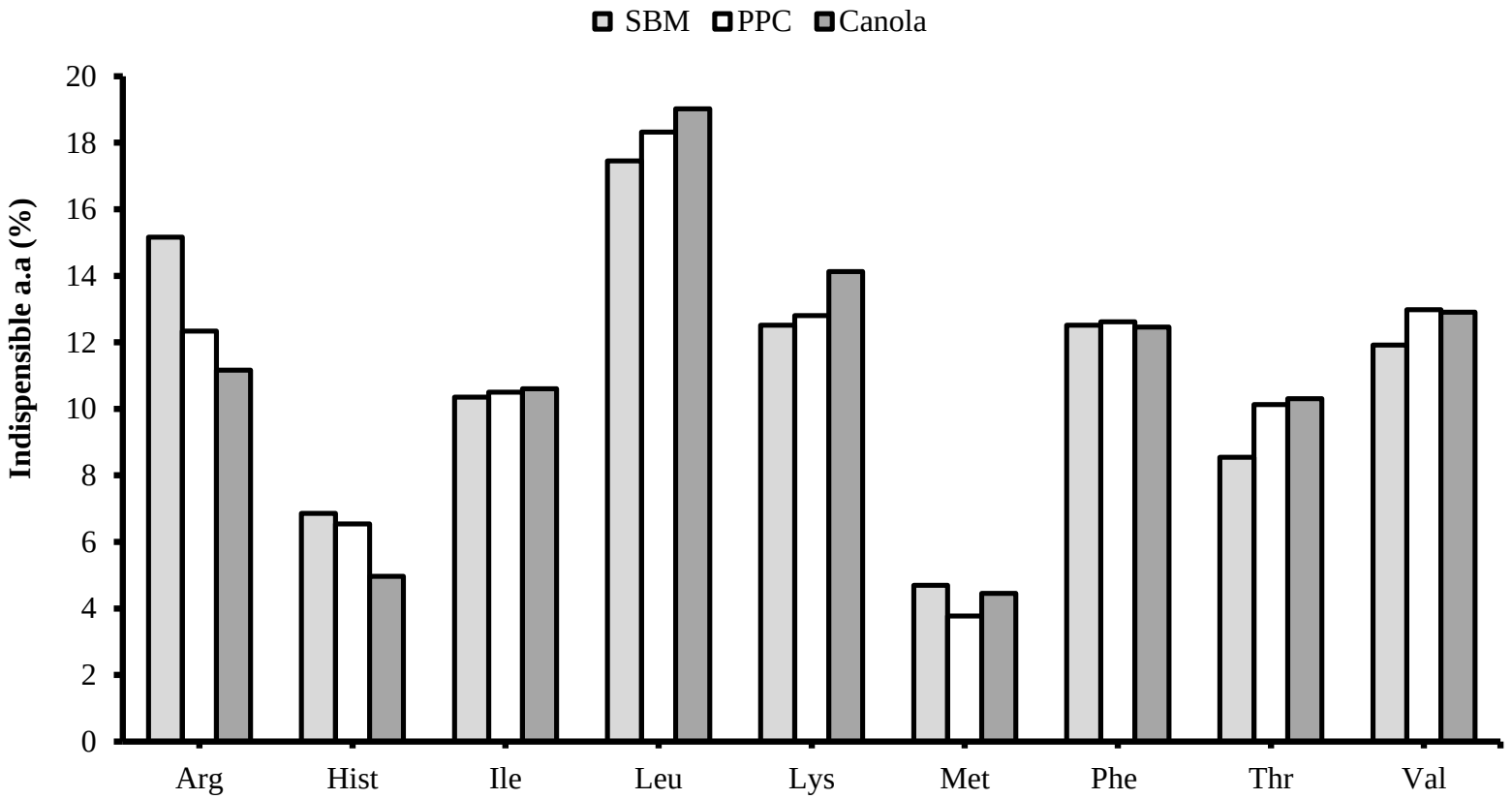

Figure 9.1: Indispensable (essential) amino acid composition of different grower diets used in experiment 2.

Arg. Arginine; His: Histidine: Ile: Iso-leucine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanin; Thr: Threonine; Val: Valine.

