Nutritional Manipulation to Minimise the Impact of Necrotic Enteritis in Broiler Chickens

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Publications

Journal papers

Keerqin, C., S.-B. Wu, B. Svihus, R. Swick, N. Morgan, and M. Choct. An early feeding regime and a high-density amino acid diet on growth performance of broilers under subclinical necrotic enteritis challenge. *Animal Nutrition* 2017; (http://dx.doi.org/10.1016/j.aninu.2017.01.002).

Keerqin, C., N. Morgan, S.-B. Wu, B. Svihus, and M. Choct. Reintroduction of microflora from necrotic enteritis resistant chickens reduces mortality and improves the performance of necrotic enteritis challenged broilers. *Journal of Applied Poultry Research*, submitted January 2017.

Keerqin, C., N. Morgan, S.-B. Wu. R. Swick, and M. Choct. Dietary inclusion of arabinoxylo-oligosaccharides in response to broilers challenged with subclinical necrotic enteritis. *Journal of British Poultry Science*, in Press since November 2016.

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Conference presentations

Keerqin, C., S.-B. Wu, B. Svihus, R. Swick, and M. Choct. Dietary supplementation of arabinoxylo-oligosaccharides improves growth performance of necrotic enteritis-challenged broilers. *Proceedings of the 28th Australian Poultry Science Symposium* 2017, 4.

Keerqin, C., S.-B. Wu, B. Svihus, and M. Choct. Effects of crude ileal and caecal flora mix on necrotic enteritis. *Proceedings of the 27th Australian Poultry Science Symposium* 2016, 64.

Keerqin, C., S.-B. Wu, and M. Choct. Early feeding and high amino acid level on performance of broilers under subclinical NE challenge. *Proceedings of the 26th Australian Poultry Science Symposium* 2015, 187.

Keerqin, C., S.-B. Wu, B. Svihus, and M. Choct. Crude microflora mix on the control of necrotic enteritis. *Proceeding of the 20th European Symposium on Poultry Nutrition* 2015, 246.

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Preface

This thesis has been written and edited in a journal article format. I have made every effort to minimise the repetition of materials between chapters. However, some overlap remains, particularly in the methodology sections. The word “broiler” has frequently been used since it is “meat chicken” in Australian term. NE stands for necrotic enteritis in poultry in most of the paragraphs.

Summary

Necrotic enteritis (NE) is an important poultry disease that is currently controlled by the use of in-feed antibiotics in many countries, including Australia. The general public demands clean and green poultry products that require the discontinuation of the use of antibiotics in feed. To achieve this goal, the broiler chicken industry must address the impact of NE without the reliance on in-feed antibiotics. The current thesis has examined a variety of nutritional strategies to minimise the impact of NE in broiler chickens using a subclinical NE model.

Chapter 1 presents the summary of literature related to NE with particular emphasis on the role of nutritional tools in alleviating losses associated with NE outbreaks. Chapter 2 examined the effect of early feeding a high amino acid density diet on performance of broilers under NE challenge. Birds fed the high amino acid diet had greater body weight by d 35 and heightened Lactobacillus content in the ileum at d 16 ($P < 0.05$). Birds that were fed the high amino acid (HAA) diet after a period of fasting post-hatch performed better regarding feed conversion ratio (FCR) performance under challenge. The findings from this study suggest there are beneficial effects of feeding high amino acid diets to birds in response to external stresses, such as post-hatch fasting and subclinical NE.

Chapter 3 investigated the effect of the reintroduction of crude ileal and caecal contents from previously NE-challenged chickens on performance, mortality and intestinal lesions of young broilers under NE challenge. Cloacal administration of both ileal and caecal crude flora inoculants significantly ($P < 0.05$) improved feed conversion efficiency and alleviated ($P = 0.049$) the severity of NE-associated lesions at d35. These preliminary findings suggest that the gut microbiome of birds plays a significant role in the susceptibility of broilers to NE.

Chapter 4 examined the prebiotic properties of arabinoxylo-oligosaccharides (AXOS) produced both in situ and in vitro for their activity against the onset of necrotic enteritis in broiler chickens. Birds fed the AXOS diet had numerically less severe gross lesions, improved feed conversion at d0-16 ($P=0.043$) and lower ileal viscosity ($P < 0.001$) at d16

compared to birds fed intact arabinoxylans. Caecal short-chain fatty acid (SCFA) concentration was higher in birds fed AXOS compared to other diets and was higher in the challenged birds compared to the unchallenged birds. The results suggest that AXOS appear to be efficacious prebiotics, as highlighted by improvements in feed conversion ratio (FCR) and increased SCFA.

Chapter 5 explored whether supplementing diets with xylanase, pectinase and protease would alleviate the effects of key predisposing factors of NE, xylans (from cereals), pectic polysaccharides (from vegetable protein sources) and undigestible proteins on bird performance, caecal volatile fatty acid concentration and ileal pH and prevalence of intestinal lesions, in NE challenged birds. The protease enabled higher weight gain compared to that fed xylanase at d0-35 ($P=0.040$) and d0-24 in the unchallenged birds ($P<0.001$). Feed intake was higher at d0-35 ($P=0.001$), and d0-24 ($P=0.011$) in birds fed protease, resulting in better feed conversion ($P=0.009$) in birds fed xylanase compared to that fed protease. Findings from this study suggest that enzyme application can be used as a tool to reduce the amount of undigested nutrients reaching the hindgut and hence lessen the growth of pathogenic bacteria.

Chapter 6 examined the effects of arabinoxylan (AX) and AXOS on net energy and nutrient utilisation in broilers. Ileal dry matter digestibility was higher in birds fed AXOS compared to that fed AX ($P=0.047$). Ileal digestible energy and total tract dry matter digestibility was higher in birds fed AXOS compared to that fed AX or AX+E ($P=0.004$ and $P=0.001$, respectively). Birds fed AXOS had higher ME intake ($P=0.049$) and nitrogen retention ($P=0.001$) and a strong trend of higher NE ($P=0.056$), NE intake ($P=0.057$) and retained energy ($P=0.054$) compared to that fed AX. Total ileal SCFA concentration, including lactic and formic acid, was higher in birds fed AXOS compared to that fed AX ($P=0.011$, $P=0.012$ and $P=0.023$, respectively). These findings indicate that AXOS generation in the gastrointestinal tract via the use of enzymes is not as efficient as feeding AXOS directly.

Declaration

I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree qualification.

I certify that any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

Chake Keerqin

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AA	Amino acid
ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
AEC	Animal Ethics Committee
ANOVA	Analysis of variance
AX	Aribinoxylan(s)
AXOS	Arabino-xylo-oligosaccharide(s)
AU	Australia
BW	Body weight
BWG	Body weight gain
CF	Crude fibre
CFU	Colony forming unit
CM	Canola meal
CP	Crude protein
Cp	*Clostridium perfringens*
CRC	Cooperative Research Centres
CSIRO	Commonwealth Scientific and Industrial Research Organisation
d	Day
D	Day
dM+C	Digestible methionine + cysteine
dArg	Digestible arginine
DDGS	Distillers dried grains with solubles
dEB	Dietary electrolyte balance
dLys	Digestible lysine
dThr	Digestible threonine
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agricultural Organization
FCR	Feed conversion ratio
FDA	Food and Drug Administration
FI	Feed intake
HAA	Higher amino acid
g	Gram
GC-MS	Gas chromatography-mass spectrometry
h	Hour, hours
kg	Kilogram, kilograms
m	Metre, metres
MBM	Meat and bone meal
min	Minute, minutes
mL	Millilitre, mililitres
netB	netB exotoxin
NSP	Non-starch polysaccharides
SBM	Soybean meal

SCFA	Short chain fatty acid
spp.	species
v/v	Volume divided by volume
VFA	Volatile fatty acids
w/w	Weigh divided by weight
XOS	Xylo-oligo-saccharides

CHAPTER 1

Introduction

1.1 Background

Availability of food products and nutrients for human consumption varies at regional and global levels (Yearbook, 2012). About 841 million people, mostly in developing countries, are still subject to chronic undernourishment from essential nutrients, especially due to lack of daily animal protein portions (Smith, 1998). It is believed that the emerging new middle class in developing countries will demand more quality animal produce (Kharas, 2010). Therefore, in order to address the potential crisis in terms of global food security, modern agriculture has been progressing to increase capacity and to improve productivity. The poultry industry is able to deliver good quality meat and eggs in a reliable and economical manner. Global poultry meat production is well over 100 million tonnes per annum (Fao, 2012). As a reflection, world's chicken meat production has experienced an 18 % increase over the last ten years, and it will account for over 50 % of growth volume of the global meat production in the next decade (FAO, 2015). For instance, by 2020, chicken meat consumption in Australia will reach 50kg per person per year (Australian Chicken Meat Federation, 2017). Such steady increase of chicken meat production has been accompanied by incredible advances and science and technology. However, the food production, in particular, the intensive livestock industries like the poultry industry, faces significant challenges, such as declining feed resources due to climate change, the availability of a skilled workforce, and discerning consumers demanding safe and ethical production. The current thesis has examined one small area of these challenges – chicken meat production without the reliance on antibiotics. It is well understood that broiler production without the use of antibiotics is highly prone to enteric diseases, the most economically important one being necrotic enteritis. A series of experiments were conducted to examine nutritional strategies to minimise the impact of necrotic enteritis in broiler chickens.

1.2 Necrotic enteritis

The most common and financially devasting bacterial disease in modern broiler flocks is necrotic enteritis (NE). Necrotic enteritis is a multifactorial, bacterial borne, clostridia disease in poultry, first described by Parish (1961). The causative agent, *C. perfringens*, is a Gram-positive, spore-forming anaerobic, ubiquitous rod bacterium that can be isolated from different environments, and it is known to produce a wide range of exotoxins and invasive enzymes responsible for several gastrointestinal disorders (Helmboldt and Bryant, 1971; Keyburn et al., 2008; Timbermont, 2011). *C. perfringens* is classified by genotypes A-E according to its 17 identified toxic exoproteins (Hofshagen and Stenwig, 1992; Myers et al., 2006). It is responsible for a number of diseases in many animals, including humans, but NE in poultry is usually caused by *C. perfringens* type A and type C strains. In addition to the existing toxin genotypes, there are few newly discovered toxins such as Beta2, NetB and TpeL. Most of the reported virulent *C. perfringens* strains are exclusively encoded with putative poultry colonization locus VR-10A (Lepp et al., 2013). *C. perfringens* is usually found below 10^2 and 10^4 colony forming units (CFU) per gram of digesta in the small intestine of healthy chickens (Kaldhusdal et al., 1999). Due to the nature of commercial broiler production and to the endemic nature of this bacterium, it is difficult to keep newly hatched chicks away from *C. perfringens* population. Initial contamination with *C. perfringens* often occurs from the egg-shell in the hatchery into the newly hatched bird. In fact, detectable levels of *C. perfringens* have been found in the young flock as early as 48 hours after hatch (Fuller, 2001; Van Immerseel et al., 2004).

Necrotic enteritis usually occurs between two to six weeks of age in commercial chickens, and its impact on the flock varies by region and by season (McDevitt, 2006; Shojadoost et al., 2012). According to the magnitude of the symptoms, NE can be classified into two broad types: clinical and subclinical. In the clinical form of NE, flocks often experience sudden mortalities without detectable early clinical signs, and significant necrosis is usually found in the small intestine of infected birds (Van Immerseel et al., 2009). The subclinical form of NE, however, causes much fewer symptoms and the infected birds exhibit slightly reduced growth, possibly due to impaired absorption and nutrient

utilisation caused by the damaged intestinal mucosa. Commonly observed symptoms of the disease vary with age of birds (van der Sluis, 2000c) but include early signs such as wet litter, diarrhoea and a small increase in mortality of less than 1%, all of which are often overlooked. However, damage to the intestine and subsequent reduction in digestion and absorption can lead to reductions in final weights of market-age broilers by an excess of 200 g (van der Sluis, 2000b) and increases in feed conversion ratios at d 35 of up to 10 points (Kaldhusdal and Løvland, 2000b). Furthermore, increased condemnations at processing due to liver lesions associated with subclinical NE can occur (Kaldhusdal and Løvland, 2000b). Failure to identify symptoms and to detect the disease often hinders its efficient and timely treatment, thus promoting the spread of the infection across the flock. As such, the subclinical form of the disease can be much more financially detrimental for the producer (Van der Sluis, 2000a; Skinner et al., 2010). It has been estimated that the total cost of clinical and subclinical NE can be as high as US$0.0625 per bird (World Poultry Magazine, 2015), or a staggering US$5-6 million dollars per annum to the global production loss of poultry and current control measures (Wade and Keyburn, 2015).

Invasive action of the *C. perfringens* is initiated by NetB toxin, secreted by virulent *C. perfringens* type A, which attacks chicken hepatocellular carcinoma cells (LMH) in the intestinal epithelial layer and forms small hydrophilic 1.6-1.8 nm pores opening on their surface (Keyburn et al., 2008; Keyburn et al., 2010b; Martin and Smyth, 2010). As a result, the intestine of NE infected birds becomes friable, distended and filled with gas, and visible gross lesions are evident on the mucosa layer (Prescott, 1979a; Keyburn et al., 2010a). The resulting damage to the plasma of epithelial cells causes leakage of cell components into the gut lumen where this protein further favours the growth of *C. perfringens* and the worsening of the disease (Van Immerseel et al., 2009). The intestinal content of affected birds can contain up to 10^7 to 10^9 CFU/g of *C. perfringens* population, which causes further damage the intestinal epithelium (Shojadoost et al., 2012). During NE infection, decreased digestibility and reduced nutrient absorption occur due to damage to the mucosa layer with subsequent diarrhoea and impaired bird performance. Intestinal gross lesions may be visible during necropsy. In severe cases, *C. perfringens* infection can cause rupture of the entire intestinal wall by damaging the integrity of the tight

junctions of the epithelial tissue. Bacteria may enter the blood stream and reach the liver ultimately leading to the death of the host (Van Immerseel et al., 2009; Timbermont, 2011).

Natural occurrence of NE in flocks is accepted as some key predisposing factors which allow an underlying low population of virulent *C. perfringens* to undergo a rapid proliferation to induce the disease (Cooper and Songer, 2009). It has become obvious that *C. perfringens* is ubiquitous in the environment and it can thrive at low levels in the normal gut flora (Timbermont et al., 2009; Lacey et al., 2016). However, *C. perfringens* alone is less likely to cause an NE unless any stress inflicted to the physical change of the intestinal tract of birds can potentially predispose birds to NE. Certain dietary components, such as the highly viscous cereals in diet, high levels of dietary animal protein, are the key risk factor for predisposing of the gut to be more vulnerable to the NE (Annett et al., 2002; Cooper and Songer, 2009). Other Damage to the intestinal mucosa through coccidial infection or fluctuations in the normal intestinal microflora related to diet change can predispose birds to the rapid proliferation of *C. perfringens* (Ficken and Wages, 1997; Moore, 2016). Alteration of feeding regimes, increase in stocking density, and immunosuppressive diseases are some of the other factors which trigger the incidence of NE in chicken flocks (Williams et al., 2003; Lee et al., 2011; Timbermont, 2011). Maternal antibodies in young birds start to disappear from 3 weeks of age onward (Heier et al., 2001), and this is also the time when the probability of NE invasion increases. However, the direct correlation between the antibodies and NE needs more clarification in future research. Furthermore, disruption of microbiota in the gut may be a parallel cause of the proliferation of *C. perfringens*. Stanley et al. (2014) and Wu et al. (2014) reported the distortion of gut flora after *Eimeria* infections in their NE challenge models. Mycotoxins are also likely to disrupt the gastrointestinal tract (GIT) microbiota predisposing birds to the NE (Antonissen et al., 2015). However, future assessments are required to verify whether the disrupted microbiota makes birds susceptible to NE, or the proliferation of virulent *C. perfringens* causes displacement of microbiota populations. Elimination of the causal links between predisposing factors to the occurrence of NE also provides possible control measures to control the disease.

1.3 In-feed antibiotics and the rationale of bans

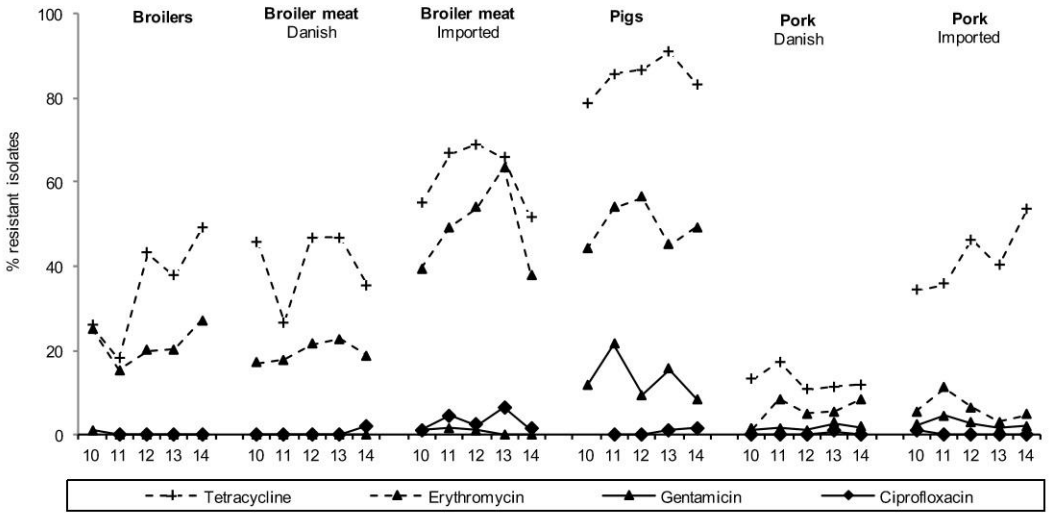
Outbreaks of NE can be effectively treated with antibiotics such as virginiamycin, bacitracin, penicillin, tylosin, flavomycin or the now banned avoparcin (Watkins et al., 1997). When used as in-feed antibiotics, the same agents can be very effective in the prevention and control of NE outbreaks. Also, included at a sub-therapeutical dosage, antibiotics can selectively modify gut flora, suppress bacterial catabolism and reduce bacterial fermentation. All these changes can result in increased nutrient availability for the animal, thereby increasing growth performance (Corpet, 1999). Effective control of coccidial infection can therefore greatly reduce the risk of NE (Williams, 2002; Collier et al., 2008). As previously discussed, mucosal damage caused by coccidial infection is a predisposing factor to the rapid proliferation of *C. perfringens* in the chicken gut (Van Immerseel et al., 2009; Wu et al., 2014; Moore, 2016). The inclusion of anti-coccidial ionophores has shown to be effective in reducing the level of *C. perfringens* in chickens (Elwinger et al., 1998). However, reports on the occurrence of vancomycin-resistant enterococci (VRE) in hospitals, which has been linked to avoparcin (Collignon, 1999), and the general apprehension of the development of antibiotic-resistant "super-bacteria" led to a reduction of antibiotics registered for use in animal feed. In 1999, the European Union (EU) placed a partial ban on the use of AGP which was then proceeded by a total withdrawal of sub-therapeutic use of antibiotics in animal production (including ionophore anticoccidials) from 2006 (Cogliani et al., 2011). Removal of in-feed antibiotics led to a massive increase in NE outbreaks in many European countries and to the widespread occurrence of ill-defined intestinal dysbacteriosis (Kaldhusdal and Løvland, 2000b; Pattison, 2002; Casewell et al., 2003; Cogliani et al., 2011). Although in countries like Australia, USA, Canada and China, antibiotics are still registered and legally allowed to use them, there has been a strong momentum driven largely by the public to phase out antibiotics in some of these countries.

Undoubtedly, the use of in-feed antibiotics improves growth performance and enhances general health of farm animals. In a review of over 12,000 studies, Rosen (1995) concluded that antibiotics improve growth and feed conversion ratio (FCR) in 72% of cases. However, the use of in-feed antibiotics has wider implications than just improving

performance (Casewell et al., 2003). They selectively modify gut flora, suppress bacterial catabolism, reduce bacterial populations and reduce the weight and length of the intestines in poultry (Tannock, 1997; Postma et al., 1999). The "thinning" of the gastrointestinal walls leads to the decreased net-energy directed to luminal tissue, meaning the energy consumption of the host may be reduced. The down scaled bacterial population in the gastrointestinal tract may also spare the vital nutrients from the host-microbes nutrient competition (Ferket, 1990; Ferket et al., 1995). All these changes lead to the enhanced health of the gastrointestinal tract, increased nutrient availability for the animal and subsequently improved growth performance (Carlson and Fangman, 2000). Improved feed utilisation means that feed resources will last longer. This is of particular relevance when feed ingredients are limited due to extreme weather conditions and poor crop yield. A more efficient use of nutrients by the use of antibiotics results in a significantly lower amount of nutrients excreted into the environment (Cromwell, 2000). One of the main reasons why in-feed antibiotics are still used at present is to protect animals against subclinical infections of clostridial infections, such as NE, *E. coli* infections (post-weaning diarrhoea in piglets) or coccidial infections. The major concern on the use of antibiotics, however, is the development of resistance to these in-feed antibiotics, in particular, against antibiotics used in human medicine. There is considerable controversy among leading scientists as to whether resistance will increase by the use of antibiotics in the feed. Veterinarians defend the use of antibiotics on the basis that there no link has been found between their use in feed and the resistance development pattern in human medicine (Cummings, 2004; Schaffer, 2004). Also, the ban also led to a precautious use of therapeutical antibiotics in human. As a result, there is a trend of increased resistance of human pathogens such as *Clostridium difficile*, *Klebsiella pneumoniae* or *Escherichia coli* to antibiotics (DANMAP, 2010). An increase in the numbers of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* was reported from clinical samples in Denmark (DANMAP, 2015). There is also considerable doubt whether a simple ban of in-feed antibiotics will reduce or eliminate resistance. Newman (2003) showed that even after a complete withdrawal of all antibiotics, the population of antibiotic-resistant bacteria could survive in a pig herd for decades. From 2008 to 2014, cases of antibiotic-resistant zoonotic bacteria remained in the animal sector in Denmark where many

antibiotics were obligated to strict prescribed administration and alternation between antimicrobial drugs to ensure resistant development (Figure 1.1)(DANMAP, 2014). Thus, a continuing monitoring of incidences of antibiotic resistance needs to be followed for the determination of mechanism behind the resistant development of the pathogens.

Possibly the most direct link between the use of in-feed antibiotics and increased resistance in human pathogens is the occurrence of vancomycin resistance enterococci (VRE) in hospitals and the general population (Collignon, 1999; Revington, 2002). Several reports have demonstrated antibiotic resistance to commonly used in-feed antibiotics (Barton and Wilkins, 2001; Mathew, 2003; DANMAP, 2015). Despite this apparent controversy on the benefits of a total ban of antibiotics in the animal feed, it seems very unlikely that any of the banned antibiotics will be reintroduced.



Note: The number of isolates varies between years (broilers: n = 86-114, Danish broiler meat: n = 34-75, imported broiler meat: n = 69-104, Pigs: n = 109-157, Danish pork: n = 84-150 and imported pork: n = 45-140).

Figure 1.1 Percentage of resistance cases in *Enterococcus faecalis* in the animal production and meat in 2014, Denmark (DANMAP, 2014)

Worldwide, the animal feed industry has been using antibiotics for over 50 years. In 1946 it was reported that the inclusion of antibiotics in chicken feed resulted in increased weight gain (Moore et al., 1946). Soon after, the advantages of the use of antibiotics in animal feed were officially recognised by the US Food and Drug Administration (FDA), and in 1951 the FDA approved the use of antibiotics in animal feed without veterinary

prescriptions (Jones and Ricke, 2003). Not until the dosing of in-feed antibiotics was banned in some countries, antibiotics had been widely used in farm animals at therapeutical levels to control diseases and at subtherapeutic levels to promote growth and feed efficiency. More recently, the use and apparent over-use of antibiotics in animal feed have been widely discussed in the scientific literature, at scientific meetings and in the general press. As mentioned earlier in this review, the main concern is the emergence of the so-called "superbugs" - antibiotic-resistant human pathogens, after the prolonged use of antibiotics in animal feed (Phillips, 1999).

As far back as in 1969, recommendations were put forward for the prudent use of antibiotics as growth promoters (Swann Committee, 1969). This Committee initiated the restriction of the use of antibiotics without veterinary prescriptions in the UK. In 1986, as a result of increased pressure from consumer groups to further reduce antibiotic use in animal feed, Sweden implemented a partial ban on their use in farm animals. In 1997, the European Union (EU) placed a partial ban on the use of in-feed antibiotics, which was replaced in 2006 by a general ban (including ionophore anticoccidials) in all animal feed. Big marketing campaigns by fast food giants have used this initiative to promote their products. For instance, one of the largest purchasers of meat globally, McDonald's Corporation, has announced that their meat source would not use medically important antibiotics as growth ionpromotants from 2003 (Dibner and Richards, 2005). Despite strong criticism and opposition for following a "precautionary principle" rather than scientific facts (Schaffer, 2004), a global ban on the use of antibiotics as growth promoter appears inevitable.

Continuingly enforced constraints on the in-feed use of antibiotics will inevitably alter the intestinal microbial ecology of modern broiler chickens (Knarreborg et al., 2002). Use of alternative methods to control NE will become inevitable (Geier et al., 2010; Huyghebaert et al., 2011; Caly et al., 2015). Data from Europe show that strict hygiene management, climate control of animal buildings and feed composition are important in maintaining production efficiency in a post-AGP era (Inborr, 2000). Nutritional management strategies, such as lowering the inclusion rate of fishmeal, wheat or barley in diets may also help prevent NE in poultry (Ficken and Wages, 1997). Modulation of the natural

bacterial population of the intestine in broilers through nutritional manipulation, such as selection of feed ingredients and/or use of alternate feed supplements can be effective tools to control NE and other clostridia diseases.

1.4 Relationship between dietary ingredients and the occurrence of NE

To find successful nutritional strategies to reduce the risk of NE, an appropriate selection of feed ingredients based on their effects on the microbial population, in particular, the growth of *C. perfringens*, is necessary. The incidence of *C. perfringens* infections is significantly higher in birds fed diets based on wheat, barley, oats or rye. The reason may be related to the fact that these grains contain a high level of indigestible soluble NSP which leads to increased digesta viscosity and decreased digesta passage rate and nutrient digestibility (Choct et al., 1996; Choct, 1999). A highly viscous intestinal environment increases the proliferation of facultative anaerobes like gram-positive cocci and enterobacteria (Bedford and Classen, 1992; Vahjen et al., 1998) and a large amount of undigested material in the small intestine together with a prolonged transit time of digesta increases the chances of rapid bacterial colonisation of the intestine (Gabriel and Mallet, 2006). Pluske (2001) showed that the incidence of porcine intestinal spirochaetosis, swine dysentery and post-weaning diarrhoea is closely related to the amount of indigestible starch and NSP in the diet and the proliferation of pathogenic bacteria in the intestine. Data by Choct and Sinlae (2000) confirmed that in three-week-old broilers, the number of total anaerobic bacteria, in particular, the number of *C. perfringens* markedly increased a day after the introduction of a wheat-based diet. The change in the microbial balance in the small intestine seems to result in dysbacteriosis, which was defined by Tice (2000) as "the presence of a qualitatively and quantitatively abnormal flora in the intestine".

Alternatively, maize is considered an excellent ingredient in broiler diets due to its high energy content and high nutrient availability. Broiler diets high in maize may also help reduce the incidence of NE in comparison to diets based on wheat or barley (Kaldhusdal and Løvland, 2000b). In an observational study attempting to link the incidence of NE with the cereal content of broiler diets in Norway, found a clear correlation between the

ratio of wheat plus barley to maize and the outbreak of NE was found (Kaldhusdal and Skjerve, 1996b). These authors showed when a high level of maize rather than wheat or barley was present in diets, the occurrence of NE reduced. Interestingly, the inclusion of maize in broiler diets also linked to reduced susceptibility to coccidiosis (Williams, 1992). It is believed that the negligible amount of the soluble fibre content of maize helps alleviate the proliferation of *C. perfringens* in the small intestine.

On the other hand, the use of poorly digestible protein sources alters the microflora and creates favourable conditions in the intestine for the proliferation of pathogens, and for the possible induction of NE (Wu et al., 2014). Naturally, the reverse is also true, and when highly digestible dietary components are fed to animals, the chance of pathogens colonising the gut is alleviated, and bird performance is increased (Gabriel and Mallet, 2006; Keerqin et al., 2017). However, the outbreak of NE is a consequence of one or multiple priming factors that evoke epithelial cell damage and cause drastic shifts in the microbial populations in the intestine of chickens (Antonissen et al., 2016). In fact, a significant portion of the poultry diet consists of protein from an animal source. Animal protein ingredients such as fishmeal or meat and bone meal are often associated with an increased risk of NE (Ficken and Wages, 1997; Rodgers et al., 2015). Many believe that the increase in the number of *C. perfringens* in the intestinal tract is directly related to the proportion of meat and bone meal, particularly in wheat-based diets (McDevitt, 2006; Rodgers et al., 2015; Moore, 2016). Most laboratory models able to successfully reproduce NE share the same principle of supplementing the excessive amount of fish meal and/or meat and bone meal before the *C. perfringens* challenge (Truscott and Al-Sheikhly, 1977; Prescott, 1979b; Wu et al., 2014). Challenge models with the inclusion of fishmeal had been more successful in reproducing NE in the lab (Rodgers et al., 2014; Stanley et al., 2014). It can be speculated that the high nutrient density, in particular, high levels of protein, alters the microflora and creates favourable conditions in the intestine for the proliferation of *C. perfringens* (Timbermont et al., 2011). Kaldhusdal and Løvland (2000a) concluded that the level of animal protein in the diet and the coupling cereal type would be a key to the incidence of NE. It is important to note that the inclusion of a high level of fishmeal alone is no guarantee for the proliferation of *C. perfringens*. The inclusion of 25% fishmeal in a wheat-based diet compared to only 9% fishmeal had no

effect on the number of *C. perfringens* or the incidence of NE in the period from 2 to 4½ weeks of age (Barnes et al., 1972). Unfortunately, these authors did not report the crude protein content of the feed. Considering that a marked increase in performance was observed when more fishmeal was added, it is likely that the protein level in the diet with only 9% fishmeal was below requirements. Hence the excess protein was in fact utilised by the chicken and did not change nutrient availability for the microflora. In a more recent study, Wu et al. (2010) showed that the number of *C. perfringens* related to the level of fishmeal included, suggesting that an excess level of protein reaching the hindgut of birds is conducive to the proliferation of the organism but not necessarily the cause.

The association of animal protein ingredients with an increased occurrence of NE together with the general ban of protein sources of animal origin in Europe has put vegetable protein sources in the spotlight (Adams, 2000). *In vitro* studies have shown that in a modified growth medium, some isolated soy proteins can stimulate the growth of *C. perfringens* (Busta and Schroder, 1971). However, to the author's best knowledge, there is no report in the literature linking the occurrence of NE with the inclusion of soybean meal or any other protein of vegetable origin. The increase of digestible amino acids in poultry diets is a topic of growing interest in recent years. Direct replacement of dietary protein by synthetic amino acids according to respective amino acid profiles may not incorporate with improvement, if not worsened, bird performance (Aletor et al., 2000). However, amino acid supplements, such as threonine, glutamine and arginine, play a key role in immune response and formation of immunoglobulins during inflammation (Le Floc'h et al., 2004). Also, an adequate amount of amino acid provision may be necessary to maintain the immune competence of the host against infectious diseases (Li et al., 2007). Kidd et al. (2004) suggested that nutritional modulation, such as amino acid addition in poultry diets, may be irrelevant to the performance of chicken; rather, it strengthens the immunity which improves disease resistance in broilers. Strengthened immunity in broiler chicken may bring about beneficial outcomes upon disease outbreaks, including NE.

It is known that low molecular weight carbohydrates such as α-galactosides present in vegetable proteins cannot be digested in the small intestine of poultry due to the absence

of endogenous α-galactosidase. Instead, they are subject to microbial fermentation in the caeca (Carré et al., 1990). Bacterial degradation of α-galactosides can lead to increased hydrogen production, impaired utilisation of nutrients and subsequently reduced performance (Saini, 1989). Despite the apparent abundance of nutrients for the intestinal microflora, there appear to be no changes in the composition of microflora (including of *C. perfringens*) when diets containing up to 80% peas compared with a corn-soy control diet are fed (Brenes et al., 1989).

So far, only a few studies investigated the effects of grinding or pelleting on microbial composition of the intestine. Branton et al. (1987) reported that the mortality attributed to NE was significantly higher in birds fed crumbles with hammer-milled wheat compared to roller-milled wheat. In contrast, Engberg et al. (2002) found no difference in the number of *C. perfringens* in the small intestine between diets with hammer-milled (fine) or roller-milled (coarse) wheat. The same study, however, found that birds fed pellets had higher counts of *C. perfringens*, total anaerobic bacteria and lactobacilli compared to those fed mash diets, albeit, roller-milling outperforms hammer milling in terms of the better uniformity of the pellet size distribution.

1.5 Dietary manipulation to control NE

Publications on the efficacy of potential alternatives to in-feed antibiotics for controlling NE have been steadily growing over recent years. These supplements can be divided into four groups according to their modes of action or strategies: 1) improvement of nutrient utilisation by the host; 2) modulation of the immune system; 3) stimulation or introduction of beneficial bacteria, and 4) direct reduction of pathogens. Within these general categories, there are hundreds of commercially-available products claiming to be as effective as antibiotics in the improvement of growth performance and animal health. In 2004, Rosen proposed animal producers to use a seven-question test so they could assess the potential value of an alternative product. Two of the central questions in this test are the number of feeding tests conducted and the frequency of positive responses. Many replacement products have been recently developed and therefore have not been tested under a wide range of conditions.

1.5.1 Enzymes

Nowadays, exogenous enzyme supplementation is virtually standard in all poultry feed. It is well-documented that the addition of feed enzymes to diets based on wheat, barley, oat or rye significantly decreases viscosity in the small intestine by partially depolymerising soluble fibre (Bedford and Classen, 1992; Choct et al., 2006). It has also been demonstrated that the inclusion of xylanases in wheat-based diets significantly reduces bacterial populations in the small intestine (Apajalahti, 1999; Choct et al., 1999; Engberg et al., 2004), in particular, the number of *C. perfringens* (Choct and Sinlae, 2000; Choct et al., 2006). The addition of enzymes is known to reduce digesta viscosity in the small intestine and increase nutrient digestion and digesta flow rate, largely diminishing the amount of substrates reaching the hindgut for the microflora (Choct et al., 1999). Despite causing profound changes in the intestinal microflora and the apparent reduction in the numbers of *C. perfringens*, feed enzymes alone cannot provide complete protection against NE (Elwinger and Teglöf, 1991; Riddell and Kong, 1992b). Enzymes change conditions in the intestine but have no direct effect on the growth of *C. perfringens*. Apajalahti and Bedford (2000) further suggested that the depolymerisation of arabinoxylans in wheat with xylanases produced xylo-oligomers and xylose which could only be partially utilised by the microflora. Subsequently, a total number of bacteria in the ileum was reduced by 60%. The inclusion of xylanase into wheat- and bran-based diet leads to an *in-situ* production of prebiotic substrates, such as arabinoxylan-oligosaccharides (AXOS), which have been widely associated with a positive modulation of intestinal microbial populations (Grootaert et al., 2007; Broekaert et al., 2011). Subsequent production of AXOS has shown to be effective against the growth of Salmonella population in the chicken gut (Eeckhaut et al., 2008). Femia et al. (2010) reported reduced intestinal lesions in rats treated with xylan-derived xylooligosaccharides. In a more recent study. De Maesschalck et al. (2015) showed that supplemental AXOS improved bird performance and enhanced butyrate production. However, it must be borne in mind that the inclusion of exogenous enzymes is only useful if diets contain the specific substrates for the enzymes to react.

1.5.2 Prebiotics

Prebiotics are low-molecular-weight carbohydrates that can be selectively utilised by beneficial microbial populations in the intestine. Prebiotics must neither be hydrolysed nor be absorbed by the host and therefore should be available to the microflora in the intestine (Gibson and Roberfroid, 1995). Nowadays, there are several commercially available prebiotic products on the market, including galactooligosaccharides, inulin and mannooligosaccharides (MOS) (Yang et al., 2009). Besides, the inclusion of substrates like fructooligosaccharides (FOS), transgalacto-oligosaccharides (TOS) or inulin can selectively stimulate the growth of beneficial microorganisms (Bifidobacteria, *Lactobacillus* spp.) in the intestine (Ziggers, 2001; Bielecka et al., 2002). Pathogens like *E. coli* or *C. perfringens* are unable to use FOS as an energy source, and consequently, the number of FOS fermenters increases, thereby creating conditions to competitively exclude unfavoured bacterial strains. The increase of beneficial bacteria not only reduces available substrates for potential pathogens but also decreases pH in the intestine due to increased fermentation activity and production of volatile fatty acids. MOS, however, work via a different mechanism to other probiotics, in that they inhibit colonisation of the gut by pathogenic bacteria through blocking type-1 fimbriae on the mucosal surface (Dawson and Pirvulescu, 1999). They also improve overall intestinal health by enhancing the gut integrity and by modulating the immune system (Iji et al., 2001; Ferket, 2002; Davis et al., 2004). Furthermore, MOS may have a direct influence on nutrient utilisation in the intestine. For example, dietary MOS have been reported to increase the number of specific populations of microbes which have enhanced fibre fermentation capacity while reducing numbers of microbes which rely on starches and sugars for their growth (Ferket et al., 2002; Kappel et al., 2004).

Substrates, such as AXOS, have been recently introduced as a potential new class of prebiotics, as they were reported to inhibit adhesion of *L. monocytogenes* to enterocytes *in vitro* (Ebersbach et al., 2012), improve bird performance (De Maesschalck et al., 2015) and decrease bacterial diversity in fish (Geraylou et al., 2013). Other authors suggest the combination of prebiotic supplements with matching probiotic products, thereby

maximising the population of beneficial microbiota against potential pathogenic colonisation (Fuller, 2001).

1.5.3 Probiotics

Microorganisms used as probiotics in animal nutrition are various, such as *Enterococcus* spp., *Saccharomyces* yeast, spore-forming *Bacillus* spp., Bifidobacterium and, to a lesser extent, *Lactobacillus* spp. (Simon et al., 2001; Fan et al., 2006). As reviewed by Fuller (2001) and Simon et al. (2001), probiotics can modify gut microflora, influence mucosa permeability, prevent binding of potential pathogens to intestinal mucosa by blocking binding sides, modulate the immune system and produce bacteriocins. In spite of the great variability of results obtained over the past decade through the use of probiotic supplements in the prevention of bacterial diseases in poultry, the consensus is still in favour of the use of probiotic supplements as an alternative to in-feed antibiotics (La Ragione et al., 2001; La Ragione and Woodward, 2003; Waititu et al., 2014). *In vitro* assays showed that the adhesion of *C. perfringens* could be reduced by lactic acid bacteria (Rinkinen et al., 2003). Growth studies without a disease challenge showed that the inclusion of *Bacillus coagulans* or Lactobacillus cultures in broiler diets significantly improve growth performance (Cavazzoni et al., 1998; Jin et al., 1998). Moreover, in a challenge study with *C. perfringens,* a commercially available probiotic significantly reduced the severity of NE (Hofacre et al., 1998; Kaldhusdal et al., 2001). The combination of probiotic with prebiotic products may be a solution to yield an optimal result (Ehrmann et al., 2002; Patterson and Burkholder, 2003; Awad et al., 2009).

1.5.4 Organic acids

Organic acids are weak acids which are known for their antimicrobial activity. Organic acids are usually half dissociated pKa profile between 3 and 5. Supplemental organic acids in the feed or water in poultry have been referred as acidifiers that could enable reduction of certain pathogenic bacteria, such as *Salmonella*, *Campylobacter* and *Escherichia coli* (Cherrington et al., 1991b; Van Immerseel et al., 2006; Gharib Naseri et al., 2012). Application of organic acids in poultry nutrition has been recently reviewed by Khan (2016). Organic acids provide a low-cost solution for inhibiting the growth of various

microbial and fungal species, and they have been widely used in food preservative purposes (Kum et al., 2010). Organic acids physically inhibit enzymatic reactions and intercellular transportation mechanisms of bacteria, which, in turn, slows down the metabolism of bacterial cells (Cherrington et al., 1991a). Simple monocarboxylic acids, including formic, acetic, propionic and butyric acids, and hydroxyl variants of carboxylic acids, such as lactic, malic, and tartaric acids have shown to have antimicrobial activity, whereas, sorbic and fumaric acids also possess antifungal activity (Dibner and Buttin, 2002). The number of *E. coli* colonies was shown to diminish when buffered propionic acid supplementation was given to birds (Izat et al., 1990). Also, organic acids were reported as bactericidal against a *Salmonella* strain (Thompson and Hinton, 1997). Microencapsulated organic acid products in poultry diets emerged in recent years. These are organic acids with a slow-digestible coating which prevents digestion and absorption of active organic acids in the upper digestive tract compared to the non-coated organic acids. Valuable active compounds are then able to withstand digestion and exhibit efficacy towards the lower gastrointestinal tract. Sodium butyrate alone could not alter growth performance of *C. perfringens* challenged birds, but the combined effect of butyrate and essential oil improved weight gain, increased intestinal villus height and reduced severity of gross intestinal lesions (Jerzsele et al., 2012).

1.6 Novel strategies to control NE

A vaccine for NE represents a potentially effective tool to prevent the disease without the use of antimicrobial drugs. Most virulent strains of *C. perfringens* have one or multiple toxin genotypes, such as the recently discovered NetB toxin (Keyburn et al., 2008). These have been meaningful targets for vaccine development against virulent *C. perfringens* (Antonissen et al., 2016). NetB is a pore-forming toxin with a heptameric structure which mode of action involves polymerisation of plasma channel protein of epithelial cells into larger single pores (Knarreborg et al., 2002; Eeckhaut et al., 2008; Keyburn et al., 2010a). The oligomerisation process of NetB is believed to be driven by cholesterol, but an effective facilitation of vaccine requires more information regarding the binding mechanisms of NetB toxin (Eeckhaut et al., 2008). The recombinant NetB protein used as antigen was reported to boost levels of anti-NetB IgY antibodies *in vitro* and

immunisation of rNetB with bacterin enabled a higher level of anti-NetB IgY antibodies, suggesting this can be a feasible approach to facilitate a vaccine against NE (Keyburn et al., 2013). Thompson et al. (2006) reported successful vaccination of *C. perfringens* α-toxin genotypes, though the result presumably interfered with another antigen. Another interesting finding is that antibodies of parental origin against a *C. perfringens* strain could passively immunise offspring birds which inherited such antibodies and showed resilience to the disease in a challenge trial (Lovland et al., 2004). Hens vaccinated against NetB were able to passively transfer antibodies and protect progenies from NE at the age of peak susceptibility to the disease (Ebersbach et al., 2012). Current application of NetB-specific vaccine is still on a preliminary research stage, but future advances in knowledge concerning NetB toxin will eventually enable affordable vaccines or subunit vaccines to be used in the field. Apart from that, live pentavalent, an attenuated anticoccidial vaccine enables birds to resist severe challenges with heterologous *E. maxima* and *C. perfringens* (Williams et al., 2003).

Bacteriophage administration has great potential against animal-borne bacterial diseases, such as NE. Bacteriophage application in human medicine can be traced back to 1915, before penicillin's discovery. Since then, and for around a century, clinical use of bacteriophages remained high in Eastern Europe, and its unique advantages over antibiotic treatments have drawn much attention in recent years (Joerger, 2003; Abedon et al., 2011). Bacteriophages are viruses that specifically infect their host bacterium. In Nature, almost every bacteria strain hosts one or multiple susceptible bacteriophage strains. Classification of bacteriophages has gone through various referencing systems according to their specific features. Lytic phage and lysogenic (temperate) phage undergo different lytic cycles, but bacteriophage application usually refers to lytic phages which enter host bacteria and reproduce phage components by using host cell machinery, thus exhausting host bacteria and eventually releasing assembled phage progenies by lysing the bacteria cell (Abedon et al., 2011). Carrillo et al. (2005) showed that a population of Campylobacter could be quickly reduced with the inclusion of Campylobacter-specific phage isolates. Miller et al. (2010) explored the inhibition of NE-causing *C. perfringens* by using a multivalent bacteriophage cocktail. The outcome showed a more effective reduction of *C. perfringens* with this cocktail in comparison to the toxoid vaccine. Parisien

et al. (2008) reviewed the potential of using bacteriophage and bacteriophage-derived substrates to control the population of *C. perfringens*. Bacteriophage gene-derived products were also shown to be effective in the elimination of susceptible host bacteria. For example, Seal (2013) designed a successful experiment to eliminate *C. perfringens* colonies using recombinant lytic enzymes derived from a field-isolated *C. perfringens* bacteriophage.

The main advantage of bacteriophage application in the prevention of NE is the host specificity of the phage which may avoid deleterious effects on other beneficial bacteria populations in the gut. Currently, there is only a handful of research available to pursue this approach in combating NE in chickens. Thus, the route of administration, efficacy of host lysis, lytic profile and shelf life of bacteriophage application would require further investigation before being approved for field applications.

1.7 Conclusion

Feed ingredients consist of numerous compounds, and it is often impossible to relate a single constituent with changes in gut microflora. Although extensive research exists on replacement of in-feed antibiotics and the alleviation of NE, only a few strategies have shown efficacy in laboratory conditions and, to date, no commercially-available product has been able to provide comprehensive protection to the gut similar to that of antibiotics. Reports from Denmark and Sweden suggest that withdrawal of antibiotics from poultry diets were followed by numerous problems associated with NE. However, Europe seems to have adapted to the situation and has shown optimism in controlling NE through nutritional manipulation and by the use of alternative supplements. Nonetheless, there are still significant gaps in the understanding of how and under what conditions these ingredients will alter numbers of *C. perfringens*, and on their role in potential outbreaks of NE.

NE is a complex, multifactorial disease and many ambiguous triggering factors influence its occurrence and the severity of outbreaks. To a certain extent, the preconditions to NE can be reverted, or the severity of NE can be minimised by optimising feeding

programmes in combination with nutritional manipulation to the gut without using in-feed antibiotic supplements.

1.8 References

Abedon, S. T., S. J. Kuhl, B. G. Blasdel, and E. M. Kutter. 2011. Phage treatment of human infections. Bacteriophage 1: 66-85.