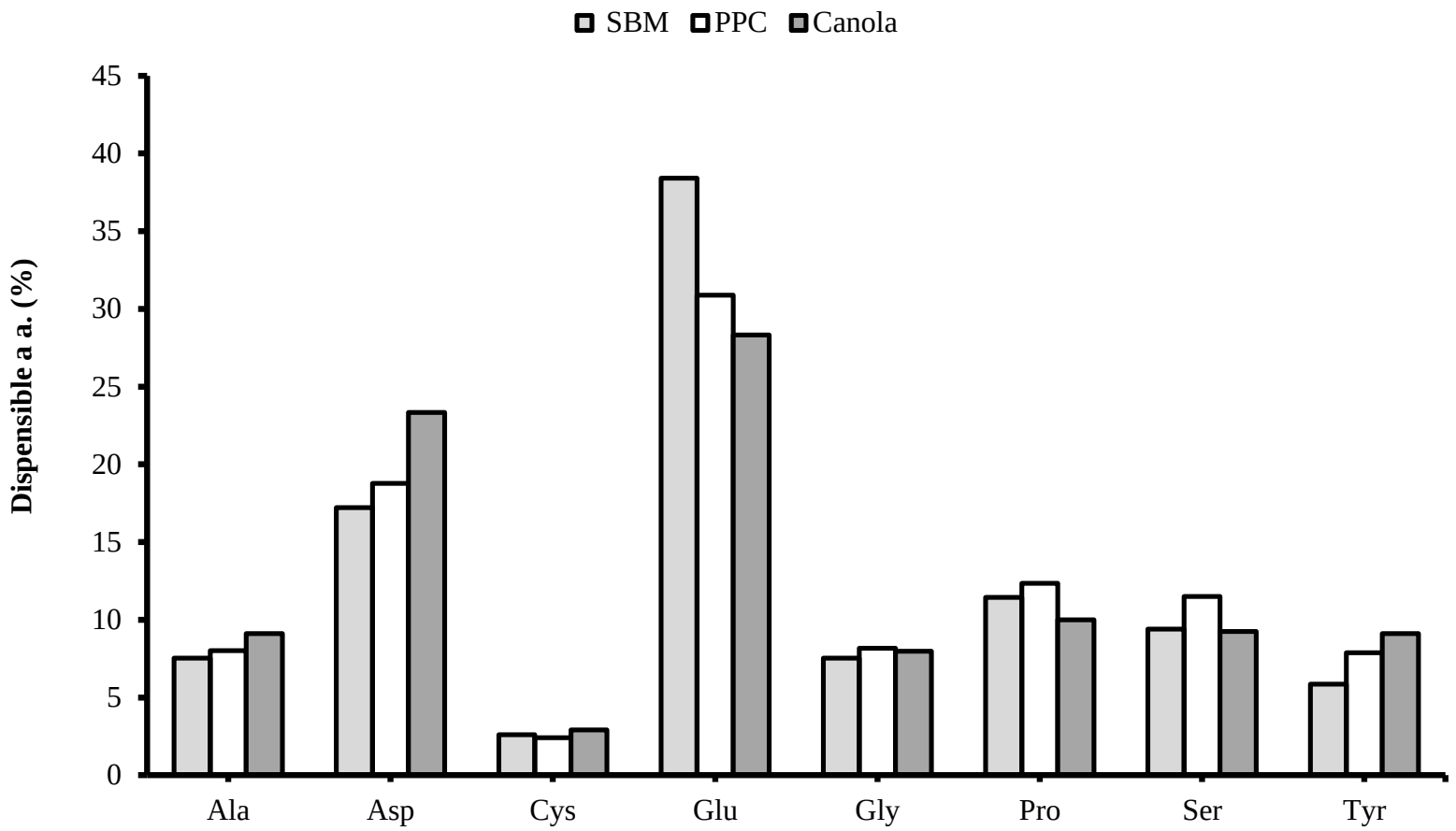

Figure 9.2 : Dispensable (non-essential) amino acid composition of different grower diets used in experiment 2.
Ala: Alanine; Asp: Aspartic acid; Cys: Cysteine; Glu: Glutamic acid; Gly: Glycine; Pro: Proline; Ser: Serine; Tyr: Tyrosine.