Adams, C. A. 2000. Nutricines as alternatives to antibiotics. In: Meeting the challenges of intensive animal production, Star City Hotel, Sydney. p 1-10.

Ahmad, I. 2006. Effect of probiotics on broilers performance. International Journal of Poultry Science 5: 593-597.

Aletor, V. A., I. I. Hamid, E. Niess, and E. Pfeffer. 2000. Low-protein amino acid-supplemented diets in broiler chickens: effects on performance, carcass characteristics, whole-body composition and efficiencies of nutrient utilisation. Journal of the Science of Food and Agriculture 80: 547-554.

Annett, C. et al. 2002. Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of Clostridium perfringens type A. Avian Pathology 31: 598-601.

Antonissen, G. et al. 2015. Fumonisins affect the intestinal microbial homeostasis in broiler chickens, predisposing to necrotic enteritis. Veterinary research 46: 98.

Antonissen, G. et al. 2016. Microbial shifts associated with necrotic enteritis. Avian Pathology 45: 308-312.

Ao, Z., A. Kocher, and M. Choct. 2012. Effects of dietary additives and early feeding on the performance, gut development and immune status of broiler chickens challenged with Clostridium perfringens. Asian-Aust. J. Anim. Sci 25: 541-551.

Apajalahti, J., and M. Bedford. 2000. Impact of dietary and envionmental factors on microbial cummunities of the avian GI tract. In: World Poultry Congress, Montreal. p S3.5.03.

Apajalahti, J. H. A. 1999. Improve bird performance by feeding its microflora. World Poultry 15: 1-3.

Awad, W., K. Ghareeb, S. Abdel-Raheem, and J. Böhm. 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poultry science 88: 49-56.

Bannam, T. L. et al. 2011. Necrotic enteritis-derived Clostridium perfringens strain with three closely related independently conjugative toxin and antibiotic resistance plasmids. MBio 2: e00190-00111.

Barnes, E. M., G. C. Mead, D. A. Barnum, and E. G. Harry. 1972. The intestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. Brit Poultry Sci 13: 311-326.

Barton, M. D., and J. Wilkins. 2001. Antibiotic Resistance. No. 01/105, RIRDC, Canberra.

Bartosch, S., A. Fite, G. T. Macfarlane, and M. E. McMurdo. 2004. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Applied and environmental microbiology 70: 3575-3581.

Bedford, M. R., and H. L. Classen. 1992. Reduction of intestinal viscosity through manipulation of dietary rye and pentosanase concentration is effected through changes in the carbohydrate composition of the intestinal aqueous phase and results in improved growth rate and food conversion efficiency of broiler chicks. Journal of Nutrition 122: 560-569.

Bielecka, M., E. Biedrzycka, and A. Majkowska. 2002. Selection of probiotics and prebiotics for synbiotics and confirmation of their in vivo effectiveness. Food Research International 35: 125-131.

Branton, S. L., F. N. Reece, and W. M. Hagler Jr. 1987. Influence of a wheat diet on mortality of broiler chickens associated with necrotic enteritis. Poultry Science 66: 1326-1330.

Brenes, A., J. Trevino, C. Centeno, and P. Yuste. 1989. Influence of peas (*Pisum sativum*) as a dietary ingredient and flavomycin supplementation on the performance and intestinal microflora of broiler chicks. British Poultry Science 30: 81-89.

Broekaert, W. F. et al. 2011. Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. Critical reviews in food science and nutrition 51: 178-194.

Broussard, C., C. Hofacre, R. Page, and O. Fletcher. 1986. Necrotic enteritis in cage-reared commercial layer pullets. Avian diseases: 617-619.

Busta, F. F., and D. J. Schroder. 1971. Effect of soy protein on the growth of Clostridium perfringens. Applied Microbiology 22: 177-183.

Caly, D. L., R. D'Inca, E. Auclair, and D. Drider. 2015. Alternatives to Antibiotics to Prevent Necrotic Enteritis in Broiler Chickens: A Microbiologist's Perspective. Frontiers in microbiology 6.

Cao, L. et al. 2012. Reduced lesions in chickens with Clostridium perfringens-induced necrotic enteritis by Lactobacillus fermentum 1.2029. Poultry science 91: 3065-3071.

Carlson, M. S., and T. J. Fangman. 2000. Swine Antibiotics and Feed Additives: Food Safety Considerations MU Guide, Agriculture, G2353 No. 2004. University of Missouri Extension, Columbia.

Carré, B., L. Derouet, and B. Leclercq. 1990. The digestibility of cell-wall polysaccharides from wheat (bran or whole grain), soybean meal, and white lupin meal in cockerels, Muscovy ducks, and rats. Poultry Science 69: 623-633.

Carrillo, C. L. et al. 2005. Bacteriophage therapy to reduce Campylobacter jejuni colonization of broiler chickens. Applied and environmental microbiology 71: 6554-6563.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. Journal of Antimicrobial Chemotherapy 52: 159-161.

Cavazzoni, V., A. Adami, and C. Castrovilli. 1998. Performance of broiler chickens supplemented with Bacillus coagulans as probiotic. British Poultry Science 39: 526-529.

Cherrington, C., M. Hinton, G. Mead, and I. Chopra. 1991a. Organic acids: chemistry, antibacterial activity and practical applications. Advances in microbial physiology 32: 87-108.

Cherrington, C. A., M. Hinton, G. Pearson, and I. Chopra. 1991b. Short-chain organic acids at pH 5.0 kill Escherichia coli and Salmonella spp. without causing membrane perturbation. Journal of Applied Bacteriology 70: 161-165.

Choct. 1999. Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chickens fed wheat. British poultry science 40: 419-422.

Choct. 2001. Alternatives to in-feed antibiotics in monogastric animal industry. ASA Technical Bulletin. AN 30.

Choct, M., and G. Annison. 1992. Anti-nutritive effect of wheat pentosans in broiler chickens: Roles of viscosity and gut microflora. British poultry science 33: 821-834.

Choct, M., R. J. Hughes, and M. R. Bedford. 1999. Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chickens fed wheat. British Poultry Science 40: 419-422.

Choct, M. et al. 1996. Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. British Poultry Science 37: 609-621.

Choct, M., and M. Sinlae. 2000. Clostridium perfringens in broiler chickens: The role of xylanase in controlling necrotic enteritis in broiler fed wheat diets without anti-microbial drugs. 0642 (to be assigned) / UNE 70A, Rural Industries Research and Development Corporation, Canberra.

Choct, M., M. Sinlae, R. Al-Jassim, and D. Pettersson. 2006. Effects of xylanase supplementation on between-bird variation in energy metabolism and the number of Clostridium perfringens in broilers fed a wheat-based diet. Crop and Pasture Science 57: 1017-1021.
Cogliani, C., H. Goossens, and C. Greko. 2011. Restricting antimicrobial use in food animals: lessons from Europe. Microbe 6: 274.
Collier, C. et al. 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth. Veterinary immunology and immunopathology 122: 104-115.
Collignon, P. J. 1999. Vancomycin-resistant enterococci and use of avoparcin in animal feed, is there a link? Medical Journal of Australia 171: 144-146.
Cooper, K. K., and J. G. Songer. 2009. Necrotic enteritis in chickens: a paradigm of enteric infection by Clostridium perfringens type A. Anaerobe 15: 55-60.
Corless, A. B., and J. Sell. 1999. The effects of delayed access to feed and water on the physical and functional development of the digestive system of young turkeys. Poultry science 78: 1158-1169.
Corpet, D. E. 1999. Mechanism of antimicrobial growth promoters used in animal feed. Comptes Rendus de l'Academie d'Agriculture de France 85: 197-205.
Cromwell, G. L. 2000. Antimicrobial and promicrobial agents. In: A. J. Lewis and L. L. Southern (eds.) Swine Nutrition. p 401-426. CRC Press, Washington.
Cummings, T. S. 2004. Antibiotic use in food animal medicine: the quiet voice of reason in the antibiotic debate. In: 5th Asia Pasific Poultry Health Conference, Sufers Paradise, Gold Coast, Austalia. p PL 3.1.
Dahiya, J., D. Wilkie, A. Van Kessel, and M. Drew. 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Animal Feed Science and Technology 129: 60-88.
DANMAP. 2010. Use of antimicorbaial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark.
DANMAP. 2014. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals food and human in Danmark.
DANMAP. 2015. Use of animicrobial agents and occurrence of antimirobial resistance in bacteria from food animals, food and humans in Denmark.
Davis, M. E. et al. 2004. Effect of phosphorylated mannans and pharmacological additions of zinc oxide on growth and immunocompetence of weanling pigs. Journal of Animal Science 82: 581–587.
Dawson, K. A., and M. Pirvulescu. 1999. Yeast-derived mannan oligosaccharides as immune modulators and alternatives to antimicrobial growth promoters. In: Alltech's Asia Pacific Lecture Tour, Sydney. p 75-83.
De Roos, N. M., and M. B. Katan. 2000. Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. The American journal of clinical nutrition 71: 405-411.
Devriese, L., G. Daube, J. Hommez, and F. Haesebrouck. 1993. In vitro susceptibility of Clostridium perfringens isolated from farm animals to growth-enhancing antibiotics. Journal of applied microbiology 75: 55-57.
Dibner, J. 1999. Feeding hatchling poultry. Avoid any delay. Feed Int 20: 30-34.
Dibner, J., and P. Buttin. 2002. Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism. The Journal of Applied Poultry Research 11: 453-463.
Dibner, J. et al. 1998. Early feeding and development of the immune system in neonatal poultry. The Journal of Applied Poultry Research 7: 425-436.
Dibner, J., and J. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poultry science 84: 634-643.

Ebersbach, T., J. B. Andersen, A. Bergström, R. W. Hutkins, and T. R. Licht. 2012. Xylo-oligosaccharides inhibit pathogen adhesion to enterocytes in vitro. Research in microbiology 163: 22-27.

Edens, F. 2003. An alternative for antibiotic se in poultry: probiotics. Revista Brasileira de Ciência Avícola 5: 75-97.

Eeckhaut, V. et al. 2008. Arabinoxylooligosaccharides from wheat bran inhibit Salmonella colonization in broiler chickens. Poultry science 87: 2329-2334.

Ehrmann, M., P. Kurzak, J. Bauer, and R. Vogel. 2002. Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. Journal of applied microbiology 92: 966-975.

Elwinger, K., E. Berndtson, B. Engström, O. Fossum, and L. Waldenstedt. 1998. Effect of antibiotic growth promoters and anticoccidials on growth of Clostridium perfringens in the caeca and on performance of broiler chickens. Acta Veterinaria Scandinavica 39: 433-441.

Elwinger, K., and B. Teglöf. 1991. Performance of broiler chickens as influenced by a dietary enzyme complex with and without antibiotic supplementation. Archiv für Geflügelkunde 55: 69-73.

Engberg, R. M., M. S. Hedemann, and B. B. Jensen. 2002. The influence of grinding and pelleting of feed on the microbial composition and activity in the digestive tract of broiler chickens. British Poultry Science 43: 569-579.

Engberg, R. M., M. S. Hedemann, S. Steenfeldt, and B. B. Jensen. 2004. Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poultry science 83: 925-938.

Fan, Y.-j., S.-j. Chen, Y.-c. Yu, J.-m. Si, and B. Liu. 2006. A probiotic treatment containing Lactobacillus, Bifidobacterium and Enterococcus improves IBS symptoms in an open label trial. Journal of Zhejiang University Science B 7: 987-991.

FAO, I. 2015. WFP (2015) The state of food insecurity in the world 2015. In: Meeting the

Fao, W. 2012. IFAD. 2012. The state of food insecurity in the world: 1-63.

Femia, A. P. et al. 2010. Arabinoxylan-oligosaccharides (AXOS) reduce preneoplastic lesions in the colon of rats treated with 1, 2-dimethylhydrazine (DMH). European journal of nutrition 49: 127-132.

Ferket, P. 1990. Effect of diet on gut microflora of poultry. In: Proceedings 1990, Georgia Nutrition Conference for the Feed Industry, Atlanta Airport Hilton, Atlanta, Georgia, November 13-15. p 122-129.

Ferket, P., J. Grimes, J. Brake, and D. Rives. 1995. Effects of dietary virginiamycin, arginine: lysine ratio, and electrolyte balance on the performance and carcass yield of turkey toms. Poult. Sci 74: 190.

Ferket, P. R. 2002. Use of Oligosaccharides and gut modifiers as replacements for dietary antibiotics. In: 63rd Minnesota Nutrition Conference, Eagan, Minnesota

Ferket, P. R., C. W. Parks, and J. L. Grimes. 2002. Mannan oligosaccharides versus antibiotics for turkeys. In: T. P. Lyons and K. A. Jacques (eds.) Biotechnology in the Feed Industry: Proceedings of Alltech's 18th Annual Symposium No. 14. p 43-63. Nottingham Press, Nottingham.

Ficken, M. D., and D. P. Wages. 1997. Necrotic enteritis. In: B. W. Calnek, H. J. Barnes, C. W. Beard, W. M. Reid and H. W. Yoder, Jr (eds.) Diseases of Poultry. p 261-265. Iowa State University Press, Ames, IA50010.

Fuller, R. 2001. The Chicken Gut Microflora and Probiotic Supplements. The Journal of Poultry Science 38: 189-196.

Gabriel, I. L., M, and S. G. Mallet, JF. 2006. Microflora of the digestive tract: critical factors and consequences for poultry. World's Poultry Science Journal 62: 499-512.

Geier, M. S. et al. 2010. Comparison of alternatives to in-feed antimicrobials for the prevention of clinical necrotic enteritis. Journal of applied microbiology 109: 1329-1338.

Geraylou, Z. et al. 2013. Effects of dietary arabinoxylan-oligosaccharides (AXOS) and endogenous probiotics on the growth performance, non-specific immunity and gut microbiota of juvenile Siberian sturgeon (Acipenser baerii). Fish & shellfish immunology 35: 766-775.

Geyra, A., Z. Uni, and D. Sklan. 2001. The effect of fasting at different ages on growth and tissue dynamics in the small intestine of the young chick. British Journal of Nutrition 86: 53-61.

Gharib Naseri, K., S. Rahimi, and P. Khaki. 2012. Comparison of the effects of probiotic, organic acid and medicinal plant on Campylobacter jejuni challenged broiler chickens. Journal of Agricultural Science and Technology 14: 1485-1496.

Gholamiandehkordi, A. R. et al. 2007. Quantification of gut lesions in a subclinical necrotic enteritis model. Avian Pathology 36: 375-382.

Gibert, M., C. Jolivet-Renaud, and M. R. Popoff. 1997. Beta2 toxin, a novel toxin produced by Clostridium perfringens. Gene 203: 65-73.

Gibson, G. R., and M. B. Roberfroid. 1995. Dietary modulation of the human clonic microbiota: Introducing the concepts of prebiotics. Journal of Clinical Nutrition 125: 1401-1412.

Gonzales, E. et al. 2003. Performance and physiological parameters of broiler chickens subjected to fasting on the neonatal period. Poultry science 82: 1250-1256.

Grootaert, C. et al. 2007. Microbial metabolism and prebiotic potency of arabinoxylan oligosaccharides in the human intestine. Trends in Food Science & Technology 18: 64-71.

Heier, B., A. Lovland, K. Soleim, M. Kaldhusal, and J. Jarp. 2001. A field study of naturally occurring specific antibodies against Clostridium perfringens alpha toxin in Norwegian broiler flocks. Avian diseases: 724-732.

Helmboldt, C., and E. Bryant. 1971. The pathology of necrotic enteritis in domestic fowl. Avian diseases: 775-780.

Hofacre, C., T. Beacorn, S. Collett, and G. Mathis. 2003. Using competitive exclusion, mannan-oligosaccharide and other intestinal products to control necrotic enteritis. The Journal of Applied Poultry Research 12: 60-64.

Hofacre, C. L. et al. 1998. Use of Aviguard and other intestinal bioproducts in experimental Clostridium perfringens-associated necrotizing enteritis in broiler chickens. Avian Diseases 42: 579-584.

Hofshagen, M., and H. Stenwig. 1992. Toxin production by Clostridium perfringens isolated from broiler chickens and capercaillies (Tetrao urogallus) with and without necrotizing enteritis. Avian diseases: 837-843.

Hume, M. et al. 2003. Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. Poultry science 82: 1100-1107.

Huyghebaert. 2011. An update on alternatives to antimicrobial growth promoters for broilers. The Veterinary Journal 187: 182-188.

Huyghebaert, G., R. Ducatelle, and F. Van Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. The Veterinary Journal 187: 182-188.

Iji, P. A., A. A. Saki, and D. R. Tivey. 2001. Intestinal development and body growth of broiler chicks on diets supplemented with non-starch polysaccharides. Animal Feed Science and Technology 89: 175-188.

Inborr, J. 2000. Swedish poultry production without in-feed antibiotics - a testing ground or a model for the future. In: Australian Poultry Science Symposium, Sydney. p 1-9.

Izat, A. et al. 1990. Effects of a buffered propionic acid in diets on the performance of broiler chickens and on microflora of the intestine and carcass. Poultry science 69: 818-826.

Jack, R. W., J. R. Tagg, and B. Ray. 1995. Bacteriocins of gram-positive bacteria. Microbiological reviews 59: 171-200.

Jerzsele, A. et al. 2012. Efficacy of protected sodium butyrate, a protected blend of essential oils, their combination, and Bacillus amyloliquefaciens spore suspension against artificially induced necrotic enteritis in broilers. Poultry science 91: 837-843.

Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin. 1998. Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing lactobacillus cultures. Poultry Science 77: 1259-1265.

Joerger, R. 2003. Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. Poultry science 82: 640-647.

Jones, F. T., and S. C. Ricke. 2003. Observations on the History of the Development of Antimicrobials and Their Use in Poultry Feeds. Poultry Science 82: 613-617.

Kaldhusdal, M., M. Hofshagen, A. Løvland, H. Langstrand, and K. Redhead. 1999. Necrotic enteritis challenge models with broiler chickens raised on litter: evaluation of preconditions, Clostridium perfringens strains and outcome variables. FEMS Immunology & Medical Microbiology 24: 337-343.

Kaldhusdal, M., and A. Løvland. 2000a. The economical impact of Clostridium perfringens is greater than anticipated. World Poult 16: 50-51.

Kaldhusdal, M., and A. Løvland. 2000b. The economical impact of Clostridium perfringens is greater than anticipated. World Poultry 16: 50-51.

Kaldhusdal, M., C. Schneitz, M. Hofshagen, and E. Skjerve. 2001. Reduced incidence of Clostridium perfringens-associated lesions and improved performance in broiler chickens treated with normal intestinal bacteria from adult fowl. Avian diseases: 149-156.

Kaldhusdal, M., and E. Skjerve. 1996a. Association between cereal contents in the diet and incidence of necrotic enteritis in broiler chickens in Norway. Preventive Veterinary Medicine 28: 1-16.

Kaldhusdal, M., and E. Skjerve. 1996b. Association between cereal contents in the diet and the incidence of necrotic enteritis in broiler chickens in Norway. Preventive Veterinary Medicine 28: 1-16.

Kappel, L. C. et al. 2004. Beneficial Effects of Mannan Oligosaccharide on Diet Component Digestibility and Fermentation Characteristics in the Dog. Journal of Animal Science in press.

Keerqin, C. et al. 2017. An early feeding regime and a high-density amino acid diet on growth performance of broilers under subclinical necrotic enteritis challenge. Animal Nutrition.

Keyburn, A. L., T. L. Bannam, R. J. Moore, and J. I. Rood. 2010a. NetB, a pore-forming toxin from necrotic enteritis strains of Clostridium perfringens. Toxins 2: 1913-1927.

Keyburn, A. L. et al. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. PLoS pathog 4: e26.

Keyburn, A. L. et al. 2013. Vaccination with recombinant NetB toxin partially protects broiler chickens from necrotic enteritis. Veterinary research 44: 1.

Keyburn, A. L. et al. 2010b. Association between avian necrotic enteritis and Clostridium perfringens strains expressing NetB toxin. Veterinary research 41: 1-8.

Kharas, H. 2010. The emerging middle class in developing countries. OECD Development Centre Paris.

Kidd, M. 2004. Nutritional modulation of immune function in broilers. Poultry science 83: 650-657.

Kidd, M. et al. 2004. Increasing amino acid density improves live performance and carcass yields of commercial broilers. The Journal of Applied Poultry Research 13: 593-604.

Knarreborg, A., M. A. Simon, R. M. Engberg, B. B. Jensen, and G. W. Tannock. 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. Applied and Environmental Microbiology 68: 5918-5924.

Kum, S., U. Eren, A. Onol, and M. Sandikci. 2010. Effects of dietary organic acid supplementation on the intestinal mucosa in broilers. Rev. Med. Vet 10: 463-468.

La Ragione, R. M., G. Casula, S. M. Cutting, and M. J. Woodward. 2001. < i> Bacillus subtilis</i> spores competitively exclude< i> Escherichia coli</i> O78: K80 in poultry. Veterinary microbiology 79: 133-142.

La Ragione, R. M., and M. J. Woodward. 2003. Competitive exclusion by< i> Bacillus subtilis</i> spores of< i> Salmonella enterica</i> serotype Enteritidis and< i> Clostridium perfringens</i> in young chickens. Veterinary microbiology 94: 245-256.

Lacey, J. A., P. A. Johanesen, D. Lyras, and R. J. Moore. 2016. Genomic diversity of necrotic enteritis-associated strains of Clostridium perfringens: a review. Avian Pathology 45: 302-307.

Le Floc'h, N., D. Melchior, and C. Obled. 2004. Modifications of protein and amino acid metabolism during inflammation and immune system activation. Livestock Production Science 87: 37-45.
Lee, D.-H., Y.-G. Zo, and S.-J. Kim. 1996. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Applied and environmental microbiology 62: 3112-3120.
Lee, K., H. Lillehoj, W. Jeong, H. Jeoung, and D. An. 2011. Avian necrotic enteritis: experimental models, host immunity, pathogenesis, risk factors, and vaccine development. Poultry science 90: 1381-1390.
Lee, Y.-K., and S. Salminen. 1995. The coming of age of probiotics. Trends in Food Science & Technology 6: 241-245.
Lepp, D. et al. 2013. Identification of accessory genome regions in poultry Clostridium perfringens isolates carrying the netB plasmid. Journal of bacteriology 195: 1152-1166.
Li, P., Y.-L. Yin, D. Li, S. W. Kim, and G. Wu. 2007. Amino acids and immune function. British Journal of Nutrition 98: 237-252.
Lovland, A., M. Kaldhusdal, K. Redhead, E. Skjerve, and A. Lillehaug. 2004. Maternal vaccination against subclinical necrotic enteritis in broilers. Avian Pathology 33: 81-90.
Martin, T. G., and J. A. Smyth. 2010. The ability of disease and non-disease producing strains of Clostridium perfringens from chickens to adhere to extracellular matrix molecules and Caco-2 cells. Anaerobe 16: 533-539.
Mathew, A. G. 2003. Development of Antibiotic Resistance in Livestock Production. In: 64th Minnesota Nutrition Conference, St. Paul, Minnesota, USA. p 1-15.
McDevitt, e. a. 2006. Necrotic enteritis; a continuing challenge for the poultry industry. World's Poultry Science Journal 62: 221-247.
Milbradt, E. et al. 2014. Use of organic acids and competitive exclusion product as an alternative to antibiotic as a growth promoter in the raising of commercial turkeys. Poultry science: PS3593.
Miller, R. W., J. Skinner, A. Sulakvelidze, G. F. Mathis, and C. L. Hofacre. 2010. Bacteriophage therapy for control of necrotic enteritis of broiler chickens experimentally infected with Clostridium perfringens. Avian diseases 54: 33-40.
Moore, P. R. A. et al. 1946. Use of sulfasuxidine, streptothricin and streptomycin in natural studies with the chick. Journal of Biological Chemistry 174: 1047-1048.
Moore, R. J. 2016. Necrotic enteritis predisposing factors in broiler chickens. Avian Pathology: 1-22.
Moran, E., and B. Reinhart. 1980. Poult yolk sac amount and composition upon placement: effect of breeder age, egg weight, sex, and subsequent change with feeding or fasting. Poultry science 59: 1521-1528.
Morishita, T. Y., P. P. Aye, B. S. Harr, C. W. Cobb, and J. R. Clifford. 1997. Evaluation of an avian-specific probiotic to reduce the colonization and shedding of Campylobacter jejuni in broilers. Avian diseases: 850-855.
Myers, G. S. et al. 2006. Skewed genomic variability in strains of the toxigenic bacterial pathogen, Clostridium perfringens. Genome Research 16: 1031-1040.
Newman, M. 2003. Effects of Antibiotic Withdrawal on Resistance. In: Mid-West Swine Nutrition Conference, Indianapolis, Indiana, USA. p 33-41.
Niewold, T. A., M. Schroyen, M. M. Geens, R. S. Verhelst, and C. M. Courtin. 2012. Dietary inclusion of arabinoxylan oligosaccharides (AXOS) down regulates mucosal responses to a bacterial challenge in a piglet model. Journal of Functional Foods 4: 626-635.
Parish, W. E. 1961. Necrotic enteritis in the fowl (Gallus gallus domesticus). I. Histopathology of the disease and isolation of the strain Clostridium welchii. Journal of Comparative Pathology 71: 377-393.
Parisien, A., B. Allain, J. Zhang, R. Mandeville, and C. Lan. 2008. Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. Journal of applied microbiology 104: 1-13.

Patterson, J., and K. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poultry science 82: 627-631.
Pattison, M. 2002. Some clinical and pathological features of enteritis in broilers - Observations on treatment in the UK. In: The Elanco Global Enteritis Symposium, Cambridge. p C1-C10.
Phillips, I. 1999. Assessing the evidence that antibiotic growth promoters influence human infections. Journal of Hospital Infection 43: 173-178.
Pluske, J. 2001. Nutritional management of the gastrointestinal tract to reduce enteric diseases in pigs. In: Recent Advances in Animal Nutrition in Australia, Armidale. p 127-134.
Postma, J., P. Ferket, W. Croom, and R. Kwakkel. 1999. Effect of Virginiamycin on intestinal characteristics of turkeys.
Prescott, J. 1979a. The prevention of experimentally induced necrotic enteritis in chickens by avoparcin. Avian diseases: 1072-1074.
Prescott, J., R. Sivendra, and D. Barnum. 1978. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. The Canadian Veterinary Journal 19: 181.
Prescott, J. F. 1979b. The prevention of experimentally induced necrotic enteritis in chickens by avoparcin. Avian Diseases 23: 1072-1074.
Revington, B. 2002. Feeding Poultry in the Post-Antibiotic Era. In: 2002 Multi-State Poultry Feeding and Nutrition Conference, Indianapolis, Indiana, USA,. p 1-14.
Riddell, C., and X.-M. Kong. 1992a. The influence of diet on necrotic enteritis in broiler chickens. Avian diseases: 499-503.
Riddell, C., and X. M. Kong. 1992b. The influence of diet on necrotic enteritis in broiler chickens. Avian Diseases 36: 499-503.
Rinkinen, M., K. Jalava, E. Westermarck, S. Salminen, and A. C. Ouwehand. 2003. Interaction between probiotic lactic acid bacteria and canine enteric pathogens: a risk factor for intestinal Enterococcus faecium colonization? Veterinary Microbiology 92: 111-119.
Rodgers, N. J. et al. 2014. A Multifactorial Analysis of the Extent to Which Eimeria and Fishmeal Predispose Broiler Chickens to Necrotic Enteritis. Avian diseases 59: 38-45.
Rodgers, N. J. et al. 2015. A Multifactorial Analysis of the Extent to Which Eimeria and Fishmeal Predispose Broiler Chickens to Necrotic Enteritis. Avian diseases 59: 38-45.
Sacranie, A. 2007. Dietary and age regulation of antiperistalsis in broiler chickens. University of New England.
Saini, H. S. 1989. Legume seed oligosaccharides. In: Recent Advances of Research in Antinutritional Factors in Legume Seeds, Wageningen. p 329-341.
Schaffer, D. A. 2004. Food Safty and antibiotics. In: 2004 Poultry Information Exchange, Surfers paradise, Australia. p 13-17.
Seal, B. S. 2013. Characterization of bacteriophages virulent for Clostridium perfringens and identification of phage lytic enzymes as alternatives to antibiotics for potential control of the bacterium1. Poultry science 92: 526-533.
Shojadoost, B., A. R. Vince, and J. F. Prescott. 2012. The successful experimental induction of necrotic enteritis in chickens by Clostridium perfringens: a critical review. Vet Res 43: 74.
Simon, O., A. Jadamus, and W. Vahjen. 2001. Probiotic feed additives - effectiveness and expected modes of action. Journal of Animal and Feed Sciences 10: 51-67.
Skinner, J. T., S. Bauer, V. Young, G. Pauling, and J. Wilson. 2010. An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. Avian Dis 54: 1237-1240.
Smith, L. C. 1998. Can FAO's measure of chronic undernourishment be strengthened? Food Policy 23: 425-445.
Stanley, D., A. L. Keyburn, S. E. Denman, and R. J. Moore. 2012. Changes in the caecal microflora of chickens following Clostridium perfringens challenge to induce necrotic enteritis. Veterinary microbiology 159: 155-162.

Stanley, D., S.-B. Wu, N. Rodgers, R. A. Swick, and R. J. Moore. 2014. Differential responses of cecal microbiota to fishmeal, Eimeria and Clostridium perfringens in a necrotic enteritis challenge model in chickens. PLoS One 9: e104739.

Swann Committee. 1969. Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine Report (Cmnd. 4190), Her Majesty's Stationary Office, London.

Tannock, G. W. 1997. Modification of the normal microbiota by diet, stress, antimicrobial agents, and probiotics. Gastrointestinal microbiology 2: 434-465.

Tech, R. 1999. Necrotic enteritis and associated conditions in broiler chickens. World Poultry 15: 44-47.

Thompson, D., V. Parreira, R. Kulkarni, and J. Prescott. 2006. Live attenuated vaccine-based control of necrotic enteritis of broiler chickens. Veterinary microbiology 113: 25-34.

Thompson, J. L., and M. Hinton. 1997. Antibacterial activity of formic and propionic acids in the diet of hens on Salmonellas in the crop. British poultry science 38: 59-65.

Timbermont, e. a. 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathology 40: 341-347.

Timbermont, L., F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathology 40: 341-347.

Timbermont, L. et al. 2009. Origin of< i> Clostridium perfringens</i> isolates determines the ability to induce necrotic enteritis in broilers. Comparative immunology, microbiology and infectious diseases 32: 503-512.

Truscott, R. B., and F. Al-Sheikhly. 1977. Reproduction and treatment of necrotic enteritis in broilers. American Journal of Veterinary Research 38: 857-861.

Tuomola, E., R. Crittenden, M. Playne, E. Isolauri, and S. Salminen. 2001. Quality assurance criteria for probiotic bacteria. The American journal of clinical nutrition 73: 393s-398s.

Vahjen, W., K. Glaeser, K. Schaefer, and O. Simon. 1998. Influence of xylanase-supplemented feed on the development of selected bacterial groups in the intestinal tract of broiler chicks. Journal of Agricultural Science 130: 489-500.

Van der Sluis, W. 2000a. Clostridial enteritis is an often underestimated problem. World Poultry 16: 42-43.

van der Sluis, W. 2000b. Necrotic enteritis (1): Clostridial enteritis a syndrome emerging worldwide. World Poultry 16: 56-57.

van der Sluis, W. 2000c. Necrotic enteritis (3): Clostridial enteritis is an often underestimated problem. World Poultry 16: 42-43.

Van Immerseel, F. et al. 2004. Microencapsulated short-chain fatty acids in feed modify colonization and invasion early after infection with Salmonella enteritidis in young chickens. Poultry science 83: 69-74.

Van Immerseel, F., J. I. Rood, R. J. Moore, and R. W. Titball. 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in microbiology 17: 32-36.

Van Immerseel, F. et al. 2006. The use of organic acids to combat Salmonella in poultry: a mechanistic explanation of the efficacy. Avian Pathology 35: 182-188.

Wade, B., and A. Keyburn. 2015. The true cost of necrotic enteritis. World Poult 31: 16-17.

Waititu, S. M. et al. 2014. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poultry science 93: 625-635.

Watkins, K. L., T. R. Shryock, R. N. Dearth, and Y. M. Saif. 1997. In-vitro antimicrobial susceptibility of clostridium perfringens from commercial turkey and broiler chickens. Veterinary Microbiology 54: 195-200.

Wenk, C. 2003. Growth promoter alternatives after the ban on antibiotics. Pig news and information 24: 11N-16N.

Williams, R. 2002. Anticoccidial vaccines for broiler chickens: pathways to success. Avian Pathology 31: 317-353.

Williams, R., R. Marshall, R. La Ragione, and J. Catchpole. 2003. A new method for the experimental production of necrotic enteritis and its use for studies on the relationships between necrotic enteritis, coccidiosis and anticoccidial vaccination of chickens. Parasitology research 90: 19-26.

Williams, R. B. 1992. Differences between the anticoccidial potencies of monensin in maize-based or wheat-based chicken diets. Veterinary Research Communications 16: 147-152.

Wu, S.-B., N. Rodgers, and M. Choct. 2010. Optimized necrotic enteritis model producing clinical and subclinical infection of Clostridium perfringens in broiler chickens. Avian diseases 54: 1058-1065.

Wu, S.-B., D. Stanley, N. Rodgers, R. A. Swick, and R. J. Moore. 2014. Two necrotic enteritis predisposing factors, dietary fishmeal and Eimeria infection, induce large changes in the caecal microbiota of broiler chickens. Veterinary microbiology 169: 188-197.

Yang, Y., P. Iji, and M. Choct. 2009. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. World's Poultry Science Journal 65: 97-114.

Yearbook, F. S. 2012. World food and agriculture. Rome: Food and Agriculture Organization of the United Nations.

Ziggers, D. 2001. TOS, a new prebiotic derived from whey. Feed Tech 5: 34-36.

CHAPTER 2

An early feeding regime and a high-density amino acid diet on growth performance of broilers under subclinical necrotic enteritis challenge

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2.1 Abstract

Broilers that have early access to feed have been shown to have enhanced immune system and gut development and heightened resilience against necrotic enteritis (NE). This study examined the effect of early feeding a high amino acid density diet on performance of broilers under a sub-clinical NE challenge model. Ross 308 broilers (n = 576) were assigned to a 2 × 2 × 2 factorial design with 2 feeding regimes (feed access either within 6 h post-hatch or after 48 h post-hatch), 2 diets (control diet or the control diet with an additional 10% highly digestible amino acids (HAA) and either presence or absence of NE challenge. Oral administrations of *Eimeria* species (d 9) and a field strain of *Clostridium perfringens* (d 14) were used to induce NE. Broiler performance was analysed on bird age d 13, 23, 30 and 35. Intestinal lesion score and bacteria count were analysed on d 16. The NE challenge reduced overall bird performance and induced severe intestinal lesions, without causing notable mortality. At d 23 bird weight was significantly lower ($P < 0.001$) in the challenged birds compared with the unchallenged birds, but by d 30 the challenged birds had recovered, and challenge no longer had an impact on bird performance. Birds fed the HAA diet had greater body weight by d 35 and heightened *Lactobacillus* content in the ileum at d 16 ($P < 0.05$). Birds that were fed the HAA diet after a period of fasting post-hatch performed better in terms of feed conversion ratio (FCR) performance under the challenge effect. The findings from this study suggest there are beneficial effects of feeding high amino acid diets to birds in response to external stresses, such as post-hatch fasting and subclinical NE.

Keywords: Early feeding, amino acid; necrotic enteritis; broiler chicken

2.2 Introduction

Necrotic enteritis (NE) is a multifactorial, bacterial borne enteric disease that causes devastating loss to poultry flocks (up to 30% mortality in an infected flock), costing approximately US$2 billion per annum worldwide (Dahiya et al., 2006; Van Immerseel et al., 2009; Wade and Keyburn, 2015). The causative agent is *Clostridium perfringens*; a Gram-positive, anaerobic, rod spore-forming bacterium that is ubiquitous and is capable of producing a variety of extracellular toxins and invasive enzymes (Gibert et al., 1997;

Keyburn et al., 2010a). Its pathogenic state can be triggered from predisposing factors that disturb the integrity of the gut mucosa; such as increased viscosity of intestinal digesta, high level of protein in the diet and coccidiosis infection (Collier et al., 2008; Rodgers et al., 2014). The clinical form of NE results in significant necrosis of the small intestine and hence catastrophic levels of mortality (Kaldhusdal and Løvland, 2000a; Van Immerseel et al., 2009). The subclinical form exhibits less obvious signs, with initial symptoms of just slightly reduced growth, due to impaired nutrient utilisation from the damaged intestinal mucosa. As a result, there is often delayed the onset of effective treatment and spreading amongst the flock, resulting in a substantial loss to production (Van der Sluis, 2000a; Skinner et al., 2010).

Clostridium perfringens strains have been shown to be responsive to various anti-microbial drugs in both *in-vitro* and *in vivo* studies (Devriese et al., 1993; Geier et al., 2010). However, worldwide concerns about the emergence of antimicrobial resistant bacteria strains mean that alternatives to in-feed antibiotics in animal production, that can both combat bacterial infections and replace antibiotic growth promotors, are desperately being sought after (Choct, 2001; Wenk, 2003; Dibner and Richards, 2005). Replacements for antibiotics that can reduce the prevalence of *C. perfringens* induced NE must, therefore, be established.

Bacterial pathogens in the gut thrive in the presence of unbalanced feed compositions. For example, a diet with high crude protein or soluble non-starch polysaccharides content causes increased gastrointestinal transit time, which induces mass proliferation of opportunistic *C. perfringens* in the microflora (Choct and Annison, 1992; Riddell and Kong, 1992a; Tech, 1999; Annett et al., 2002; Wu et al., 2014; Moore, 2016). A refined nutritional formula that makes the broiler gut environment less susceptible to *C. perfringens* domination is, therefore, critical for maintaining optimal health and performance in broiler chickens and reducing production costs (Croom et al., 2000; Dahiya, 2006; Moore, 2016; Van Immerseel et al., 2009).

The amount of time between the hatch and access to feed has been shown to greatly influence survivability and performance in young birds (Geyra et al., 2001). Due to the nature of the broiler industry, birds often do not get access to water and feed for up to 48

h post-hatch (Dibner et al., 1998). Early nutrient availability, especially access to essential amino acids, could play a significant role in immune development (Kidd, 2004; Li et al., 2007). Ao et al. (2012) reported that birds that had early access to feed and water post-hatch had improved immune development and better performance in the presence of NE than birds that had been fasted for 48 h post-hatch. Essential amino acids have been shown to be important components in the development of the immune system in birds, and amino acid utilisation is prioritised towards tissues involved in immune response and inflammation (Li et al., 2007) Le Floc'h et al. (2004). Kidd et al. (2004) reported that healthy broilers responded positively to high dietary amino acid inclusion, and the positive effect on performance was more evident in birds exposed to the dietary treatment immediately post-hatch. The impact of amino acid supplementation in young broilers is still however under debate, and the relationship between amino acids and NE is poorly understood. The aim of this study was to examine the benefit of early access to feed and of feeding starter diets with a high amino acid density on the response of birds to NE challenge.

2.3 Materials and methods

2.3.1 Experimental design and feeding treatments

A total of 576 day-old male broiler chickens (Ross 308) were procured from Baiada Country Road Hatchery (Tamworth, NSW, Australia) at the day of hatch. Chicks were randomised by weight and placed in floor pens (approx. area 120 × 75 cm^2 per pen), bedded on clean wood shavings. Birds were vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease at the hatchery before arrival. The lighting regimen used was 23 h, 18 h and 23 h of light during days 0 to 24, 25 to 30 and 31 to 35, respectively. The floor bedding temperature was maintained at 34 to 35 °C from d 0 to 3 and was then gradually decreased by 3 °C per week onward until 22 to 24 °C was reached by d 21. The rearing facility at the University of New England meets the Australian standards, and the area was sterilised before bird arrival. All experimental procedures involved in this study were approved by the Animal Ethics Committee of the University of New England. The study had a 2 × 2 × 2 factorial design, resulting in 8 treatments; 2

feeding regimes of either immediate access to feed (FED) or access to feed delayed by 48 h post-hatch (HELD), 2 starter diets with (control) or without amino acid density 10% above the recommended level (HAA) and either exposed to NE challenge or not (Challenged or Unchallenged). Birds were allocated to 48 pens with 12 birds per pen and 6 replicates per treatment (72 birds/treatment). Birds were evenly distributed to ensure that there were no statistical differences between initial starting pen weights.

Birds in the FED treatment group had *ad libitum* access to feed on arrival (within 6 h post-hatch), whereas the birds in the HELD treatment group had access to only water on arrival and feed was then introduced after 48 h post-hatch. The starter diets were fed as crumble until d 7 and then as pellets (⌀2 to 3 mm). The diets were wheat, sorghum, meat meal and soybean based and were formulated based on Aviagen Ross 308 (2012) nutritional specification guidelines. The HAA starter diet was the same as the control diet but with an additional 10% digestible amino acids (Table 2.1). From d 13 all birds were fed the same grower diet until d 24 and then the same finisher diet from d 24 to d 35, both fed as pellets (⌀3 to 3.5mm).

Table 2.1 Starter diet formulation and nutrient composition

Item	Control	HAA
Ingredient, %		
Wheat	30	30
Sorghum	26.6	21
Soy Bean Meal	30.1	36.1
Canola meal solvent extracted	3	1
Meat and bone meal	2	3.8
Canola oil	4.3	4.7
Limestone	1.2	1
Dical Phos 18P/21Ca	1.476	1.089
NaCl	0.128	0.101
Na bicarb	0.2	0.2
UNE vitamin premix[1]	0.05	0.05
UNE trace mineral premix[2]	0.075	0.075
Choline Cl 70%	0.038	0.045
Analysed composition, %		
ME, kcal/kg	3,025	3,027
Crude protein	22.94	25.34
Crude fat	6.21	6.65
Crude fibre	3.00	3.03
Digestible Amino acids		
Isoleucine	1.01	1.11
Arginine	1.34	1.52
L-lysine	1.27	1.40
DL-methionine	0.60	0.67
Methionine and cystine	0.94	1.03
Tryptophan	0.23	0.26
L-threonine	0.83	0.91
Valine	0.94	1.03

HAA = high amino acid starter diet (10% more essential amino acid over the Aviagen recommendations); UNE = University of New England. [1] Vitamin premix per kg contains: vitamin A, 12 MIU; vitamin D, 5 MIU; vitamin E, 75 mg; vitamin K, 3 mg; nicotinic acid, 55 mg; pantothenic acid, 13 mg; folic acid, 2 mg; riboflavin, 8mg; cyanocobalamin, 0.016 mg; biotin, 0.25 mg; pyridoxine, 5 mg; thiamine, 3 mg; antioxidant, 50 mg. [2] Mineral premix per kg contains: Cu, 16 mg as copper sulphate; Mn, 60.

2.3.2 Necrotic enteritis challenge

On day 9, each bird in the NE-challenge group was given 1 mL *per os* vaccine strain of *Eimeria* (Bioproperties Pty Ltd., Sydney, Australia). Each 1 mL gavage included phosphate buffered saline (PBS) suspension of approximately 5,000 oocysts each of *E. acervulina* and *E. maxima*, and 2,500 oocysts of *E. brunetti*. To the unchallenged group, 1 mL of sterile PBS was administered as a stress treatment. On d 14 and 15, a field strain of *C. perfringens* EHE-NE18 producing a netB toxin (approx. 10^8 CFU/mL) (CSIRO

Livestock Industries, Geelong, Victoria, Australia) in 2 mL thioglycolate broth was administered *per os* per bird. A sterile thioglycollate broth was administered to the non-challenged group. The *C. perfringens* count in the inoculant had been quantified on *perfringens* tryptose-sulfite-cy-closerine (TSC) selective agar (Oxoid) following serial dilutions.

2.3.3 Parameters analysed

The parameters analysed in the present study were bird weight, feed intake, feed conversion ratio, intestinal lesion score and intestinal bacteria count.

2.3.4 Bird weight, feed intake and feed conversion ratio

Pen weight and cumulative pen feed intake were recorded on days 0, 13, 23, 30 and 35 and used to calculate mean bird weight, feed intake and FCR (corrected for mortality).

2.3.5 Post-mortem and lesion scoring

On day 16, 2 birds were randomly selected from each pen and euthanised by cervical dislocation. The duodenal loop, the jejunum (from the end of the duodenal loop to the Meckel`s diverticulum) and the ileum (from the Meckel`s diverticulum to the ileo-ceaco-colonic junction) were excised. Caecal and ileal digesta were collected into sterile 30 mL sample tubes, one tube per bird per section of the gastrointestinal tract. The entire length of the section of small intestine underwent a lesion scoring process, based on a previously reported lesion scoring system that ranges from 0 to 4 (Prescott et al., 1978; Broussard et al., 1986). Score 0 referred to intestine of healthy appearance, 1 referred to gas-filled intestine with evidence of at least two necrotic lesions, 2 referred to ballooned, friable, foul-smelling intestine with evidence of necrotic lesions, 3 referred to intestines that displayed all the above along with a yellow pseudomembrane (often described as having a “Turkish towel” or flannelette blanket-like appearance) and 4 referred to prevalence of above description with ruptures of the intestinal epithelial layer, blood filled intestine or multiple petechial haemorrhages. Three experienced personnel, with no knowledge of the trial design, were involved in the scoring process.