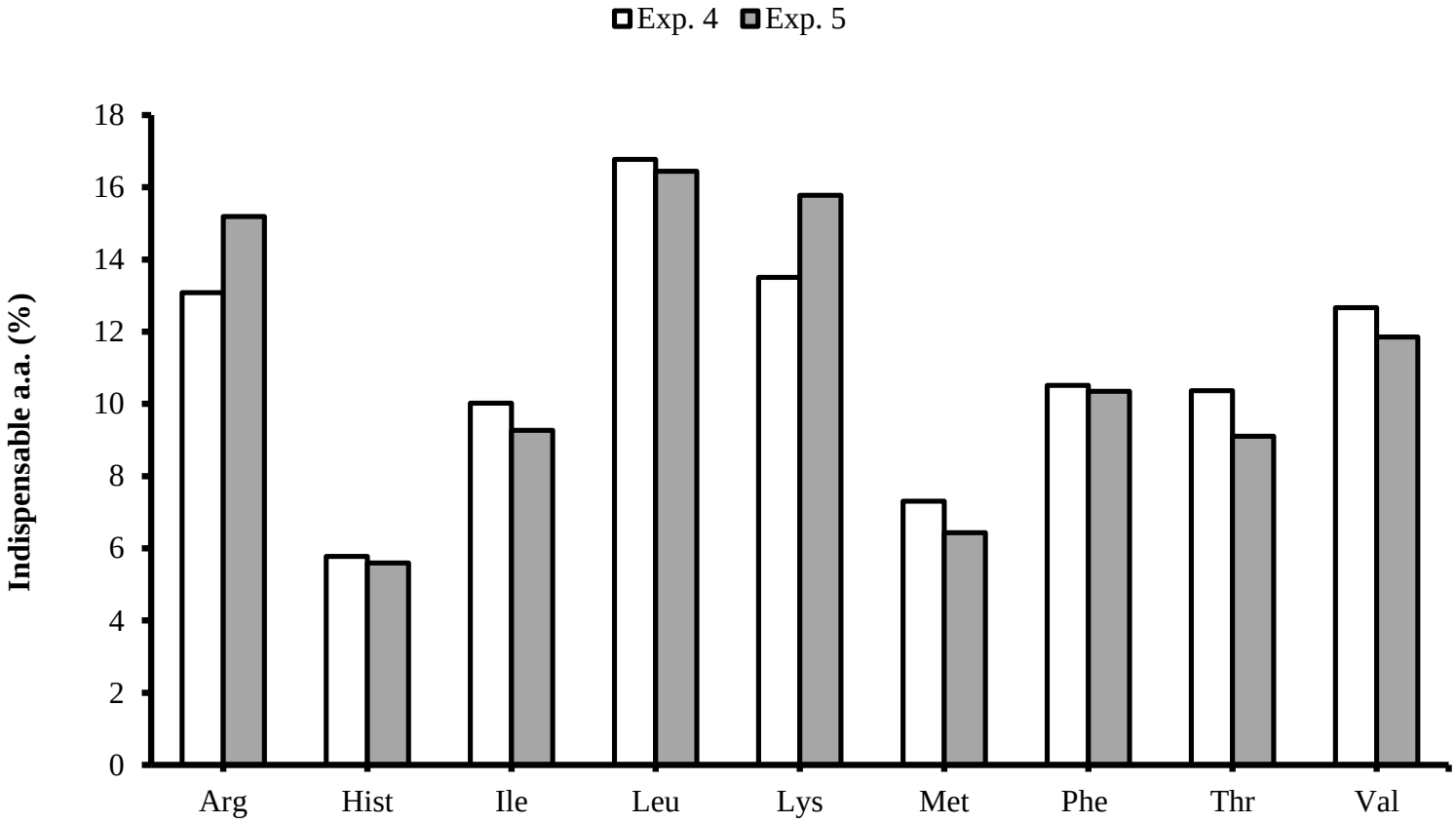

Figure 9.3: Indispensable (essential) amino acid composition of grower diets used in experiment 4 and experiment 5.
Arg. Arginine; His: Histidine: Ile: Iso-leucine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanine; Thr: Threonine; Val: Valine