2.3.6 Enumeration of bacteria using quantitative PCR

To quantitatively measure intestinal bacterial population, a section of the ileum (approximately 3 cm long), directly adjacent to the Meckel`s diverticulum, was excised. And 1 g of digesta from this section of ileum was aseptically transferred into a 2 mL Eppendorf safe-lock tube, snap-frozen in liquid nitrogen and stored at -20 °C until DNA extraction was performed. PCR amplification of 16S ribosomal DNA was used to determine the chromosomal DNA counts of the subject bacterium. Total gut bacterium, *Lactobacillus* spp., *C. perfringens* and *Enterobacteria* spp. were quantified. Template DNA samples were prepared from the ileal digesta using Bio line Isolate II Plant DNA Kit. Approximately 200 mg of ileal digesta was accurately weighed and vigorously shaken with 0.2 g of ⌀0.1 mm glass beads prior to the extraction step. For DNA preparation of the caecal digesta, 60 mg of caecal digesta was processed by a Qiaxtractor automated DNA extractor robot (Qiagen, Australia). A NanoDrop ND-8000 UV spectrophotometer was used to assess the DNA purity (Thermo Fisher Scientific, Waltham, USA). Only DNA elutions emitting ratios of between 1.6 and 1.8 in 260/280 nm wavelength were used in the following analysis. The quantitative PCR analysis was performed on a Rotorgene-6500 real-time PCR machine (Corbett, Sydney, Australia). A total volume of 10 µL was used in each PCR reaction, with duplicate reactions of each sample. SensiMix SYBR No-ROX (Bioline, Meridian Life Science, Memphis, USA) was used to amplify the 16S ribosomal DNA for analysis of the total bacteria, *Lactobacillus* spp. and *Enterobacteria* spp. The annealing primers involved were maintained at a concentration of 300 nm (total bacteria: 5'-CGG YCC AGA CTC CTA CGG G-3' and 5'-TTA CCG CGG CTG CTG GCA C-3'; *Lactobacillus*: 5'-CAC CGC TAC ACA TGG AG-3' and 5'-AGC AGT AGG GAA TCT TCC A-3'; *Enterobacteria*: 5'-CAT TGA CGT TAC CCG CAG AAG-3' and 5'-CTC TAC GAG ACT CAA G-3';) (Lee et al., 1996; Bartosch et al., 2004). SensiFAST SYBR NO-ROX (Bioline, Meridian Life Science, Memphis, USA) and TaqMan probe were used in the qPCR assay to quantify 16S ribosomal DNA of *C. perfringens* (5'-GCA TAA CGT TGA AAG ATG G-3' and 5'-CCT TGG TAG GCC GTT ACC C-3'). Serial dilutions of linearised plasmid DNA (pCR4-TOPO Vector, Life Technologies, Carlsbad, USA) inserted with respective amplicons were used to construct a standard curve. A threshold cycle average from the replicate samples was assigned for quantification analysis. The number of target DNA copies was calculated from the mass of the DNA,

taking into account the size of the amplicon insert in the plasmid. Mean value of replicates were calculated. While the bacterial numbers were expressed as $\log_{10}$ genomic DNA copy number per gram of digesta (wet weight).

2.3.7 Statistical analysis

All data were analysed using the JMP 11 statistical programme (SAS Institute Inc. Cary, NC). FCR calculations took into account the weights of sample birds and mortalities. ANOVA analysed data with treatment as the individual variance. When differences among variances were significant, Duncan's multiple range test was then used to compare the means. Statistical significance was declared at $P \leq 0.05$.

2.4 Results

2.4.1 Bird performance

Total mortality in this trial was 3.5%, but none of the observed mortalities was induced by the presence of the NE challenge, based on necropsy analysis. As illustrated in Table 2.2, birds fed the HAA diet had significantly improved BW at d 13 ($P < 0.001$), d 30 ($P = 0.029$) and d 35 ($P = 0.005$) compared with birds fed the control diet. An interaction between challenge and regime was observed at d 13 ($P < 0.001$), showing that the challenged birds fed immediately post-hatch had the heaviest body weights, followed by the unchallenged birds that were fasted post-hatch. At d 30, birds offered the diets immediately post-hatch had lower body weight ($P = 0.007$) compared with those that were not offered feed until 48 h post-hatch. Birds from the unchallenged group were well feathered, whereas birds that were subjected to the NE challenge had ruffled feathers. A regime × challenge interaction suggested that FED and challenged combination effect produced the heaviest BW while the FED and the non-challenge combination produced the lightest BW (data not shown). Significant interactions between regime and diet ($P = 0.014$) and challenge and diet ($P = 0.045$) were observed at d 23. Birds fed the HAA diet after fasting or the control diet immediately post-hatch had heavier body weights compared with the other treatments, and birds fed the control diet after fasting had the lowest average body weight. By d 30, the effect of NE challenge on BW was diminished.

At d 35, the main effect of HAA diet yielded higher body weights compared with those of the control diet. Also a significant ($P = 0.020$) full factorial interaction between challenge, regime and diet were observed at d 35. This interaction showed that body weight was lower in the unchallenged birds fed the control diet immediately post-hatch compared with the unchallenged birds fed either diet after fasting, the challenged birds fed the HAA diet after fasting or control diet immediately post-hatch.

Table 2.3 illustrates that challenge had a significant impact on feed intake at d 13 ($P = 0.028$), d 23 ($P < 0.001$) and d 30 ($P < 0.001$). There was also a full factorial interaction between challenge, diet and regime on feed intake at d 35, showing that feed intake was highest in the challenged birds fed the HAA diet immediately post-hatch and the control diet after fasting, and was lowest in the unchallenged birds fed the control diet. Birds that were fed immediately post-hatch had higher feed intake at d 13 compared with birds that were fasted 48 h ($P < 0.001$). At d 23, there was an interaction between regime and diet ($P = 0.044$) which showed that feed intake was greater in birds fed the control diet compared with the HAA diet when birds were fed immediately post-hatch, but when birds were fasted feed intake was comparatively greater in birds fed the HAA diet (data not shown).

Interactions between challenge and regime on feed conversion were observed at d 13 ($P < 0.001$) which showed that when birds were fed immediately post-hatch, FCR was significantly higher in the unchallenged birds compared with the challenged birds, but there was no significant difference between the challenged and unchallenged birds when they were fasted post-hatch. At d 23 there was also an interaction between challenge and regime on feed conversion ($P = 0.042$), illustrating that FCR was lowest in the unchallenged birds that were fasted and was significantly higher in the challenged birds compared with the unchallenged birds. At d 30 challenge ($P < 0.001$) and regime ($P < 0.001$) had a significant effect on FCR, showing that FCR was higher in the challenged birds compared with the unchallenged birds, and was higher in birds that were fed immediately post-hatch compared with those that were fasted. A full factorial interaction ($P = 0.037$) was detected at d 35, showing that FCR was higher in the challenged birds compared with the unchallenged birds and that the challenged birds fed

the HAA diet after fasting had lower FCR than the challenged birds fed the control diet after fasting or HAA diet immediately post-hatch.

Table 2.2 Interaction of necrotic enteritis challenge, feeding regime and diet on broiler body weight.[1]

Treatments			D 13	D 23	D 30	D 35
Unchallenged	HELD	Control	489.0 ± 8.2	1301.0 ± 18.4	2022.3 ± 31.2	2586.2 ± 44.0 ^{abc}
		HAA	518.8 ± 15.4	1375.2 ± 15.3	2103.8 ± 29.8	2667.7 ± 39.0 ^{abc}
	FED	Control	464.2 ± 16.6	1221.5 ± 49.3	1883.8 ± 74.8	2434.8 ± 75.0 ^{d}
		HAA	492.0 ± 12.4	1282.5 ± 35.6	1989.2 ± 57.1	2624.0 ± 54.1 ^{abc}
Challenged	HELD	Control	476.8 ± 8.7	1192.8 ± 16.4	1961.2 ± 23.5	2494.7 ± 45.8 ^{cd}
		HAA	513.2 ± 2.8	1183.7 ± 15.5	2110.2 ± 31.2	2685.2 ± 29.4 ^{abc}
	FED	Control	563.5 ± 16.6	1218.0 ± 24.3	2012.5 ± 28.3	2585.5 ± 42.3 ^{abc}
		HAA	594.5 ± 12.5	1194.3 ± 34.9	1958.7 ± 49.2	2540.3 ± 56.1 ^{bcd}
Mean of main effects						
Unchallenged			490.2 ± 6.2^{b}	1293.1 ± 15.0^{a}	2000.9 ± 23.7	2578.1 ± 26.4
Challenged			542.9 ± 6.7^{a}	1196.8 ± 16.1^{b}	2011.0 ± 25.4	2560.0 ± 28.3
HELD			501.2 ± 6.2^{b}	1270.0 ± 15.0	2062.7 ± 23.7^{a}	2613.6 ± 26.4
FED			531.9 ± 6.7^{a}	1219.9 ± 16.1	1949.3 ± 25.4^{b}	2524.5 ± 28.3
CONTROL			504.9 ± 6.9^{b}	1239.1 ± 16.5	1983.4 ± 26.1^{b}	2524.3 ± 29.1^{b}
HAA			528.2 ± 6.0^{a}	1250.8 ± 14.5	2028.6 ± 23.0^{a}	2613.9 ± 25.6^{a}
P = values						
Challenge			< 0.001	< 0.001	0.729	0.961
Regime			0.002	0.101	0.007	0.085
Diet			0.001	0.215	0.029	0.005
Challenge × Regime			< 0.001	0.014	0.226	0.324
Challenge × Diet			0.786	0.045	0.466	0.380
Regime × Diet			0.837	0.735	0.158	0.370
Challenge × Regime × Diet			0.925	0.987	0.076	0.020

HAA = high amino acid starter diet (10% more essential amino acid over the Aviagen recommendations); FED = access to feed with 6 h post-hatch; HELD = access to feed after 48 h post-hatch; Challenge = necrotic enteritis induced by oral administrations of Eimeria species and a field strain of *Clostridium perfringens*.

[a, b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

[1] Means represent the average individual body weight of 12 birds per pen, 6 pens per treatment (72 birds/treatment).

Table 2.3 Interaction of necrotic enteritis challenge, feeding regime and diet on broiler feed intake.[1]

Treatments			D13	D 23	D 30	D 35
Unchallenged	HELD	Control	512.0 ± 8.1	1800.2 ± 37.7	2851.8 ± 38.2	3741.3 ± 31.4^{cd}
		HAA	519.0 ± 6.9	1932.8 ± 32.5	3020.5 ± 37.7	3960.2 ± 51.1^{c}
	FED	Control	539.3 ± 22.7	1815.2 ± 71.8	2785.7 ± 116.9	3570.7 ± 123.8^{d}
		HAA	551.2 ± 14.1	1903.2 ± 38.2	2968.3 ± 46.0	3893.0 ± 86.5^{c}
Challenged	HELD	Control	519.2 ± 9.2	2008.8 ± 18.6	3048.8 ± 33.4	4216.7 ± 58.1^{b}
		HAA	530.7 ± 1.5	2137.8 ± 42.5	3228.7 ± 50.3	4476.7 ± 64.5^{a}
	FED	Control	581.8 ± 13.6	2226.7 ± 108.4	3294.7 ± 105.3	4529.2 ± 156.7^{a}
		HAA	569.8 ± 11.6	2079.7 ± 28.8	3135.7 ± 53.4	4328.2 ± 58.1^{ab}
Main effects						
Unchallenged			531.5 ± 6.6^{b}	1868.7 ± 22.3^{b}	2910.9 ± 32.4^{b}	3783.5 ± 37.5^{b}
Challenged			545.9 ± 7.0^{a}	2084.2 ± 23.9^{a}	3148.6 ± 34.7^{a}	4349.8 ± 40.2^{a}
HELD			521.9 ± 6.6^{b}	1982.3 ± 22.3	3053.1 ± 32.4	4117.3 ± 37.5
FED			555.5 ± 7.0^{a}	1970.6 ± 23.9	3006.4 ± 34.7	4016.1 ± 40.2
Control			534.6 ± 7.2	1944.1 ± 24.5	2981.3 ± 35.6	3989.6 ± 41.3^{b}
HAA			542.9 ± 6.4	2008.8 ± 21.5	3078.1 ± 31.3	4143.8 ± 36.3^{a}
P-values						
Challenge			0.028	< 0.001	< 0.001	< 0.001
Regime			< 0.001	0.353	0.857	0.768
Diet			0.605	0.197	0.058	0.021
Challenge × Regime			0.235	0.265	0.162	0.114
Challenge × Diet			0.585	0.130	0.090	0.060
Regime × Diet			0.598	0.044	0.096	0.159
Challenge × Regime × Diet			0.425	0.142	0.071	0.029

HAA = high amino acid starter diet (10% more essential amino acid over the Aviagen recommendations); FED = access to feed with 6 h post-hatch; HELD = access to feed after 48 h post-hatch; Challenge = necrotic enteritis induced by oral administrations of Eimeria species and a field strain of *Clostridium perfringens*.

[a, b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

[1] Means represent the average individual body weight of 12 birds per pen, 6 pens per treatment (72 birds/treatment).

Table 2.4 Interaction of necrotic enteritis challenge, feeding regime and diet on broiler feed conversion.1

Treatments			D 13	D 23	D 30	D 35
Unchallenged	HELD	Control	1.052 ± 0.027	1.392 ± 0.038	1.406 ± 0.019	1.448 ± 0.017^{c}
		HAA	1.006 ± 0.022	1.405 ± 0.031	1.436 ± 0.016	1.485 ± 0.014^{c}
	FED	Control	1.161 ± 0.022	1.488 ± 0.031	1.478 ± 0.016	1.466 ± 0.014^{c}
		HAA	1.135 ± 0.024	1.507 ± 0.034	1.507 ± 0.017	1.473 ± 0.015^{c}
Challenged	HELD	Control	1.085 ± 0.024	1.685 ± 0.034	1.553 ± 0.017	1.709 ± 0.015^{a}
		HAA	1.035 ± 0.022	1.808 ± 0.031	1.530 ± 0.016	1.667 ± 0.014^{b}
	FED	Control	0.963 ± 0.031	1.732 ± 0.043	1.578 ± 0.022	1.702 ± 0.019^{ab}
		HAA	0.961 ± 0.024	1.750 ± 0.034	1.607 ± 0.017	1.724 ± 0.015^{a}
Main effects						
Unchallenged			1.088 ± 0.012^{a}	1.448 ± 0.017^{b}	1.457 ± 0.008^{b}	1.468 ± 0.007^{b}
Challenged			1.011 ± 0.013^{b}	1.744 ± 0.018^{a}	1.567 ± 0.009^{a}	1.701 ± 0.008^{a}
HELD			1.044 ± 0.012	1.573 ± 0.017	1.481 ± 0.008^{b}	1.577 ± 0.007
FED			1.055 ± 0.013	1.619 ± 0.018	1.543 ± 0.009^{a}	1.591 ± 0.008
Control			1.065 ± 0.013	1.574 ± 0.018	1.504 ± 0.009	1.581 ± 0.008
HAA			1.034 ± 0.011	1.618 ± 0.016	1.520 ± 0.008	1.587 ± 0.007
P-values						
Challenge			< 0.001	< 0.001	< 0.001	< 0.001
Regime			0.522	0.0635	< 0.001	0.212
Diet			0.080	0.085	0.206	0.598
Challenge × Regime			< 0.001	0.042	0.421	0.323
Challenge × Diet			0.774	0.276	0.299	0.145
Regime × Diet			0.328	0.322	0.323	0.435
Challenge × Regime × Diet			0.690	0.263	0.303	0.037

HAA = high amino acid starter diet (10% more essential amino acid over the Aviagen recommendations); FED = access to feed with 6 h post-hatch; HELD = access to feed after 48 h post-hatch; Challenge = necrotic enteritis induced by oral administrations of Eimeria species and a field strain of *Clostridium perfringens*.

[a, b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$).

[1] Means represent the average individual body weight of 12 birds per pen, 6 pens per treatment (72 birds/treatment).

2.4.2 Lesion score and intestinal bacterial enumeration

Necropsy of the small intestine revealed healthy intestines in birds from the unchallenged group, in contrast to birds from the challenged group which had enlarged gas-filled small intestines and prevalence of gross lesions. As expected, the necrotic lesions in the proximal duodenum, jejunum, and ileum were significantly ($P < 0.001$) more severe in the birds subjected to the NE challenge compared with those that were not challenged (Fig. 1 and 2). In this study feeding regime and diet had no significant impact on intestinal lesion scores.

Table 2.5 shows there were interactions between challenge and diet on measured *Lactobacillus* ($P = 0.047$) and *Enterobacter* ($P = 0.028$) populations in the ileum digesta. In the unchallenged birds, the number of *Lactobacillus* was lower in birds fed the HAA diet compared with those fed the control diet. The *Enterobacter* population was greater in the challenged birds fed the control diet compared with the unchallenged birds fed the HAA diet. The number of *Lactobacillus* observed in the ileum was significantly lower ($P = 0.008$) in digesta from the challenged birds compared with the unchallenged birds, but the number of *C. perfringens* in the ileum was markedly higher ($P < 0.001$) in the challenged birds. In the challenged birds, feeding the HAA diet resulted in a higher *Lactobacillus* population compared with feeding the control diet. *Enterobacter* population in the caeca was significantly higher ($P = 0.006$) in the challenged birds compared with the unchallenged birds. *C. perfringens* colonisation in the caecum of the unchallenged birds was below the threshold of the detectable level but was detectable in the caeca of the challenged birds.

A B

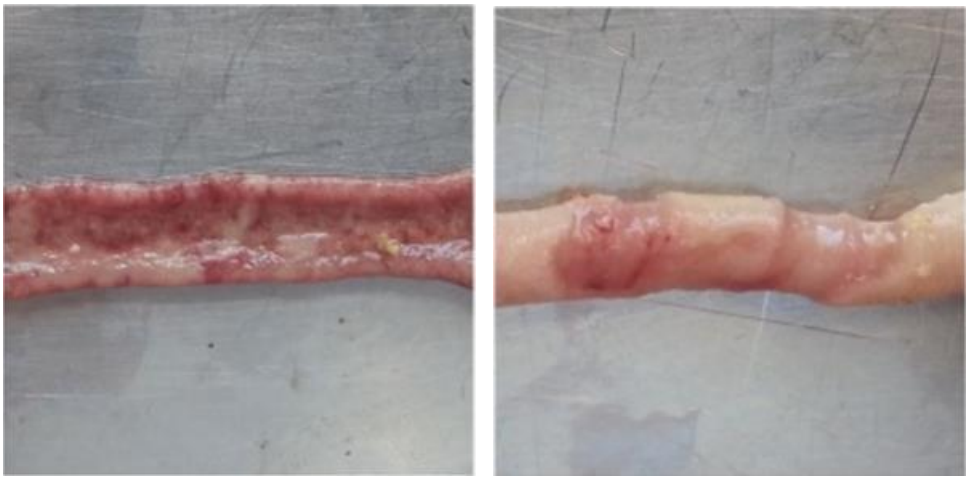

Figure 2.1 Examples of necropsy of gross lesions observed in the small intestine of 16-day-old birds exposed to necrotic enteritis challenge. (A) Ileum section (score 3) and (B) jejunum section (score 2).

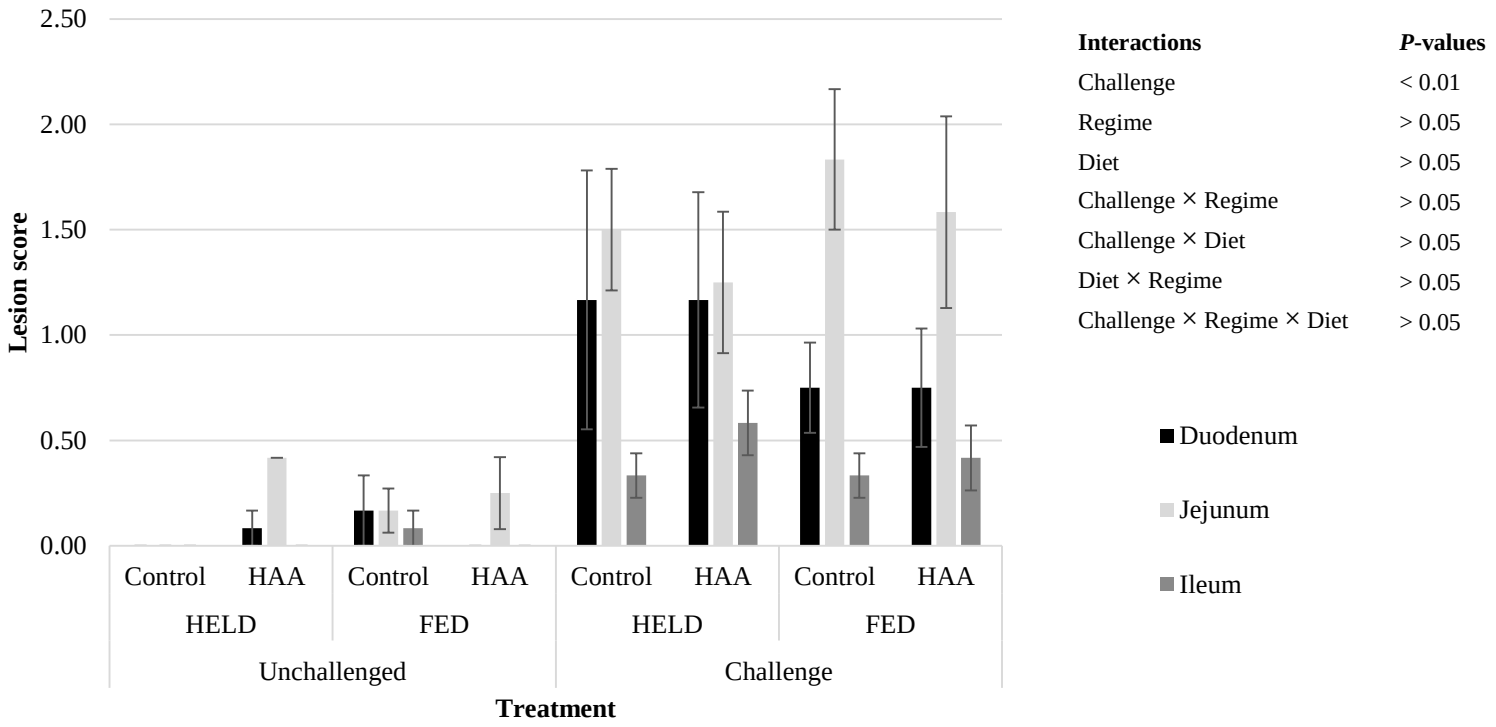

Figure 2.2 Interaction of necrotic enteritis challenge, feeding regime and diet on lesion score in 16-day-old broilers in the duodenum, jejunum and ileum. HAA = High amino acid starter diet; HELD = access to feed after 48 h post-hatch; FED = access to feed with 6 h post-hatch; Challenge = necrotic enteritis induced by oral administrations of Eimeria species and a field strain of *Clostridium perfringens*

Table 2.5 Interaction of necrotic enteritis challenge, feeding regime and diet on Log10 DNA enumeration of gut bacteria in ileum and cecum digesta from d16 broilers, using 16S-rDNA qPCR quantification.[1]

Challenge	Regime	Diet	Ileal digesta				Caecal digesta			
			Lac [2]	Ent [2]	Tot [2]	Cp [2]	Lac [2]	Ent [2]	Tot [2]	Cp [2]
Unchallenged	HELD	Control	8.7 ± 0.2	6.6 ± 0.2	8.2 ± 0.2	7.2 ± 0.4	9.1 ± 0.2	7.5 ± 0.4	10.3 ± 0.1	-
		HAA	8.7 ± 0.4	6.8 ± 0.1	8.3 ± 0.4	7.3 ± 0.5	9.4 ± 0.1	7.5 ± 0.3	10.4 ± 0.1	-
	FED	Control	9.0 ± 0.3	6.6 ± 0.2	8.6 ± 0.4	7.7 ± 0.1	9.3 ± 0.1	6.9 ± 0.3	10.4 ± 0.0	-
		HAA	8.5 ± 0.2	6.9 ± 0.2	8.0 ± 0.2	8.4 ± 0.1	9.2 ± 0.2	7.3 ± 0.3	10.4 ± 0.0	-
Challenged	HELD	Control	8.0 ± 0.2	7.0 ± 0.2	8.3 ± 0.2	10.3 ± 0.3	9.2 ± 0.1	8.0 ± 0.3	10.2 ± 0.1	11.6 ± 0.2
		HAA	8.8 ± 0.4	6.6 ± 0.1	8.7 ± 0.1	10.2 ± 0.4	9.2 ± 0.1	8.0 ± 0.2	10.3 ± 0.0	11.0 ± 0.6
	FED	Control	7.6 ± 0.2	6.9 ± 0.1	7.8 ± 0.1	9.9 ± 0.2	9.4 ± 0.1	7.5 ± 0.3	10.2 ± 0.1	11.5 ± 0.2
		HAA	8.2 ± 0.3	6.6 ± 0.2	8.4 ± 0.2	10.5 ± 0.1	9.5 ± 0.1	8.0 ± 0.2	10.3 ± 0.0	11.8 ± 0.1
P-values										
Challenge			0.008	0.848	0.837	<0.001	0.452	0.006	0.254	-
Regime			0.274	0.918	0.384	0.155	0.181	0.164	0.567	0.362
Diet			0.302	0.653	0.360	0.151	0.206	0.325	0.135	0.700
Challenge × Regime			0.201	0.767	0.295	0.120	0.184	0.678	0.682	-
Challenge × Diet			0.047	0.028	0.056	0.776	0.601	0.759	0.559	-
Regime × Diet			0.399	0.642	0.540	0.241	0.231	0.307	0.569	0.207
Challenge × Regime × Diet			0.696	0.883	0.242	0.917	0.273	0.916	0.488	-

HAA = high amino acid starter diet (10% more essential amino acid over the Aviagen recommendations); FED = access to feed with 6 h post-hatch; HELD = access to feed after 48 h post-hatch; Challenge = necrotic enteritis induced by oral administrations of Eimeria species and a field strain of *Clostridium perfringens*.

[1] means represent the average individual feed intake of 12 birds per pen, 6 pens per treatment (72 birds/treatment).

[2] Lac, Ent, Tot and Cp refer to bacteria genus *Lactobacillus*, *Enterobacter*, total bacteria enumeration, and *Clostridium perfringens* species, respectively.

"-" means below detection level.

2.5 Discussion

The NE challenge model (Wu et al., 2010; Rodgers et al., 2014) employed in the current study successfully induced subclinical NE, which was characterised by a significant deterioration of bird performance without affecting mortality. It was surprising that the challenge led to average lesions score of more than 1.5 in the jejunum, but the affected birds gradually recovered by d 30, as illustrated by the mean weight of the birds in the challenge treatment being similar to that of the unchallenged birds. Significant loss of feed efficiency and severe gross lesions, but without observable mortality, all indicates that subclinical NE outbreaks can indeed be economically devastating to a broiler flock (Skinner et al., 2010).

It is well documented that an unbalanced diet that contains excessive amounts of dietary ingredients, such as barley, wheat, oat, rye or fishmeal, can predispose the intestinal environment to *C. perfringens* proliferation, possibly leading to NE outbreak in broilers (Kaldhusdal and Løvland 2000). However, to the best of our knowledge, the current study was the first to examine the potential beneficial implications of feeding a high density of digestible amino acids to NE challenged birds. Indeed, the current study used a high-quality diet that met or exceeded the nutrient requirements recommended for Ross 308 broilers (Aviagen, 2012). Throughout the study, the body weight of the flock was higher than the Ross 308 broiler standard. For instance, at d 13, the body weights of all the groups ranged from 476 to 600 g (Ross 308 male bird standard is approximately 428 g) and at d 35 it ranged from 2,435 g to 2,685 g (Ross 308 male standard is approximately 2,250 g). Previously, the same challenge model with the same organism was used and resulted in up to 30% NE-related mortalities (Mikkelsen et al., 2009; Wu et al., 2010), whereas in the current study, despite the presence of severe NE lesions, no NE-related mortalities was recorded. The only observed difference between the current study and previous studies using the same study design is the diet. Supplementing 10% extra digestible amino acids over the recommended level proved to have long-lasting benefits to a bird that was subjected to multiple stress conditions. Although bird weight in the HAA and control diets did not differentiate in full factorial setup, the mean bird weight of birds fed the HAA diet was greater by the end of the trial. Moreover, when the main effects of challenge and non-

challenge were separately analysed, post-hatch fasted, i.e., the HELD birds fed the diet with HAA performed better in terms of the FCR performance under the challenge effect. This finding may constitute the beneficial effect of high amino acid supplement to birds in response to external stresses, for instance, post-hatch fasting and the subclinical NE. Traditionally, the majority of essential amino acids, such as arginine, histidine, glycine and lysine, have been perceived as a critical resource for immune function and cytokine production (Kidd, 2004; Li et al., 2007). Therefore, it is possible that demand for essential amino acids would increase in the presence of inflammation or immune stress (Le Floc'h et al., 2004). However, studies on the mere characterisation of nutrient utilisation on carcass weight suggest that additional digestible amino acid supplementation should not exceed the level of recommendation in the healthy flock if the crude protein had been adequately provided, since the consumed nitrogen composition in excess would not be utilised and would hence be excreted (Aletor et al., 2000). Abiding to the hypothesis of the present study, the role of excess amounts of essential amino acids, such as lysine and arginine, may have induced improvements in birds that are recovering from disease stress, although the excess likely did not instigate improvements in the healthy flock (Aletor et al., 2000; Li et al., 2007). However, due to limited evidence provided in the present study, further investigation might be required to confirm the potential benefit of excess amino acid density.

Any negative change to dietary compositions may be accompanied by drastic changes in the gut microflora, which may potentially predispose the gut to pathogenic infection (Mikkelsen et al., 2009; Wu et al., 2014; Standley et al., 2014). *C. perfringens* is considered highly opportunistic, and its population rapidly changes depending on alterations to the host gut environment (Lee et al., 2011; Timbermont, 2011). *C. perfringens* strains also possess the capability to secrete proteinaceous enzymes, which act as invasive toxic compounds to closely related species and competitively exclude bacterial strains (Jack et al., 1995; Bannam et al., 2011). Pathogenic *C. perfringens* strains can take advantage of high viscosity and high protein environment in the digestive tract to further proliferate, which inevitably results in an NE outbreak (Van Immerseel et al., 2009; Timbermont, 2011). It is not surprising that in this study a significantly larger numbers of *C. perfringens* were present in the gut in the challenged birds compared with

the unchallenged birds, which resulted in a decrease of *Lactobacillus* present in the ileum. *Lactobacillus* is a beneficial gut commensal; it lowers the pH of the gastrointestinal environment and balances gut microflora in the lower intestine (Jin et al., 1998; Waititu et al., 2014). Cao et al. (2012) reported a successful example of a decreased number of *C. perfringens* colonies as a result of dietary induction of *Lactobacillus* in chickens. In this study, the HAA starter diet heightened the presence of *Lactobacill*i in the ileum of the challenged birds, but it did not affect the *Lactobacillus* population in the unchallenged birds. The effect of NE challenge on the gut microbiota is extremely complex. Stanley et al. (2014) reported vastly different gut flora in broilers with or without NE challenge. It is thus difficult to explain the interaction detected in the current study. One interesting finding was that higher numbers of *Enterobacter* were found in the caecum digesta of the challenged birds. It is difficult to determine the exact cause of the increased proportion of *Enterobacter* reacting to enlarged *C. perfringens* population, but it appears that the drastic change to the gut microflora induced by NE positively instigated *Enterobacter* proliferation. *Enterobacter* is a facultative anaerobe and hence may be able to retain growth over other strict anaerobes, after the environmental change caused by the exceeding population of *C. perfringens* in the caecum (Gabriel and Mallet, 2006).

It is reported that delayed access to diet post-hatch generates production loss when the bird is older (Dibner, 1999; Gonzales et al., 2003). The yolk is capable of sustaining the bird for the first few days post-hatch, and it contains maternal antibodies that strengthen immune development (Dibner et al., 1998; Dibner, 1999). During a nutrient scarcity, it is possible that vital antibodies may be degraded to meet the demand of amino acids for physical development of rapidly growing chickens, rather than functioning as a component of passive immunity (Dibner, 1999). Parental generated antibodies that induce passive immunity against NE challenge have been shown to pass on to the offspring (Lovland et al., 2004). This hypothesis indicates the important role of early feed access in broiler chickens in preserving the vital role of immune function of the yolk (Gonzales et al., 2003). Thus, fasting in the early post-hatch period may not only impose harm on immune development but it also possibly suppresses the physiological development of the gut, thereby further implementing reduced development and performance as the bird ages (Geyra et al., 2001; Kidd et al., 2004). Geyra et al. (2001) reported that early feed

restriction resulted in negative impacts on intestinal and critical organ development in broilers. A similar observation was found by Corless and Sell (1999) in fasted young turkey poults. However, their findings indicate that it is the period of fasting as opposed to the act of fasting itself that causes potentially depressed growth in the older bird (Corless and Sell, 1999), and the impact of the period of fasting is largely dependent on the nutrient quality of the yolk (Moran and Reinhart, 1980).

2.6 Conclusion

The subclinical form of NE-induced in this study resulted in significant loss of feed efficiency and severe gross lesions, but without observable mortality. This indicates that subclinical NE outbreaks can indeed be economically devastating to a broiler flock. Supplementing 10% extra digestible amino acids over the recommended level proved to have long-lasting benefits to birds that were subjected to multiple stress conditions, namely post-hatch fasting and NE. This was illustrated in this study by the fact the mean weight of birds fed the HAA diet was greater by the end of the trial compared with those fed the control diet, and post-hatch fasted birds fed the HAA diet performed better in terms of FCR performance under the challenge effect. This was likely because demand for essential amino acids is increased in the presence of inflammation or immune stress. The HAA diet also appeared to promote intestinal *Lactobacillus* population in the lower small intestine. Further investigation is required to assess the potential benefit of feeding broilers diets with excess amino acid density in different husbandry conditions and diets. Further investigation is however warranted into determining the threshold period of post-hatch fasting that affects the susceptibility of birds to NE. In this study, a limited number of bacteria species were enumerated, so it may be advantageous to characterise the complete range of bacteria species in the NE challenged birds in order to fully understand its impact on the gut microflora community.

2.7 References

Aletor VA, Hamid II, Niess E, Pfeffer E. Low-protein amino acid-supplemented diets in broiler chickens: effects on performance, carcass characteristics, whole-body composition and efficiencies of nutrient utilisation. J Sci Fd Agri 2000; 80:547-554.

Annett C, Viste J, Chirino-Trejo M, Classen HL, Middleton D, Simko E. Necrotic enteritis: Effect of barley, wheat and corn diets on proliferation of Clostridium perfringens type A. Avian Path 2002; 31:598-601.

Ao Z, Kocher A, Choct M. Effects of dietary additives and early feeding on the performance, gut development and immune status of broiler chickens challenged with Clostridium perfringens. Asian-Aust J Anim Sci 2002; 25:541-551.

Aviagen. Ross 308 Broiler: Nutrition Specification. 2012. http://www.aviagen.com.

Bannam TL, Yan XX, Harrison PF, Seemann T, Keyburn AL, Stubenrauch C, Weeramantri LH, Cheung JK, McClane BA, Boyce JD. Necrotic enteritis-derived Clostridium perfringens strain with three closely related independently conjugative toxin and antibiotic resistance plasmids. MBio 2011; 2:e00190-00111.

Bartosch S, Fite A, Macfarlane GT, McMurdo ME. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. AEM 2004; 70:3575-3581.

Bedford M. Removal of antibiotic growth promoters from poultry diets: Implications and strategies to minimise subsequent problems. Worlds Poult Sci J 2000; 56:347-365.

Broussard C, Hofacre C. Page R, Fletcher O. Necrotic enteritis in cage-reared commercial layer pullets. Avian Dis 1986; 617-619.

Cao L, Yang X, Li Z, Sun F, Wu X, Yao J. Reduced lesions in chickens with Clostridium perfringens-induced necrotic enteritis by Lactobacillus fermentum 1.2029. Poult Sci 2012; 91:3065-3071.

Choct M. Alternatives to in-feed antibiotics in monogastric animal industry. ASA Technical Bulletin 2001. AN. 30.

Choct M, Annison G. Anti-nutritive effect of wheat pentosans in broiler chickens: Roles of viscosity and gut microflora. Brit Poultry Sci 1992; 33:821-834.

Collier C, Hofacre C, Payne A, Anderson D, Kaiser P, Mackie R, Gaskins H. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth. Vet Immunol Immunop 2008;122:104-115.

Corless AB, Sell J. The effects of delayed access to feed and water on the physical and functional development of the digestive system of young turkeys. Poult Sci 1999; 78:1158-1169.

Croom J, Edens F, Ferket P. The impact of nutrient digestion and absorption on poultry performance and health. In Proc. 27th Ann. Carolina Poultry Nutrition Conference, Carolina Feed Industry Association, Research Triangle Park, November 2000.16: 65-73.

Dahiya JP. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Anim Feed Sci Tech 2006; 129:60-88.

Devriese L, Daube G, Hommez J, Haesebrouck F. In vitro susceptibility of Clostridium perfringens isolated from farm animals to growth-enhancing antibiotics. J Appl Microbiol 1993; 75:55-57.

Dibner J. Feeding hatchling poultry. Avoid any delay. Feed Int 1999; 20:30-34.

Dibner J, Knight C, Kitchell M, Atwell C, Downs A, Ivey F. Early feeding and development of the immune system in neonatal poultry. J Appl Poult Res 1998; 7:425-436.

Dibner J, Richards J. Antibiotic growth promoters in agriculture: History and mode of action. Poult Sci 2005; 84:634-643.

Ferket PR. Alternatives to antibiotics in poultry production: responses, practical experience and recommendations. In: Nutritional Biotechnology in the Feed and Foods Industries. Nottingham Univ Press, 2004; 20:54-67.

Gabriel I, Lessire M, Mallet S, Guillot JF. Microflora of the digestive tract: critical factors and consequences for poultry. Worlds Poult Sci 2006; 62:499-512.

Geier MS, Mikkelsen LL, Torok VA, Allison GE, Olnood C, Boulianne M, Hughes RJ, Choct M. Comparison of alternatives to in-feed antimicrobials for the prevention of clinical necrotic enteritis. J Appl Microbiol 2010; 109:1329-1338.

Geyra A, Uni Z, Sklan D. The effect of fasting at different ages on growth and tissue dynamics in the small intestine of the young chick. Br J Nutr 2001; 86:53-61.

Gibert M, Jolivet-Renaud C, Popoff MR. Beta2 toxin, a novel toxin produced by Clostridium perfringens. Gene 1997; 203:65-73.

Gonzales E, Kondo N, Saldanha E, Loddy M, Careghi C, Decuypere E. Performance and physiological parameters of broiler chickens subjected to fasting on the neonatal period. Poult Sci 2003; 82:1250-1256.

Huyghebaert G. An update on alternatives to antimicrobial growth promoters for broilers. Vet J 2011; 187:182-188.

Jack, RW, Tagg JR, Ray B. Bacteriocins of gram-positive bacteria. Microbiol Rev 1995; 59:171-200.

Jin LZ, Ho YW, Abdullah N, Jalaludin S. Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing Lactobacillus cultures. Poult Sci 1998; 77:1259-1265.

Kaldhusdal M., Løvland A. The economical impact of Clostridium perfringens is greater than anticipated. World Poult 2000; 16:50-51.

Keyburn AL, Bannam TL, Moore RJ, Rood JI. NetB, a pore-forming toxin from necrotic enteritis strains of Clostridium perfringens. Toxins. 2010; 2:1913-1927.

Kidd M. Nutritional modulation of immune function in broilers. Poult Sci 2004; 83:650-657.

Kidd M., McDaniel C, Branton S, Miller E, Boren B, Fancher B. Increasing amino acid density improves live performance and carcass yields of commercial broilers. J Appl Poult Res 2004;13:593-604.

Le Floc'h, N, Melchior D, Obled C. Modifications of protein and amino acid metabolism during inflammation and immune system activation. Livest Prod Sci 2004; 87:37-45.

Lee D-H., Zo Y-G, Kim S-J. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Appl Environ Microbiol 1996; 62:3112-3120.

Lee K, Lillehoj H, Jeong W, Jeoung H, An D. Avian necrotic enteritis: experimental models, host immunity, pathogenesis, risk factors, and vaccine development. Poult Sci 2011; 90:1381-1390.

Li P, Yin Y-L, Li D, Kim SW, Wu G. Amino acids and immune function. Br J Nutr 2007; 98:237-252.

Lovland A, Kaldhusdal M, Redhead K, Skjerve E, Lillehaug A. Maternal vaccination against subclinical necrotic enteritis in broilers. Avian Path 2004; 33:81-90.

Mikkelsen LL, Vidanarachchi JK, Olnood GG, Bao YM, Selle PH, Choct M. Effect of potassium diformate on growth performance and gut microbiota in broiler chickens challenged with necrotic enteritis. Brit Poult Sci 2009; 50:66-75.

Moore RJ. Necrotic enteritis predisposing factors in broiler chickens. Avian Path 2016; 1-22.

Moran E, Reinhart B. Poult yolk sac amount and composition upon placement: effect of breeder age, egg weight, sex, and subsequent change with feeding or fasting. Poult Sci 1980; 59:1521-1528.

Prescott J, Sivendra R, Barnum D. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. Can Vet J 1978; 19:181.

Riddell C, Kong X-M. The influence of diet on necrotic enteritis in broiler chickens. Avian Dis 1992; 499-503.

Rodgers NJ, Swick RA, Geier MS, Moore RJ, Choct M, Wu S-B. A multifactorial analysis of the extent to which eimeria and fishmeal predispose broiler chickens to necrotic enteritis. Avian Dis 2014; 59:38-45.

Skinner JT, Bauer S, Young V, Pauling G, Wilson J. An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. Avian Dis 2010; 54:1237-1240.

Stanley D, Wu SB, Rodgers N, Swick R, Moore RJ. Differential responses of cecal microbiota to fishmeal, Eimeria and Clostridium perfringens in a necrotic enteritis challenge model in chickens. PLoS One 2014; 9:104739

Tech R. Necrotic enteritis and associated conditions in broiler chickens. World Poult 1999; 15:44-47.

Timbermont EA. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Path 2011; 40:341-347.

Van der Sluis W. Clostridial enteritis is an often underestimated problem. World Poult 2000; 16:42-43.

Van Immerseel F, Rood JI, Moore RJ, Titball RW. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens.Trend Microbiol 2009; 17:32-36.

Wade B, Keyburn A.. The true cost of necrotic enteritis. World Poult 2015; 31:16-17.

Waititu SM, Yitbarek A, Matini E, Echeverry H, Kiarie E, Rodriguez-Lecompte JC, Nyachoti CM. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poultry Sci 2014; 93:625-635.

Wu S-B, Rodgers N, Choct M. Optimized necrotic enteritis model producing clinical and subclinical infection of Clostridium perfringens in broiler chickens. Avian Dis 2010; 54:1058-1065.

Wu S-B, Stanley D, Rodgers N, Swick RA, Moore RJ. Two necrotic enteritis predisposing factors, dietary fishmeal and Eimeria infection, induce large changes in the caecal microbiota of broiler chickens. Vet Microbiol 2014; 169:188-197.

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

TYPE OF WORK	PAGE NUMBER
TABLE 2.1	36
TABLE 2.2	42
TABLE 2.3	43
TABLE 2.4	44
TABLE 2.5	48
Figure 2.1	46
Figure 2.2	47

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CHAPTER 3

Re-introduction of microflora from necrotic enteritis resistant chickens reduces gross lesions and improves performance of necrotic enteritis challenged broilers

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3.1 Summary

Probiotics feature one or multiple strains of viable bacteria that beneficially modulate the intestinal health of the host. The key criteria for probiotics, like all feed additives, are that they must be safe and efficacious to use. One of the efficacious measures for probiotics used in poultry feed is their ability to act as an alternative to in-feed antibiotics against economically important diseases like necrotic enteritis (NE). The current study investigated the effect of the reintroduction of crude ileal and caecal contents from previously NE-challenged chickens on performance, mortality and intestinal lesions of young broilers under NE challenge. An expected, a subclinical NE challenge depressed bird performance ($P < 0.001$) and caused intestinal damage. Cloacal administration of both ileal and caecal crude flora inoculants significantly ($P < 0.05$) improved feed conversion efficiency at d 35. The severity of NE-associated intestinal lesions was also alleviated ($P = 0.049$), where caecal flora inoculant markedly reduced lesion score in challenged birds and the ileal crude flora inoculum numerically improved lesion score. These preliminary findings suggest that the gut microbiome of birds plays a significant role in the susceptibility of broilers to NE and more work is required to elucidate the mechanisms by which this occurs.

3.2 Description of Problem

There is a strong global move to phase out the prophylactic use of antibiotics from animal industries. For broiler production, necrotic enteritis (NE) remains a key threat to the viability of the industry if in-feed antibiotics are completely removed. Indeed, NE is prevalent in all poultry producing areas of the world, and it is costly when an outbreak occurs (Timbermont, 2011). NE can be classified into two broad types: 1) acute clinical NE, where birds suffer from severe disease symptoms and flock mortality can be very high, and 2) subclinical NE, where birds do not usually display obvious symptoms, nor there is a noticeable increase in mortality (Dahiya et al., 2006; Van Immerseel et al., 2009; Skinner et al., 2010).

Skinner et al. (2010) estimated that subclinical NE alone could cost as much as US$0.05 per bird on average. It is suggested that the subclinical form of NE can be more financially

devastating than the clinical form because of morbidity, rather than mortality, that drains feed efficiency without visible symptoms prompting swift treatment. A more recent estimate by the World Poultry Magazine (October 2015) put the figure at US$0.0625 per birds, or close to US$6 billion per year globally.

In broilers, NE is caused by *Clostridium perfringens* type A and, in rare cases, by type C strains. However, *Clostridium perfringens* exists in the normal intestinal flora of healthy chickens (Kaldhusdal et al., 1999; Tech, 1999; Dahiya et al., 2006). Natural occurrence of NE in chicken production is usually associated with predisposing factors, such as unbalanced diet composition, increased intestinal viscosity and *Eimeria* infections (Choct and Annison, 1992; Kaldhusdal and Skjerve, 1996a; Kaldhusdal et al., 1999; Collier et al., 2008; Rodgers et al., 2015). Maintenance of a balanced diet composition and settlement of healthy intestinal microflora can alleviate NE outbreaks in broiler chickens (Choct, 2001; Gabriel and Mallet, 2006).