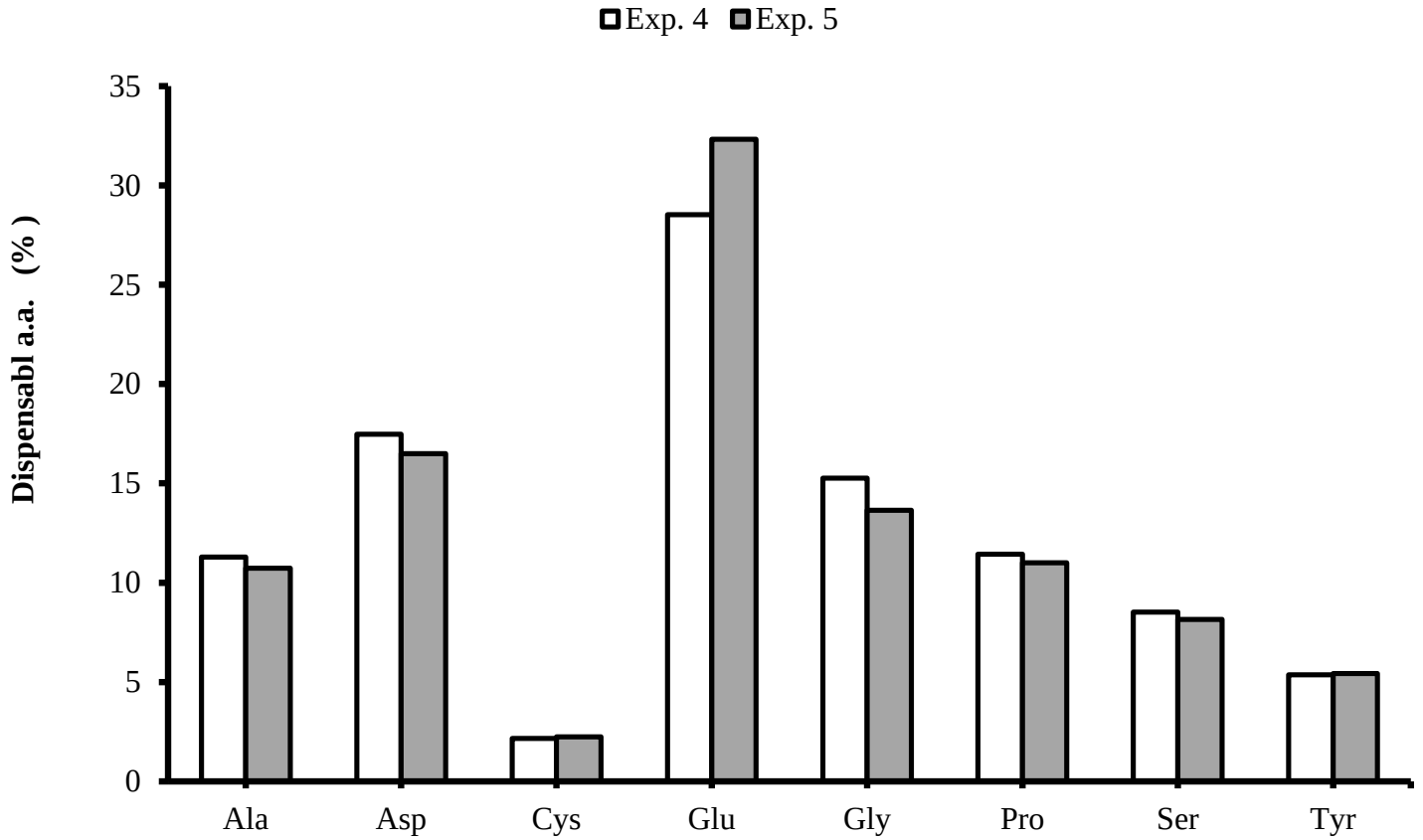

Figure 9.4: Dispensable (Non-essential) amino acid composition of grower diets used in experiment 4 and experiment 5. Ala: Alanine; Asp: Aspartic acid; Cys: Cysteine; Glu: Glutamic acid; Gly: Glycine; Pro: Proline; Ser: Serine; Tyr: Tyrosine.

9.3 Impact of low and high doses of *C. perfringens* through in feed challenge on experimental induction of sub-clinical NE

Experiments 4a and 4b, detailed in Chapter 5, drew on the information gained from the preceding experiments (Saleem *et al*., 2011a; Saleem *et al*., 2011b). Both experiments were designed to develop an experimental disease model for sub-clinical NE using wheat-based diets incorporating high amounts of fish meal. Birds were challenged;

- With low in-feed doses of *C. perfringens* (10^2 cfu/g), together with higher doses of coccidial vaccine (Experiment 4a)

- With higher and repeated in-feed doses of *C. perfringens* (10^9 cfu/g), together with higher doses of coccidial vaccine (Experiment 4b).

As different broiler strains have varying levels of susceptibility to infectious diseases, possibly due to genetic differences (Lamont, 1998; Zekarias *et al.*, 2002), experiment 4b compared two breeds (Ross 308 & Hubbard) in their resistance to induction of sub-clinical NE under the same experimental conditions.

Experiment 4a failed to induce the disease. In experiment 4b lesions were seen in only a few birds. Both experiments applied some of the predisposing factors, such as incorporation of more than 50% of wheat in the diet (Branton *et al.*, 1987; Riddell & Kong, 1992), and high levels of fish meal (30% on top of the basic diet) in the grower diet from days 7 to 16 (Prescott *et al.*, 1978a). Diet was offered in the form of mash rather than pellets as Engberg *et al*. (2002) found higher numbers of *C. perfringens* in the caecum of birds fed a mash compared to a pelleted diet. These experiments showed that lower concentrations of *C. perfringens* in gavage (10^2), predisposing factors like higher doses of coccidial vaccine and high levels of fish meal in the diet were unable to produce sub-clinical NE. The results also show that even excessively high challenges of *C. perfringens* with high levels of fishmeal diets and high doses of coccidial vaccination, may not result in consistent production of gross lesions of sub-clinical NE. Further study is required to determine whether the lower number of Hubbard birds with gross lesions (1 out of 15) compared to Ross birds (2 out of 15) indicates a higher level

of resistance to NE between these species. Although Prescott *et al. (*1978b) reported that high levels of fish meal in the diet may provoke NE, the failure to produce NE in these two experiments emphasizes that other factors than a fish meal addition to the diet are likely to be involved.

9.4 Experimental disease model for sub-clinical NE

NE is difficult to reproduce experimentally with *C. perfringens* alone although some authors (Collier *et al.*, 2008; Park *et al.*, 2008) reported lesions by challenge with *C. perfringens* alone. This research clearly shows that the presence of *C. perfringens* in the intestinal tract of broilers or inoculation of birds with relatively higher doses of *C. perfringens* does not always lead to the development of sub-clinical NE.

It can be concluded that sub-clinical NE can be successfully induced under experimental conditions (Experiment 5 Chapter 7) by feeding broiler diets containing higher levels of fishmeal, using higher doses of paracox vaccine and gavaging *C. perfringens* for four consecutive days. Therefore the challenge model can be recommended for study of sub-clinical NE under controlled environmental conditions. However feeding higher levels of dietary proteins, feed withdrawal, higher levels of dietary wheat - each of these factors alone did not result in successful induction of the disease. Olkowski *et al.* (2006a) observed similar findings, although feed withdrawal periods were not mentioned in their study. Olkowski *et al.* (2006b) examined the responses of broilers to oral administration of C. *perfringens* isolated from field cases of NE. None of their challenged trials produced overt clinical signs of the disease. High dietary protein content of animal origin (fish meal or meat meal), fasting, a high wheat diet or abrupt change of diet form did not increase the risk of NE. Many birds showed pronounced changes in their intestinal tissue. Although all these changes appeared to be subtle when compared to field cases of NE, yet they had a significant, negative effect on the digestive physiology of the intestinal mucosa.