There are two broad options available to mitigate the risk of NE outbreaks in broilers. One is the use of vaccines. But it appears that a highly efficacious vaccine against NE is yet to be developed [13]. The second option is related to the use of neutraceuticals, such as prebiotics, probiotics, symbiotics and plant extracts, which works towards creating a healthy gut environment [14, 15] Manipulation of the gut microflora to skew the populations towards beneficial microorganisms can result in competitive exclusion of pathogens, thereby preventing disease outbreaks (Morishita et al., 1997; Patterson and Burkholder, 2003; Ahmad, 2006). Prebiotic and probiotic combinations have also been explored in the search for an optimal outcome (Ehrmann et al., 2002; Patterson and Burkholder, 2003). Although the results for probiotic application in the prevention of bacterial diseases have varied in laboratories, probiotics are regarded as a viable alternative to antibiotics in poultry production (Edens, 2003; Patterson and Burkholder, 2003; Huyghebaert, 2011; Waititu et al., 2014). The variation in the efficacy of probiotics stems, in part, from differences in individual birds, in diet composition and husbandry conditions (Fuller, 2001; Gabriel and Mallet, 2006). Kaldhusdal et al. (2001) reported improved performance and reduction of NE incidences in 3 to 4 week-old birds after administration of formulated probiotics, which closely resembled the natural flora of adult

chickens. Moreover, various commercially-available prebiotic and probiotic products, such as mannan-oligosaccharides and *Lactobacillus*-based products, reported to improve bird performance and alleviate the severity of bacterial diseases, such as NE (La Ragione et al., 2001; Hofacre et al., 2003; Milbradt et al., 2014).

The current study examined whether cloacal administration of the ileal or caecal contents of chickens, which had not succumbed to NE challenge in a previous experiment, could offer protection against NE in young birds that were given NE challenge.

3.3 Materials and Methods

All experimental procedures involved in this study were approved by the Animal Ethics Committee of the University of New England, Armidale, Australia.

3.3.1 Treatments

The present study employed a 2 x 3 factorial arrangement with exposure to NE challenge (Challenged vs. Unchallenged) and inoculation of crude caecal flora, ileal flora or a sterile saline (Caecal vs. Ileal vs. Control) as independent variables. This resulted in 6 treatments, with 6 replicates per treatment and 11 birds per replicate. A total of 396 Ross 308 day-old male broilers were raised from day 0.

Table 3.1 Experimental diets

Ingredient %	Starter	Grower	Finisher
Wheat, Australian	30.00	28.00	33.80
Sorghum whole	26.60	34.70	30.00
SBM	30.10	24.10	20.20
Canola meal, solvent extracted	3.00	2.00	3.00
Meat and bone meal	2.00	3.00	5.00
Canola oil	4.30	5.00	5.60
Limestone	1.20	0.80	0.50
Dical Phos 18P/21Ca	1.50	1.10	0.70
Salt	0.10	0.10	0.10
Na bicarb	0.20	0.20	0.20
UNE vitamin and trace mineral premix[1]	0.20	0.20	0.20
Choline Cl 70%	0.04	0.04	0.03
Available phosphorous%	0.50	0.45	0.45
Metabolisable energy, kcal/kg	3025	3150	3200
Crude Protein, %	22.90	21.00	21.10
Crude Fat, %	6.20	7.10	7.90
Crude Fiber, %	3.00	2.80	2.70
Insoluble NSP, %	1.44	1.61	1.58
Isoleucine, %	1.01	0.90	0.87
Arginine	1.34	1.18	1.14
Lysine	1.27	1.11	1.06
Methionine	0.60	0.53	0.53
Methionine and Cysteine	0.94	0.84	0.83
Tryptophan	0.24	0.20	0.20
Threonine	0.83	0.73	0.72
Valine	0.94	0.85	0.83

[1]Premix per kg contains vitamins: vitamin A, 12 MIU; vitamin D, 5 MIU; vitamin E, 75 mg; vitamin K, 3 mg; nicotinic acid, 55 mg; Pantothenic acid, 13 mg; folic acid, 2 mg; Riboflavin, 8mg; cyanocobalamin, 0.016 mg; Biotin, 0.25 mg; pyridoxine, 5 mg; thiamine, 3 mg; antioxidant, 50 mg. And contains trace minerals per kg: Cu, 16 mg as copper sulphate; Mn, 60 mg as manganese sulphate; Mn, 60 mg as manganous oxide; I, 0.125 mg as potassium iodide; Se, 0.3 mg; Fe, 40 mg, as iron sulphate; Zn, 50 mg as zinc oxide; Zn, 50 mg as zinc sulphate.

At d 6 post-hatch, birds were given cloacally the crude ileal and caecal flora inoculants with sterile crop needles (18 gauge) and syringes. Two doses (0.2 ml each) of caecal flora, ileal flora or sterile saline were administered with a 2-hour interval. Cloacal route of administration referred to a procedure previously described by Sacranie [28] regarding anti-peristalsis, migration of the flora due to reflux of contents from the colon to the caeca and distal intestinal tract could be expected.

The ileal and caecal contents were obtained from chickens that remained unaffected despite challenged with NE at d 19. Birds with healthy appearance were selected by experienced personnel, and the health status of the intestinal tract was confirmed by necropsy. Then, intestinal contents of these birds were aseptically collected and immediately suspended in a CO_2-flushed anaerobic broth media culture. After that, flora

suspension was stored at minus 20°C with sterile glycerin addition to 15% (v/v). Broth cultures were pooled and filtered through a sterile 0.5 mm mesh filter and supernatants were collected for use in the current experiment. The 16S ribosomal DNA sequence of bacterial populations, *Lactobacillus* spp., *Bifidobacterium* spp. and *Clostridium perfringens* from both inoculants underwent quantitative PCR measurement. The qPCR assay used quantitative amplification of 16S ribosomal DNA of each subject bacterium in relative prospect to the standard curve of the corresponding bacterial species. Serial dilutions of linearized plasmid DNA (pCR®4-TOPO Vector, Life Technologies, Carlsbad, USA) inserted with respective amplicons were used to construct a standard curve [29]. DNA extraction protocol followed the standard procedure of the Qiagen Stool Mini Kit (Qiagen, Australia). A NanoDrop ND-8000 UV spectrophotometer (Thermo Fisher Scientific, Waltham, USA) assessed the measurement of DNA mass and purity, allowing DNA samples are emitting ratios of between 1.6 and 1.8 in 260/280 nm wavelength to be used in following qPCR assay. A Rotorgene-6500 real-time thermocycler (Corbett, Sydney, Australia) assessed the quantitative analysis of the target DNA copies. The SensiMix™ SYBR® No-ROX (Bioline, Meridian Life Science, Memphis, USA) master mix was used to amplify the 16S ribosomal DNA of *Lactobacillus* spp. [30] and *Bifidobacterium* spp. [31]. The SensiFAST™ SYBR® NO-ROX (Bioline, Meridian Life Science, Memphis, USA) with TaqMan probe were used in the qPCR assay to amplify 16S ribosomal DNA of *C. perfringens* [32]. A threshold cycle average from duplicate amplicons was assigned for quantification analysis (Rotor-Gene 6000 Series Software 1.7, Corbett, Sydney, Australia). The quantity of target DNA copies was calculated from the mass of the DNA, taking into account the size of the amplicon insert in the plasmid. The results are presented in Table 3.2 as the base-10 logarithm copy per milliliter of inoculant.

Table 3.2 Selected organisms present in the inoculants

Inoculant	*Lactobacilli* count (log_{10}/ml)	*Bifidobacterium* count (log_{10}/ml)	*C. perfringens* count (log_{10}/ml)
Caecal	7.0 ± 0.1	3.9 ± 0.0	8.6 ± 0.0
Ileal	5.6 ± 0.1	ND	5.6 ± 0.1

Bacterial count was expressed in the base-10 logarithm transformation of the bacterial chromosomal DNA copy per ml. ND means not detected.

The NE challenge model used in the present study had been previously described by Wu et al. (2010). The model consisted of a single oral dose of *Eimeria* (Bioproperties Pty Ltd., Sydney, Australia) at d 9 of age followed by a single oral inoculation of freshly prepared *C. perfringens* type A (approx. 10^8 CFU/mL) (CSIRO Livestock Industries, Geelong, Victoria, Australia) in thioglycolate-starch broth suspension at d 14. Each 1 mL gavage contained approximately 5000 oocysts each of *E. acervulina* and *E. maxima*, and 2500 oocysts of *E. brunetti* suspended on sterile PBS. The unchallenged group received one-dose of 1 ml of sterile PBS as a stress equivalent treatment. Similarly, unchallenged treatments were subject to 2 mL doses of sterile thioglycolate broth per bird as a sham treatment at d 14. *C. perfringens* count was performed on perfringens tryptose-sulfite-cycloserine (TSC) selective agar (Oxoid) following serial dilutions to ensure the adequate concentration of 10^8 CFU/ml. After the NE challenge, all dead birds were necropsied, and intestinal swab samples were taken to confirm the cause of death.

3.3.2 Post-Mortem and Lesion Scoring

Two birds randomly selected from each pen were euthanized by cervical dislocation at d 16 of age. A blind procedure of lesion scoring was conducted. Personnel involved in the scoring procedure had no knowledge of treatment allocation of birds. The entire length of the small intestine was visually inspected for lesions as described in previous reports (Broussard et al., 1986). The magnitude of lesions was rated from 0 to 4. A healthy intestine was given score 0 = none; a gas-filled intestine with evidence of at least two necrotic lesions was scored 1 = mild; a ballooned, friable, foul-smelling intestine with moderate necrotic lesions was scored 2 = moderate; an intestine with yellow pseudomembrane appearance (also known as “Turkish towel”) with either appearance of score 1 or 2 was regarded as score 3 = marked/severe; an intestine with ruptured epithelial

layer and blood filled intestine, or above description and preceived petechial haemorrhages along the small intestine were associated with score 4 = very severe.

3.3.3 Bird Performance and Statistical Analysis

Performance parameters were recorded at days 13, 24 and 35. Weight gain (WG), feed intake (FI), feed conversion ratio (FCR) are presented in Table 3.3. FCR was corrected for mortality. WG, FI, FCR were analyzed with an individual pen as the independent variable. The score of intestinal lesions was summarized with mean lesion score from the entire length of the small intestine of individual sample bird as the independent variable. Statistical analysis was conducted using the JMP® statistical software (2015 version 12 ed. SAS Inst. Inc., Cary, NC). When difference among comparisons was significant, comparisons of means were separated using Tukey's test. Statistical significance was declared at $P \leq 0.05$.

3.4 Results and Discussion

The NE challenge model [33, 34] employed in this study produced a subclinical NE outbreak which did not induce a sharp increase in mortality but resulted in a significant deterioration of bird performance and prevalence of lesions in the small intestine. Mortality occurred throughout the experimental period. Among the 23 birds that died, 13 belonged to NE challenged treatments and 10 in the unchallenged treatments, and there was no effect of treatment on mortality (Table 3.4). However, necropsies were conducted out on all dead birds, and it was clear that the NE challenge resulted in obvious lesions in the challenged birds after d 14 of age.

In the current study, the ileal and caecal inoculants were obtained from birds that remained unaffected after an NE challenge. The inoculants were then cloacally administered to another batch of NE challenged birds to see if they provided protection against NE. The idea was that the gut microflora of these birds might have played a key role in protecting them from NE infection. If so, the "protective flora" might be transferred to other birds. The reason for using crude flora was to directly transfer the bacterial strains that possessed probiotic effects. The result shows that both ileal and caecal inoculants contained *Lactobacillus* spp. in similar profiles (5.6 and 7.0 $\log_{10}$/ml respectively), but the

Bifidobacterium spp. was detected only in the caecal inoculant up to 3.9 $\log_{10}$/ml. *Lactobacilli* and *Bifidobacteria* are widely regarded as beneficial bacteria in poultry [36-38].

In commercial broiler chicken production, *C. perfringens* colonization of the gastrointestinal tract (GIT) of chickens naturally occurs as early as from the hatchery [39]. Previous work suggests that differentiation between probiotic populations from naturally occurring indigenous strains via culturing methods could be a challenging task (Fuller, 2001). Besides, microflora in the GIT changes frequently depending on rearing conditions (Gabriel and Mallet, 2006), especially from stress factors to the intestinal environment or significant changes in dietary composition (Hume et al., 2003). Therefore, a well-balanced natural gut flora may mean a mix of beneficial microbial populations that represent a healthy flora. It is followed that such a flora has elements and integrity to provide the necessary defence against the proliferation of pathogenic bacteria (Jin et al., 1998; Cao et al., 2012), thereby improving the overall intestinal environment of the chicken. As it was not known whether the "protective flora" would come from the ileum or the caeca, we made separate preparations to answer this question. In general, commercially available probiotics are usually grown in the laboratory media, in which case the typical characters of the probiotic organisms, such as the mucosal adhesive properties and metabolism, will be different from those grown in the natural intestinal contents or intestinal mucosa (De Roos and Katan, 2000; Tuomola et al., 2001). We also speculate that numerous other characteristics, such as bacterium-to-bacterium communication, bacterial interaction with the host immune system, and essential bacterial metabolites (bacteriocins and defencins), may be "filtered" out by the artificial culture media, whereas in theory, the crude gut contents from the same species would be more likely to contain all the essential protective elements of the flora. Also, probiotic microorganisms must have an adequate level of tolerance and resistance to survive the passage through the stomach and small intestine (Lee and Salminen, 1995). To avoid this particular issue of delivering crude flora inoculants orally, the present study used cloacal administration as per the procedure developed by Sacranie (2007). Precautions were taken so that voiding of the inoculants via bird defecation was carefully avoided by priming the birds using lukewarm sham carrier over a period of time. It was clear that the

administration was successful because the effects of the inoculants on NE lesions and bird performance were significant.

Among the 72 randomly selected birds at d 16, 44 % of NE-challenged birds presented NE lesions, whereas only 11 % of unchallenged birds showed very mild lesions. As expected, challenged birds had significantly more severe ($P = 0.001$) intestinal lesions than non-challenged birds (Table 3.3). In the present study, the magnitude of lesion scores also varied depending on the inoculant given although both inoculants reduced the severity of intestinal lesions resulting from NE challenge. In particular, the caecal flora preparation markedly ($P < 0.05$) decreased lesion score to the level found in the unchallenged control birds. A possible explanation for this preventive effect of the caecal flora against NE could be due to the introduction of organisms that contained the necessary protective mechanisms. Changes in the caecal microflora were drastic after NE challenge, where most butyrate-producing bacteria were reduced in challenged birds [46]. It is possible that two things may have happened in the current study. First, a "protective flora" transferred to the birds was, in fact, probiotics specifically selected against NE. Some probiotic organisms may have antagonistic effects on *C. perfringens*, possibly via competitive exclusion and the production of molecules such as bacteriocins [47]. However, such an assumption would require further validation. The major challenge is to understand the roles of various microorganisms harbored in the chicken gut in both normal chickens and chickens that are NE challenged. Despite the advent of sequencing technology, this task retains costly and difficult. Secondly, it has been shown that the gut microflora of chickens does not mature during the first two weeks of life, and it slowly starts to stabilize from d 15 onwards [48]. This period in broiler production often coincides with feed change in many countries that may lead to NE outbreaks [49]. It is speculated that the crude flora from birds that did not get sick after NE challenge may also have brought about a vaccination-like effect. This means that at d 6, the birds obtained a "mature flora" that was protective, eliminating the susceptibility of birds from NE challenge at d 13-16.

Regardless of the mechanisms, in this pilot study, birds given at day 6 the crude caecal and ileal flora preparations from birds that survived NE challenge had better FCR at d 35

(Table 3.4). To be specific, birds that received ileal and caecal flora possessed approximately 2 FCR points of advantage (P = 0.021) over the control treatment in the FCR at d 35 (Table 3.4). Necrotic enteritis challenge hampered the performance of birds on weight gain and FCR at d 24 and d 35. But the statistical analysis did not show any interaction between challenge effect and the inoculation of crude flora in any of the performance parameters. Although bird performance improvements from crude flora were small in magnitude, it is intriguing to find how a single dose of crude flora at day 6 of age led to an improvement in FCR that sustained to d 35.

Table **3.3** Effect of necrotic enteritis challenge and cloacal inoculation with ileal or caecal flora on small instestinal lesion scores[1] at d 16 post hatch[2] and total mortality for the tiral period

Treatments		Mortality d6-13	Mortality d13-24	Mortality d24-35	Mean Lesion Score[2]
Unchallenged	Caecal	0	1	1	0.11[b]
	Ileal	0	0	2	0.00[b]
	Control	2	4	0	0.11[b]
Challenged	Caecal	2	3	2	0.11[b]
	Ileal	1	2	0	0.44[ab]
	Control	1	2	0	0.61[a]
P- Value					
Challenge		0.223	0.445	0.990	0.001
Inoculation		0.313	0.296	0.667	0.077
Challenge × Inoculation		0.601	0.555	0.320	0.048

[1] Lesion scores range from 0 to 4. Score 0 - intestine of healthy appearance; score 1 - gas-filled intestine with evidence of at least two necrotic lesions; score 2 - ballooned, friable, foul-smelling intestine with evidence of necrotic lesions; score 3 – intestine displaying all the above along with a yellow pseudomembrane (often described as "Turkish towel"); score 4 - prevalence of ruptures of the intestinal epithelial layer and blood-filled intestine.
[2]Means represent the average of 6 replicates of 2 birds per treatment (12 birds/treatment). Challenged birds were exposed to a subclinical necrotic enteritis challenge, induced by oral administration of *Eimeria* and *C. perfringens*. Unchallenged birds received oral administration of sterile thioglycolate broth. Flora was derived from either the caeca or ileum of birds previously challenged with necrotic enteritis was administered by cloacal reverse inoculation. Control birds were inoculated with sterile saline.

Table 3.4 Effect of necrotic enteritis challenge and cloacal inoculation with ileal or caecal flora on broiler body weight, feed intake and feed conversion ratio at 24 and 35 post hatch[1]

Treatments		Weight gain (g)		Feed intake (g)		Feed conversion ratio	
		d 24	d 35	d 24	d 35	d 24	d 35
Unchallenged	Caecal	1145.2	2230.3	1527.9	3167.5	1.334	1.420
	Ileal	1173.4	2290.3	1553.7	3235.7	1.325	1.413
	Control	1177.9	2195.9	1573.3	3182.9	1.335	1.450
Challenged	Caecal	1088.1	2186.8	1518.5	3191.2	1.396	1.459
	Ileal	1093.9	2173.5	1521.4	3180.1	1.391	1.464
	Control	1064.4	2091.1	1496.5	3095.3	1.405	1.480
SEM		24.1	50.1	34.1	72.2	0.012	0.010
Challenge[2]							
Unchallenged		1165.5[a]	2238.8[a]	1551.6	3195.4	1.331[b]	1.428[b]
Challenged		1082.1[b]	2150.5[b]	1512.1	3155.5	1.398[a]	1.468[a]
SEM		13.9	35.5	19.7	41.7	0.007	0.006
Inoculation[3]							
Caecal		1116.6	2208.6	1523.2	3179.3	1.365	1.440[b]
Ileal		1133.6	2231.9	1537.5	3207.9	1.358	1.439[b]
Control		1121.1	2143.5	1534.9	3139.1	1.370	1.465[a]
SEM		17.0	35.5	24.1	51.0	0.008	0.007
P- Value							
Challenge		<0.001	0.039	0.167	0.504	<0.001	<0.001
Inoculation		0.767	0.205	0.905	0.637	0.575	0.021
Challenge × Inoculation		0.506	0.738	0.609	0.732	0.945	0.559

[1] Means represent the average of 6 replicates of 11 birds per treatment (66 birds/treatment)

[2] Challenged birds were exposed to a subclinical necrotic enteritis challenge, induced by oral administration of *Eimeria* and *C. perfringens*. Unchallenged birds received oral administration of sterile thioglycollate broth.

[3] Flora derived from either the caeca or ileum of birds previously challenged with necrotic enteritis was administered by cloacal reverse inoculation. Control birds were inoculated with sterile saline.

3.5 Conclusions and Applications

3.5.1 Crude ileal and caecal flora inoculants from birds remained healthy after NE challenge reduced the frequency and magnitude of NE lesions in another group of broilers challenged with NE; the inoculants also improved FCR by d 35.

3.5.2 The cloacal route of administration is a useful technique to transfer gut microflora between birds, which will be useful for the further elucidation of the role of the gut microbiota in poultry health and nutrition.

3.5.3 Future research should focus on the determination of the composition of the resulting intestinal flora and the mechanisms whereby it offers protection against NE in broilers.

3.6 References and Notes

1. Timbermont, L., F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Path. 40:341-347.
2. Skinner, J. T., S. Bauer, V. Young, G. Pauling. and J. Wilson. 2010. An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. Avian Dis. 54:1237-1240.
3. Dahiya, J. P., D. C. Wilkie, A. G. Van Kessel, and M. D. Drew. 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Ani. Feed Sci. and Tech. 129:60-88.
4. Van Immerseel, F., J. I. Rood, R. J. Moore, and R. W. Titball. 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends Micro. 17: 32-36.
5. Tech, R. 1999. Necrotic enteritis and associated conditions in broiler chickens. World Poult. 15:44-47.
6. Kaldhusdal, M., M. Hofshagen, A. Løvland, H. Langstrand, and K. Redhead. 1999. Necrotic enteritis challenge models with broiler chickens raised on litter: evaluation of preconditions, Clostridium perfringens strains and outcome variables. FEMS Imm. & Med. Micro. 24:337-343.
7. Kaldhusdal, M., and E. Skjerve. 1996. Association between cereal contents in the diet and incidence of necrotic enteritis in broiler chickens in Norway. Pre. Vet. Med. 28:1-16.
8. Choct, M., and G. Annison. 1992. Anti- nutritive effect of wheat pentosans in broiler chickens: Roles of viscosity and gut microflora. Brit. Poul. Sci. 33:821-834.
9. Rodgers, N.J., R. A. Swick, M. S. Geier, R. J. Moore, M. Choct, and S-B. Wu. 2015. A Multifactorial Analysis of the Extent to Which Eimeria and Fishmeal Predispose Broiler Chickens to Necrotic Enteritis. Avian Dis. 59:38-45.

10. Collier, C. T., C. L. Hofacre, A. M. Payne, D. B. Anderson, P. Kaiser, R. I. Mackie, and H. R. Gaskins. 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth. Vet. Imm. Imm. 122:104-115.
11. Gabriel, I. L. M., and S. G. J. F. Mallet. 2006. Microflora of the digestive tract: critical factors and consequences for poultry. Wor. Poult. Sci. J. 62:499-512.
12. Choct, M. 2001. Alternatives to in-feed antibiotics in monogastric animal industry. ASA Tech. Bull. AN. 30.
13. Keyburn, A. L., R. W. Portela, K. Sproat, M. E. Ford, T. L. Bannam, X. Yan, J. I. Rood, and R. J. Moore. 2013. Vaccination with recombinant NetB toxin partially protects broiler chickens from necrotic enteritis. Vet. Res. 44:1.
14. Ferket, P. R. 2004. Alternatives to antibiotics in poultry production: responses, practical experience and recommendations. Nutr. Biotech. Feed Food Indus. 57-67.
15. Huyghebaert, G., R. Ducatelle, and F. Van Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. Vet. J. 187:182-188.
16. Morishita, T.Y., P. P. Aye, B. S. Harr, C. W. Cobb, and J. R. Clifford. 1997. Evaluation of an avian-specific probiotic to reduce the colonization and shedding of Campylobacter jejuni in broilers. Avian Dis. 850-855.
17. Patterson, J., and K. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82:627-631.
18. Ahmad, I. 2006. Effect of probiotics on broilers performance. Int. J. Poult. Sci. 5:593-597.
19. Ehrmann, M. A., P. Kurzak, J. Bauer, and R. F. Vogel. 2002. Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. J. App. Micro. 92:966-975.
20. Edens, F. W. 2003. An alternative for antibiotic se in poultry: probiotics. Revi. Bras. Ciên. Aví. 5:75-97.
21. Waititu, S. M., A. Yitbarek, E. Matini, H. Echeverry, E. Kiarie, J. C. Rodriguez-Lecompte, and C. M. Nyachoti. 2014. Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poult. Sci. 93:625-635.
22. Huyghebaert, G., R. Ducatelle, and F. Van Immersell. 2011. An update on alternatives to antimicrobial growth promoters for broilers. Vet. J. 187:82-188.
23. Kaldhusdal, M., C. Schneitz, M. Hofshagen, and E. Skjerve. 2001. Reduced incidence of Clostridium perfringens-associated lesions and improved performance in broiler chickens treated with normal intestinal bacteria from adult fowl. Avian Dis. 149-156.
25. La Ragione, R. M., G. Casula, S. M. Cutting, M. J. Woodward. 2001. Bacillus subtilis spores competitively exclude Escherichia coli O78: K80 in poultry. Vet. Micro. 79:133-142.
26. Milbradt, E. L., A. S. Okamoto, J. C. Z. Rodrigues, E. A. Garcia, C. Sanfelice, L. P. Centenaro, and R. L. Andreatti Filho. 2014. Use of organic acids and competitive exclusion product as an alternative to antibiotic as a growth promoter in the raising of commercial turkeys. Poult. Sci. PS3593.
27. Hofacre, C. L., T. Beacorn, S. Collett, and G. Mathis. 2003. Using competitive exclusion, mannan-oligosaccharide and other intestinal products to control necrotic enteritis. J. of Appl. Poult. Res. 12:60-64.
28. Sacranie, A. 2007. Dietary and age regulation of antiperistalsis in broiler chickens.: Univ New England. Australia.
29. Six replications of ileal and caecal inoculants with each replication containing 20 ml were spun down at 11,000 ×g to form pellets. Resulting pellets were dispensed into 200 µl of lysis buffer from the Qiagene Stool Mini Kit for 30 minutes.