In several of the previously described models of NE, birds were inoculated with a cocktail of various strains of *C. perfringens* (McReynolds *et al.*, 2004b; McReynolds *et al.*, 2008; Cooper *et al.*, 2009). However, the model described in the present study only used one strain of *C. perfringens*. Some models described in the literature are based on populations of *C. perfringens* in the ileum and caecum rather that the presence

of necrotic lesions in the intestine (Wilkie *et al.*, 2005). However, the model used in this thesis is based on lesions not mortality since lesions are a more sensitive indicator of disease.

9.5 Biochemical markers of sub-clinical NE

Under field conditions carcass rejection is the stage at which most cases of sub-clinical NE are identified (Kaldhusdal & Lovland, 2000), although the numbers involved are not necessarily reflected in the statistics of economic loss. At present the degree of host responses to NE can only be assessed by scoring gross pathological lesions within the intestine, although there is currently no validated lesion scoring system to characterize the disease condition in poultry. In addition, identification is made more complex by the use of different lesion scoring systems (Shane *et al.*, 1985; Prescott, 1979; Lovland *et al.*, 2004). It has been suggested that sub-clinical NE cannot be diagnosed solely on gross observations (Olkowski *et al.*, 2006a).

The need to develop reliable tests to quantify the problem is widely recognised particularly in the absence of AGPs. A more objective and quantifiable approach would be to determine the levels of various acute phase proteins (APP) and measure the level of expression of different genes, up or down, regulated in response to *C. perfringens* challenge in the intestinal tract of broiler chickens. This work is the first to provide evidence on the identification of novel bio-markers for sub-clinical NE in terms of acute phase protein levels in blood as well as the up/down expression of different genes regulated in response to *C. perfringens* challenge in broilers.

9.5.1 Acute phase proteins response to sub-clinical NE

A host's natural systemic reaction, to neutralize the effect of pathogen, trauma and immune disorder is alteration in the levels of APPs released as a result of an acute phase response (Murata *et al.*, 2004; Chamanza *et al.*, 1999). The response of three main APPs, Ceruloplasmin, PIT 54 and OTF in response to *C. perfringens* challenge was investigated to determine whether they can be used as diagnostic biochemical markers for sub-clinical NE in poultry (Chapter 8a). Greater insight into the pathogenesis of sub-clinical NE will also be provided by the host's response in terms of APPs. The results showed that there was a marked increase in serum ceruloplasmin

concentration in chickens challenged with *C. perfringens* compared to unchallenged birds, although no differences were seen in PIT 54 and OVT.

An elevation of ceruloplasmin concentration evidenced an APP response that was induced in broiler chickens experimentally infected with sub-clinical NE. Haptoglobin like protein; PIT 54 in chicken is still not completely elucidated. Determination of plasma Ceruloplasmin concentrations can help in early diagnosis of sub-clinical infection, possibly even prior to the actual appearance of necrotic lesions on intestinal walls. Further studies are needed to confirm levels of these APPs in sub-clinical NE for use as a tool to monitor chicken health. A better understanding of the kinetics of APP in serum definitely improves knowledge of the kinetics of this disease.

Experimental models of sub-clinical NE are increasingly required for studies of disease progress and the effect of novel interventions products. However reliable, easily quantifiable indicators of sub-clinical NE are not readily available. There is great variety in the scoring system(s) for disease characterization (Prescott, 1979; George *et al.*, 1982; Lovland *et al.*, 2004). Acute phase proteins, as an indicator of the host's response to early inflammation can represent a potentially useful marker of bacterial infection (Chamanza *et al.*, 1999). Economically important diseases, especially sub-clinical forms which are difficult to detect by clinical signs or even by post-mortem examination, may be diagnosed by levels of APPs, since an acute phase response in chickens is linked to growth depression and decreased production (Klasing & Korver, 1997). The effect of sub-clinical NE on the concentration of various APPs has not been assessed in broilers. The present model analyses the effect of experimental challenge on three important APPs and their relation to attempted induction of sub-clinical NE.

The disease was successfully produced in the present study. The appearance of typical lesions of sub-clinical NE following *C. perfringens* challenge confirmed the presence of infection in the gut. The initial phase of an inflammatory process induced by infection, involves the release of acute phase proteins (Gruys *et al.*, 1994). The present study indicates that serum levels of the positive acute phase protein ceruloplasmin increased in challenged birds compared to unchallenged controls probably due to gut lesions caused by bacterium. These findings are in agreement with previous studies which reported significant elevations of ceruloplasmin concentrations (Georgieva, 2010). Mazur-Gonkowska *et al.* (2004) found significant increases in

concentrations of ceruloplasmin in turkeys infected with *E. coli*, with levels remaining elevated until day 10 post infection, indicating that ceruloplasmin is an important APP in poultry response to bacterial infection.

The purpose of experiments described in Chapter 8 was to identify potential biochemical markers in terms of APP and gene expression so that these can give an indication of the severity of sub-clinical NE in the absence of grossly visible gut lesions. The expression of these APPs has yet to be fully determined through further investigation in experimental models. It is noteworthy that a proportion of birds in the control, unchallenged groups developed lesions of sub-clinical NE. This could explain the difference in the levels of PIT 54 and OTF compared to ceruloplasmin (O'Reilly, Personnel communication). However this is the first study to measure/analyse the response of APPs in broiler chickens challenged with *C. perfringens.* In conclusion, our results show that ceruloplasmin may be considered as moderate APP in sub-clinical NE in chickens, as it increased around 3 fold, suggesting that monitoring serum ceruloplasmin concentration can indicate the presence of *C. perfringens* infection in poultry. However further research confirming its role and concentrations in sub-clinical NE in order to establish a cut-off point to allow the optimal balance of clinical specificity and sensitivity is needed to provide a diagnostic and prognostic marker for flock health and welfare to ultimately help in better understanding of the pathophysiology of sub-clinical NE. Determination of a receiver operator curve (roc) with well defined populations of healthy and subclinically infected would be required to determine the best cut-off point for diagnostic use (Jesnsen and Kjelgaard-Hansen, 2006).

9.5.2 Expression pattern of host gene in response to C. perfringens challenge

Athanasiadou *et al.* (2011) have identified the pathways that regulate early host responses to *C. perfringens* toxin in the duodenum of broilers by using a genome-wide transcriptomic analysis and an *in situ* broiler gut loop model. In earlier work, qPCR confirmed a wide range of up to 11 genes associated with innate immune responses following genome-wide transcriptomic analysis of the duodenum of broilers infused with crude *C. perfringens* α-toxin, compared to controls (Athanasiadou *et al.*, 2011). Six of the eleven genes confirmed by qPCR, were chosen for analysis. With the objective of obtaining a better understanding of the immune competence of two genetically different

commercial broiler species (Ross and Hubbard) the expressions of 6 gene transcripts were analysed in broilers experimentally challenged with *C. perfringens* (1.54 x 10^9 cfu/g of feed).

Chapter 8 demonstrated there was no alteration in the expression level of different gene transcripts, *fas*, *BL-A, GIMAP8* and *NBL1,* following challenge with higher in-feed doses of *C. perfringens,* even though these genes had been up and down regulated after infusion of crude toxin, indicating the difference of host response in term of gene expression, to toxin and *C. perfringens* per se, and/ or different stages in pathogenesis of *C. perfringens* infection.

The present experiment showed no differential gene expression pattern for the genes analysed between the two different commercial species of broilers. Unfortunately very limited information is available on the effect of bird breed on susceptibility to NE (Hong *et al.*, 2012). In one UK epidemiological study one, un-named breed of chicken was more susceptible than the other employed (Hermans & Morgan, 2007). Though not experimentally verified, Olkowski *et al.* (2006a) speculated that currently broiler breeds may be more resistant to NE than those used 30 or more years ago. That could provide a reason for the difficulties experienced in developing a model of NE for disease induction.

Hong *et al. (*2012) found differential gene expression patterns for avian ß-defensins when analysing Ross and Cobb broilers response following *E. maxima* and *C. perfringens* co-infection. However only 8 out of 14 ß-defensins of the genes investigated were expressed differentially in the jejunum of two broiler species. Sumners *et al.* (2012) documented that the established trend of pathogen susceptibility is not always followed by genetically divergent species in their resistance to *C. perfringens* infection. Broilers with a low antibody response have been observed to have a lower resistance to *C. perfringens* infection compared to broilers with a high antibody response. The low antibody response species were observed to have a lower up-regulated expression of various cytokines, IFN-γ, IL-8, IL-15 during exposure to *C. perfringens* α-toxin (Sumners *et al.*, 2012).

However in the experiment (Chapter 6b) only a small number of birds (3/30) developed lesions. So it would be useful for further gene expression studies to be done

where higher numbers of birds develop gut lesions, in order for a better understanding of the gene responses, whether they change or remain the same. Changes in host gene expression response can reflect cellular and molecular biological processes during host response to *C. perfringens* infection in broilers. More extensive gene expression analysis is necessary to further build on the work of this preliminary study so that an experimental disease model can be refined to provide a better understanding of host pathogen interactions.

10 CONCLUSION

The present thesis confirms that NE develops when *C. perfringens* establishes and multiplies in the intestinal tract of chickens in the presence of multiple predisposing factors such as: feed withdrawal, IBD vaccination, coccidiosis and high levels of dietary fish meal. Sub-clinical NE is a complex multi-factorial disease so successful reproduction requires careful incorporation of all these factors that, this researcher's experiments suggest, act synergistically in inducing grossly visible gut damage.

The ideal model to study the pathogenesis of sub-clinical NE is one in which a statistically significant proportion of challenged birds develop grossly visible lesions, but without clinical signs or associated mortality. The disease model used in the present study incorporates the different predisposing factors that commercial poultry operations are likely to face on a day to day basis. Most of the challenged models used a mixture of *C. perfringens* strains to induce NE (McReynolds et al., 2005; McReynolds et al., 2008), although only one strain was used in the present study. Ficken & Wages, (1997) suggested that factors such as coccidiosis, IBD and dietary stress along with high doses of *C. perfringens* would favour experimental induction of NE in chickens. The present study confirms this finding. More importantly this model will be more acceptable to animal welfare activists since there is no mortality associated with this *C. perfringens* challenge so there would be minimal ethical issues to consider. It is, however, recommended to repeat this experiment several times to establish the repeatability of the model. Of the predisposing factors tested by the present project, the role of coccidiosis is clearly crucial in the establishemnt of NE. Control of coccidia through anticoccidials or by vaccination may become more critical under commercial conditions particulary in those parts of the world that no longer use antimicrobial growth promoters. This research has indicated that control of coccidiosis is likely to be the most effective way of preventing NE.

The overall findings of this thesis showed that, of the factors tested, feed withdrawal, co-infection with coccidiosis, stress and dietary ingredients (fish meal and CM) are the most important in the development of sub-clinical NE. Understanding of the disease would benefit from a challenge model that will enable further, more effective investigation to combat it.

11 FUTURE WORK /RECOMENDATIONS

This thesis reports work, as often occurs during scientific research, that generates more questions than it answers. This creates scope for several avenues of further study.

- Future work is required to confirm the reproducibility of the disease model (Experiment 5, Chapter 7).

- Additional research is needed to determine the extent and involvement of the other contributory, predisposing factors. Such research could start from the final experiment (Chapter 7), successively removing individual predisposing factors in order to isolate the role of the crucial predisposing factor(s).

- Continue to explore the differences between two genotypes in their differing susceptibility to NE.

- Further study needs to be undertaken, keeping all variables constant, to determine whether feed or gavage is the most appropriate for an NE model.

- Levels of APPs as a potential tool for early diagnosis of sub-clinical NE needs further study. Pathophysiology of APP in serum of chickens challenged with *C. perfringens* is necessary to characterize various molecular and biochemical events that lead to pathogenesis of this complex disease.

- Future work on confirmation of these novel markers and then use of these markers to identify and test diet related strategies to control NE.

- Once confirmed its reproducibility, the present model can be used for testing various potential strategies to combat NE include Probiotics or Direct fed microbials (Fuller, 1989; Fukata et al., 1991; Hofacre et al., 1998a), Prebiotics (Gibson & Roberfroid 1995; Tako et al., 2008), Feed

enzymes (Ferket 1993; Choct et. al., 1999; Dahiya et al., 2006), Organic acid (Adam 2004; Koscher et al., 2004; Kmet' et al., 1993), Herbs (Dahiya et al., 2006), spices and essential oils (Wilkinson et al., 2003; Mitsch et al., 2004a; Timbermont et al., 2010; Abildgaard et al., 2010), Hen egg antibodies (Wilkie et al., 2006), Vaccination (Kulkarni et al., 2007), and bacteriophages therapy (Miller et al., 2010). However, in this researcher's opinion, the first and most important potential alternative that needs to be considered is directly fed microbials (probiotics). There are several such products on the global market due to growing interest in the potential of novel probiotics to not only promote gut health but also prevent the rise of NE. However before they can be reliably regarded as a valuable solution to maintain healthy gut flora there is a crucial need for them to be tested for their impact on proliferation of *C. perfringens*. Combined with good management practices and careful incorporation of feed ingredients, the application of antibiotic alternatives have the potential to be effective in achieving some control of NE. This could optimise production without the costly impact inevitable if NE is not controlled.

Using modern biotechnology tools, such as host gene analysis and APP, superior models for disease reproduction can be developed enabling resolution of the aetiology of this multifactorial disease. Ultimately this will lead to novel approaches to disease control, a gateway to gut health for all chickens within the industry. This thesis has confirmed the complexity of this disease. Although it is understandable that there is presently no clear single answer for the aetiology of NE, this thesis has clarified the importance of recognising the need for a holistic approach that looks at the whole range of predisposing factors involved in the pathology of the disease.

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13 APPENDICES:

13.1 Appendix A

Haematoxylin and Eosin staining technique for histopathological sections

Slides were placed in a slide holder for the purpose of staining. The stain was applied according to the following timetable

Pure alcohol	2 minutes
Industrial spirit (IMS)	1minute
Gills Haemotoxylin	5minute
Wash in water	1 minute
Scotts tap water substitute (STWS)	10-20seconds
Wash in water	10-15 seconds
Eosin	10-15 seconds
Wash in water	10 seconds
Pure alcohol	1 minute
Pure alcohol	1 minute
Organic solvent	30 seconds

The purpose of these steps was to remove any wax from the sections and rehydrate them to allow them to take up the stain.

13.2 Appendix B

Gram staining technique for *C. perfringens* identification

Gram staining technique for bacterial identification

A gram stain for morphology was carried out

Place the slide with bacterial smear made from TSC colony on a staining rack

Stain the slide with crystal violet for 1 minute

Pour off the stain

Flood the slide with Gram's iodine for 1 minute

Pour off the iodine

Decolorize by washing the slide briefly with acetone (2-3 seconds)

Wash slide with water to remove the acetone

Flood slide with counter stain

Wash the slide with water

Blot excess water.

After drying, slides were examined under oil immersion and the structure and appearance of cells noted. Gram positive cultures will appear purple black in colour while Gram negative cultures appear pink.

13.3 Appendix C

Growth strain 56 of *C. perfringens* on different media over different time

The experiment described in this appendix was conducted to find out when the growth of *C. perfringens* is at its peak as well as determining which medium best supports the growth of *C. perfringens* strain 56 and the associated α-toxin to ensure the best medium is used for growing *C. perfringens* to be used for the challenge studies. We studied two commonly used media for growing up *C. perfringens*, i.e. Brain heart infusion broth (BHI) and thioglycollate broth (TG), and bacterial counts were determined at 0, 2, 4, 8, 10, 12, and 24 hrs.

Frozen beads of *C. perfringens* strain 56, isolated from the intestine of a broiler chicken with severe necrotic gut lesions were streaked onto the Tryptose sulphite cyclerserine (TSC) agar plate. These plates were invertedly incubated in an anaerobic jar with CO_2 generating gas packs (AnaeroGen™, AN0025A, Oxoid Ltd, UK). The jar was placed in an incubator at 37°C for 24hrs before inoculation into sterile respective tubes in brain heart infusion (BHI) broth (CM 1032, Oxoid, Ltd, UK). After overnight incubation, 3 colonies (Figure 13.1) were taken from TSC agar plates and mixed with further (BHI) broth (Oxoid Ltd, UK). Up to 3-4 colonies from overnight cultures of TSC plates (Figure 13.1) were taken and inoculated into bijou bottles with BHI broth. Brain heart infusion broth was incubated at 37ºC, shaking at 60rmp anaerobically overnight (18-24hrs).

Thirty two sterile tubes containing 100ml of respective media (BHI and TG) were used in an *in vitro* experiment. Each tube was inoculated with 0.2ml of BHI broth containing an actively growing culture of *C. perfringens*. At 0, 2, 4, 6, 8, 12 and 24 hour intervals two tubes were removed. After mixing, 1ml of media was taken and mixed with 9ml of maximum recovery diluent. For enumeration of *C. perfringens*, dilutions were plated on TSC agar plates, incubating the plates at 37°C overnight, prior to counting the number of black presumptive *C. perfringens* colonies.

From the above *in vitro* experiment little difference was observed in *C. perfringens* counts on TSC agar plates suggesting there was also little difference the

between the growth of *C. perfringens* on BHI or TG media over the time periods. It was also observed that the *C. perfringens* count was at its peak between 8 -12 hrs. As the TG media is known to quickly deteriorate under aerobic conditions, it was decided to use an 8-12 hrs BHI culture of *C. perfringens* for challenging birds in subsequent experiments.

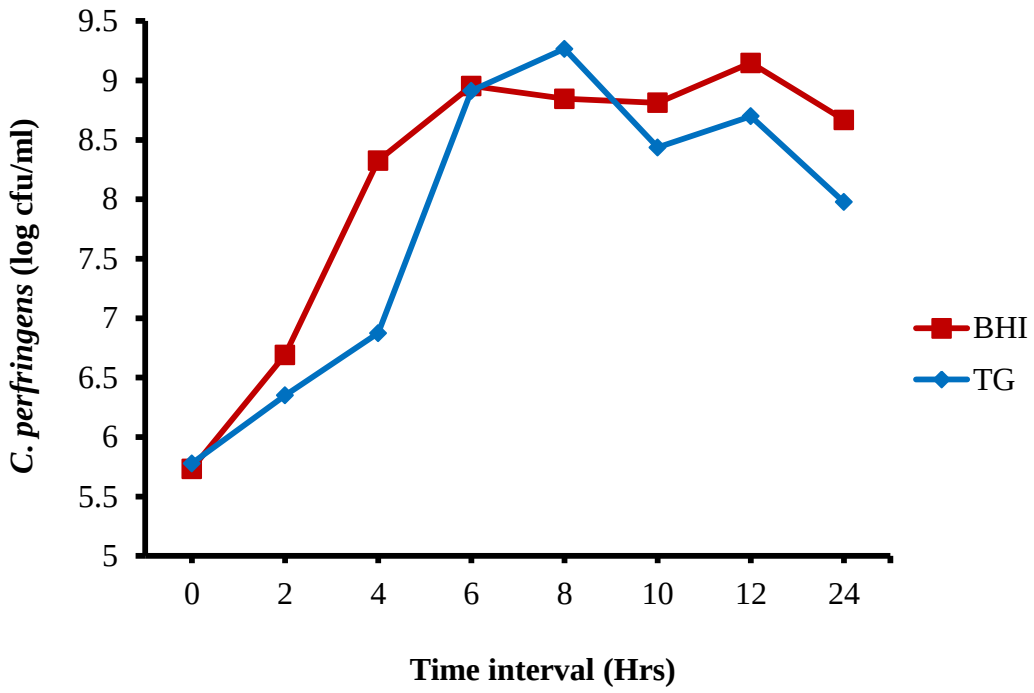

Figure 13.1: Growth of *C. perfringens* in different media. BHI: Brain heart infusion broth; TG, thioglycolate at different intervals of time.

13.4 Appendix D

13.4.1 RLT BUFFER

Add 10µl of β-merceptoethanol (β-ME) in 1ml of buffer RLT

13.4.2 70% ETHANOL

Add 70ml of ethanol in graduated flask and make up to 100 ml by adding distilled water.