30. Primers for Lactobacillus: 5′-CAC CGC TAC ACA TGG AG-3′ and 5′-AGC AGT AGG GAA TCT TCC A-3′.
31. Primers for Bifidubacterium: 5′-GCG TCC GCT GTG GGC-3′ and 5′-CTT CTC CGG CAT GGT GTT G-3′.
32. Primers for C. perfringens: primers: 5′-GCA TAA CGT TGA AAG ATG G-3′ and 5′-CCT TGG TAG GCC GTT ACC C-3′.
33. Wu, S-B., N. Rodgers, and M. Choct. 2010. Optimized necrotic enteritis model producing clinical and subclinical infection of Clostridium perfringens in broiler chickens. Avian dis. 54:1058-1065.
34. Rodgers, N. J., R. A. Swick, M. S. Geier, R. J. Moore, M. Choct, and S-B. Wu. 2015. A Multifactorial Analysis of the Extent to Which Eimeria and Fishmeal Predispose Broiler Chickens to Necrotic Enteritis. Avian Dis. 59:38-45.
35. Prescott, J. F. 1979. The prevention of experimentally induced necrotic enteritis chickens by avoparcin. Avian Dis. 1072-1074.
36. Fuller, R. 2001.The Chicken Gut Microflora and Probiotic Supplements. Poult. Sci. 38:189-196.
37. Fan, Y. J., S. J. Chen, Y. C. Yu, J. M. Si, and B. Liu. 2006. A probiotic treatment containing Lactobacillus, Bifidobacterium and Enterococcus improves IBS symptoms in an open label trial. J. of Zhejiang Uni. Sci. 7:987-991.
38. Mountzouris, K. C., P. Tsirtsikos, E. Kalamara, S. Nitsch, G. Schatzmayr, and K. Fegeros. 2007. Evaluation of the efficacy of a probiotic containing Lactobacillus, Bifidobacterium, Enterococcus, and Pediococcus strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poult. Sci. 86: 309-317.
39. Creven, S. E., N. J. Stern, J. S. Bailey, and N. A. Cox. 2001. Incidence of Clostridium perfringens in broiler chickens and their environment during production and processing. Avian dis. 887-896.
40. Hume, M. E., L. F. Kubena, T. S. Edrington, C. J. Donskey, R. W. Moore, S. C. Ricke, and D. J. Nisbet. 2003. Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. Poult. Sci. 82:1100-1107.
41. Cao, L., X.. J. Yang, Z. J. Li, F. F. Sun, X. H. Wu, and J. H. Yao. 2012. Reduced lesions in chickens with Clostridium perfringens-induced necrotic enteritis by Lactobacillus fermentum 1.2029. Poult. Sci. 91:3065-3071.
42. Jin, L.Z., Y. W. Ho, N. Abdullah, and S. Jalaludin. 1998. Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing Lactobacillus cultures. Poult. Sci. 77:1259-1265.
43. Tuomola, E., R. Crittenden, M. Playne, E. Isolauri, and S. Salminen. 2001. Quality assurance criteria for probiotic bacteria. Amer. J. Clinical Nutr. 73:393s-398s.
44. De Roos, N.M., and M.B. Katan. 2000. Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. Amer. J. Clinical Nutr. 71:405-411.
45. Lee, Y. K., and S. Salminen. 1995. The coming of age of probiotics. Tr. Food Sci & Tech. 6:241-245.
46. Stanley, D., A. L. Keyburn, S. E. Denman, and R. J. Moore. 2012. Changes in the caecal microflora of chickens following Clostridium perfringens challenge to induce necrotic enteritis. Vet. Micro. 159:155-162.
47. Caly, D. L., R. D`Inca, E. Auclair, and D. Drider. 2015. Alternatives to Antibiotics to Prevent Necrotic Enteritis in Broiler Chickens: A Microbiologist's Perspective. Front. Micro. 6.
48. Ranjitkar, S., B, Lawley, G. Tannock, and R. M. Engberg. 2016. Bacterial Succession in the Broiler Gastrointestinal Tract. Appl. Env. Micro. 82:2399-2410.

49. Choct, M. 2006. Enzymes for the feed industry: past, present and future. World Poult. Sci. J. 62:5-16.

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STATEMENT OF ORIGINALITY

We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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TABLE 3.1	65
TABLE 3.2	67
TABLE 3.3	71
TABLE 3.4	72

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STATEMENT OF AUTHORS' CONTRIBUTION

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the Statement of Originality.

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CHAPTER 4

Dietary inclusion of arabinoxylo-oligosaccharides in response to broilers challenged with subclinical necrotic enteritis

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4.1 Abstract

1. This study investigated the prebiotic properties of arabinoxylo-oligosaccharides (AXOS) produced both *in situ* and *in vitro* for their activity against the onset of necrotic enteritis in broiler chickens.

2. A 2 × 3 factorial arrangement was applied, including necrotic enteritis challenge (challenged/unchallenged) and three dietary treatments from d 10-21. A wheat-soy commercial type basal grower diet was fed with 2% of the wheat proportion replaced by the same amount of either arabinoxylan (AX), AXOS produced from hydrolysing AX with 16,000 BXU xylanase *in vitro*, or AX fed with a 16,000 BXU xylanase (AX + E). Necrotic enteritis (NE) challenge was induced by orally infecting birds with a vaccine strain of *Eimeria* oocysts at d 9 of age followed by oral gavage of a freshly prepared *C. perfringens* broth at d 14.

3. The challenge depressed growth performance ($P < 0.001$), induced gross lesions and reduced ileal viscosity ($P < 0.001$) at d10-21. Birds fed the AXOS diet had numerically less severe gross lesions, improved feed conversion at d0-16 ($P=0.043$) and lower ileal viscosity ($P < 0.001$) at d16 compared to birds fed AX. Weight gain of the unchallenged birds ranked as follows in terms of the diets: AXOS > AX + E > AX. AX+E produced a lower ileal viscosity ($P < 0.001$) compared to the AX treatment but only led to marginal improvements in performance and intestinal lesion scores.

4. Caecal short-chain fatty acid (SCFA) concentration was higher in birds fed AXOS and AX+E compared to that fed AX, and was higher in the challenged birds compared to the unchallenged birds. Gizzard pH was lower in birds fed AX+E compared to those fed AXOS at d16. Challenged birds had lower ileum pH compared to the unchallenged birds at d16 and d21.

5. Results of this study suggest that AXOS appear to be efficacious prebiotics, as highlighted by improvements in feed conversion ratio (FCR), increased SCFA production and reduction in severity of intestinal lesions. Future studies are warranted to elucidate the types of AXOS that are most active against NE and the mechanisms by which different levels of AXOS enhance bird performance.

Keywords: Xylo-oligosaccharides, AXOS, necrotic enteritis, broilers, nutrition

4.2 Introduction

There is a strong global move to phase out in-feed antibiotics, due to concern that their prolonged use in feed leads to the development of resistance by organisms, eventually rendering those antibiotics ineffective in humans. As a result, animal industries worldwide have started to implement strategies that either completely ban the prophylactic use of antibiotics or reduce reliance on antibiotics through the use of alternative products such as enzymes, probiotics, prebiotics and plant extracts (Yang *et al.*, 2008).

One of the key barriers to complete withdrawal from antibiotic use in the case of broiler production is necrotic enteritis (NE). NE is a bacterial borne enteric disease prevalent in all poultry producing areas of the world (Wu *et al.*, 2010; Moore, 2016). There are two broad types of NE; a) the clinical form which causes a sudden increase in flock mortality (up to 50%) without premonitory signs (Helmboldt and Bryant, 1971; Riddell and Kong, 1992a) and b) the subclinical form in there is reduced performance but birds do not display obvious symptoms, nor is there a noticeable increase in mortality. The subclinical form is more financially devasting because the lack of obvious symptoms means there is delayed instigation of an effective treatment, resulting in and substantial loss in flock performance and reduced feed efficiency (Kaldhusdal et al., 1999; Timbermont, 2011). Natural occurrence of NE in chicken production is usually associated with predisposing factors that encourage proliferation of *C.Perfringens*, such as unbalanced diet composition and increased intestinal viscosity caused by the presence of dietary grains containing high levels of soluble non-starch polysaccharides ((Annett et al., 2002; Timbermont, 2011; Ao et al., 2012; Rodgers et al., 2014). This suggests that NE outbreaks can be alleviated by maintaining balanced diet compositions and healthy intestinal microbiota. Currently, antibiotics are the only known agent to effectively eliminate *C. perfringens*, the causal agent of necrotic enteritis (Dahiya et al., 2006; Moore, 2016), but there is much interest in using neutraceuticals, such as prebiotics, probiotics, symbiotics and plant extracts, and enzymes as an alternative tool, which can aid towards creating a healthy gastrointestinal environment.

Prebiotics are low-molecular-weight carbohydrates that act as substrates for beneficial gut microflora. There is a number of prebiotics currently on the market, including galactooligosaccharides, inulin and mannooligosaccharides (Yang *et al.,* 2008), that are included in the feed as additives to modulate organisms harboured in the gut of poultry. Arabinoxylo-oligosaccharides (AXOS), the products resulting from arabinoxylan degradation by xylanase, act as prebiotics in the gut ecosystem, by selectively stimulating beneficial organisms and suppressing the growth of pathogenic organisms. The potential prebiotic effects of AXOS include optimising colon function, increasing or changing the composition of short-chain fatty acids (SCFAs) and stimulating immunity. Courtin et al. (2008) reported that benefits observed on feed conversion efficiency in broiler chickens induced by 0.5% of dietary XOS were similar to that obtained by xylanase supplementation, suggesting that these low-molecular-weight carbohydrates act as prebiotics. The mechanisms by which XOS exhibit their beneficial effects in humans and animals appear to be complex; for example, Ebersbach et al. (2012) reported that XOS inhibit pathogen adhesion to enterocytes *in vitro* and Geraylou et al. (2013) demonstrated that XOS improved the non-specific immunity and changed gut colonization in fish.

Preferably, specific XOS could be produced and hence prebiotic activities tailored for use in poultry. However, in practice, there are a number of hurdles that prevent this from happening. Firstly, knowledge on the NSP substrates present in different diets remains poor as it, in turn, is constrained by research capacities in the area of carbohydrate chemistry expertise and the lack of rapid methods to detect substrates in real time. Secondly, the understanding of gut microbiota in animals regarding their functions, activity and substrate requirement is at a very early stage of development despite the advent of molecular technologies in microbiology. However, this is an area of immense potential as it holds the key to a sustainable animal production without the reliance on in-feed antibiotics.

Thus the current study investigated whether AXOS produced in the laboratory and used as a dietary supplement had a positive effect in broilers challenged with necrotic enteritis in comparison with a diet containing intact arabinoxylans as well as one that contained the same arabinoxylans plus a xylanase.

4.3 Materials and Methods

Day-old male Ross 308 chickens (n = 180) were procured from a local hatchery and immediately allocated to 30 individual floor pens of six birds per pen. Each pen had a dimension of 120 x 75 cm^2 and contained dry wood shavings as bedding material. All birds received vaccination against Marek`s disease, infectious bronchitis and Newcastle disease at the hatchery under Australian code of practice for the distribution of broiler chickens. A 2 x 3 factorial arrangement using three diets with or without necrotic enteritis challenge; there was a total of 30 pens comprised of 6 treatments of 5 pens per treatment and 6 birds per pen. Birds were given water and feed *ad libtum*. Temperature settings followed Ross 308 recommendations of 34-35°C (50-60% relative humidity) upon arrival up to d 3 of age; then the temperature was gradually decreased by 3°C until a temperature of 22-24 °C was reached by d 21. Birds were weighed at d 10 at the beginning of dietary treatments allocation to ensure a uniform distribution of pen weights across the entire flock. All procedures used in this experiment were approved by the Animal Ethics Committee of the University of New England, Armidale, Australia.

A common starter diet followed by three grower treatment diets were used (Table 4.1). From d 0 to d 10, all birds were raised on a wheat-soybean starter crumble with commercial specifications. From d 10, the basal diet with 2% (weight) of its wheat composition replaced by either arabinoxylans (AX), arabinoxylan-oligosaccharides (AXOS) or arabinoxylans + xylanase (AX+E) (16,000 BXU/g) (Econase® XT 25, AB Vista Feed Ingredients, Marlborough, UK) was fed, until d21. The arabinoxylan was isolated from a starch-milling by-product that contained 191g arabinoxylans per kg dry matter and was extracted by ethanol precipitation. AXOS was prepared *in vitro* by hydrolysing the arabinoxylans with 16,000 BXU/g xylanase in 50°C citrate buffer (50mM, pH 5.4). The diets, composed of wheat, sorghum and soybean meal, were formulated to the Ross 308 nutrient specifications and were cold-pelleted (3-3.5mm; 50-70^oC). Performance parameters during the treatment period from d 10 to d 21 were recorded, which included weight gain (WG), feed intake (FI) and the feed conversion ratio (FCR corrected for mortality and sampled birds).

Table 4.1 Nutrient composition of the starter and grower diets

Ingredient, g/kg	Starter	Grower
Wheat	568	631
Soybean meal 45.2%	261	199
Canola meal 37%	80	100
Meat and Bone Meal 53%	35	25
Tallow	35	24
Limestone	7.32	7.66
Dicalcium phosphate (18P:21Ca)	0. 39	0. 59
Salt	1.99	1.59
Sodium Bicarbonate	1.50	1.50
Premix[1]	2.00	2.00
Choline Cl 60%	0.74	0.68
L- Lysine HCl 78.4%	2.57	2.45
DL- Methionine	2.92	2.32
L- Threonine	1.74	1.38
Phytase 5000 U/g[2]	0.10	0.10
Calculated Nutrient Composition		
Crude Protein (g/kg)	24.1	22.2
ME (MJ/kg)	12.6	12.6
Crude Fat (g/kg)	5.6	4.5
Calcium (g/kg)	9.6	8.6
Available P (g/kg)	4.8	4.3
Insoluble NSP (g/kg)	71.67	73.64
Soluble NSP (g/kg)	8.39	8.71

[1]Premix per kg contains vitamins: vitamin A, 12 MIU; vitamin D, 5 MIU; vitamin E, 75 mg; vitamin K, 3 mg; nicotinic acid, 55 mg; Pantothenic acid, 13 mg; folic acid, 2 mg; Riboflavin, 8mg; cyanocobalamin, 0.016 mg; Biotin, 0.25 mg; pyridoxine, 5 mg; thiamine, 3 mg; antioxidant, 50 mg. And contains trace minerals per kg: Cu, 16 mg as copper sulphate; Mn, 60 mg as manganese sulphate; Mn, 60 mg as manganous oxide; I, 0.125 mg as potassium iodide; Se, 0.3 mg; Fe, 40 mg, as iron sulphate; Zn, 50 mg as zinc oxide; Zn, 50 mg as zinc sulphate. [2]Quantum® Blue phytase.

To carry out the necrotic enteritis challenge, a model previously described by Rodgers et al. (2015) and Wu et al. (2010) was employed. In brief, half of the birds (Challenged) were orally given 1ml PBS suspension containing a vaccine strain of *Eimeria* (Bioproperties Pty Ltd., Sydney, Australia) at d 9 of age, while the other half were given sterile PBS (Non-challenged) simultaneously. At d 14, challenged birds were orally infected with a freshly prepared field strain of *C. perfringens* type A EHE-NE18 broth (approx. 10^8 CFU/ml), whereas the unchallenged birds received a sterile broth culture as a sham treatment. The *C. perfringens* count in the inoculant had been quantified on perfringens tryptose-sulfite-cy-closerine (TSC) selective agar (Oxoid) following serial dilutions. After the challenge, dead birds were immediately examined to determine the cause of death. On d 16, 2 birds per pen were randomly chosen and euthanised to examine the intestinal lesion score, collect ileal digesta samples for viscosity analysis and measure

pH in the gizzard, ileum and caeca. Determination of lesion scores ranged from 0 to 4 as per Prescott (1979): no lesion: 0, mild: 1, moderate: 2, severe: 3 to very severe: 4. Scoring was done by two experienced people who were trained for the procedure. On d21, 2 birds per pen were randomly chosen and euthanised to collect caeca digesta samples for short chain fatty acid analysis and to again measure pH in the gizzard, ileum and caeca.

Immediately post-euthanasia on d16 and d21, the gizzard, ileum and caeca were removed intact and a digital pH meter (Ecoscan, Eutech Instruments, Singapore) with a spear tip piercing pH electrode (Sensorex S175CD) was directly inserted into the digesta in the lumen, whilst ensuring the pH electrode did not touch the intestinal wall, and pH was recorded. This was repeated 3 times, putting the probe in different areas of the section of tract each time. The probe was then rinsed with ultra-pure water.

Viscosity was measured on the ileal samples that were collected on d 16. Upper proximal part of the ileum (one inch downstream from the Meckel`s diverticulum) was cut, and its contents were collected into a 2 ml Eppendorf tube. Collected tubes were placed on ice until transferred to a -20 °C freezer. To measure the viscosity, Eppendorf tubes containing the digesta samples were thawed to room temperature and were centrifuged at 10,000 x g for 10 minutes to obtain the supernatant, from which a 0.5 ml aliquot was taken to measure viscosity (Brookfield DVIII Rheometer, Chatswood, NSW, Australia).

The short-chain fatty acid (SCFA) and lactic acid content of the caeca digesta collected on d21 were analysed. Briefly, 1 mL of internal standard (0.01 M ethyl butyric acid) was added to approximately 2 g of fresh homogenized digesta sample, and the solution was then mixed and centrifuged at 38625 × g at 5°C for 20 minutes. Approximately 1 mL of the resulting supernatant, 0.5 mL of concentrated HCl and 2.5 mL of ether were then combined. An internal standard solution and a blank were also prepared using 1 mL of the standard acid mixture and 1 mL of water respectively in place of the supernatant. The mixture was then centrifuged at 2000 ×g at 5°C for 15 minutes, and 400 µL of the resulting supernatant was combined with 40 µL of N-tert-butyldimethylsilyl-N-methyl trifluoroacetamide (MTBSTFA). The samples were then heated at 80 °C for 20 minutes, left at room temperature for 48 hours and were then analysed on a Varian CP3400 CX Gas Chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA). Total SCFA

concentration was derived as the sum of all the SCFAs measured in the sample, expressed as µmol/g digesta after log10+1 transformation.

The total anaerobic bacteria and lactobacilli concentration in the caeca digesta was analysed. Approximately 1g of digesta was snap-frozen in liquid nitrogen immediately post-collection and stored at -20°C for DNA extraction. The chromosomal DNA counts of the total microb iota and *Lactobacillus spp* was quantified by PCR amplification of 16S ribosomal DNA. Bioline Isolate II Plant DNA Kit (Bioline, Alexandria, NSW, Australia) was used to prepare template DNA samples from the digesta. Approximately 60 mg of digesta was processed by a Qiaxtractor automated DNA extractor robot (Qiagen, Australia). A NanoDrop ND-8000 UV spectrophotometer was used to assess the purity of the DNA (Thermo Fisher Scientific, Waltham, USA). Only DNA elutions that emitted ratios of between 1.6 and 1.8 in 260/280 nm wavelength were used for PCR analysis. The quantitative PCR analysis was performed on a Rotorgene-6500 real-time PCR machine (Corbett, Sydney, Australia). Duplicate samples of 10 µl were used in each PCR reaction. SensiMix™ SYBR® No-ROX (Bioline, Meridian Life Science, Memphis, USA) was used to amplify the 16S ribosomal DNA for analysis, and a SensiMix™ SYBR® No-ROX Kit was used to quantify the bacteria and lactobacilli. Species-specific 16 rRNA annealing primers, which were maintained at 300Nm, were used Lactobacillus spp. F: CACCGCTACACATGGAG and R: AGCAGTAGGGAATCTTCCA and total bacteria F: CGGYCCAGACTCCTACGGG and R: TTACCGCGGCTGCTGGCAC. Serial dilutions of linearized plasmid DNA (pCR®4-TOPO Vector, Life Technologies, Carlsbad, USA) inserted with respective amplicons were used to construct a standard curve. A threshold cycle average from the replicate samples was assigned for quantification analysis. The number of target DNA copies was calculated from the mass of the DNA, taking into account the size of the amplicon insert in the plasmid. Bacteria numbers were expressed as $\log^{10}$ (genomic DNA copy number)/g digesta.

4.4 Results and Discussion

Any study attempting to examine the interaction between nutrition and disease requires subclinical disease model that does not produce mass mortality, yet produces enough

damage regarding feed efficiency. The current experiment successfully induced subclinical necrotic challenge, as highlighted by significantly reduced body weight ($P < 0.001$) (Table 4.3) in the challenged birds compared to the unchallenged birds. AXOS treatment halved the lesion score in the challenged birds, albeit numerically (Table 4.2). This is in agreement with the work of Femia et al. (2010) which described beneficial effects of AXOS in rats, in that it reduced preneoplastic lesions in the colon. Also, preliminary research on the intestinal health of piglets showed that AXOS could up-regulate the inflammatory and antibacterial protein pancreatitis associated protein (PAP) and exert a modulatory effect on the intestinal innate immune system (Niewold et al., 2012).

Table 4.2 Effect of diets containing either arabinoxylan (AX), arabinoxylo-oligosaccharides (AXOS) or arabinoxylan + xylanase (AX+E) and necrotic enteritis challenge[1] on intestinal lesion scores

Necrotic enteritis challenge	Diet	Lesion score*	Mean lesion score
Unchallenged	AX	2	0.11
	AXOS	0	0.00
	AX+E	2	0.11
Challenged	AX	4	0.23
	AXOS	2	0.11
	AX+E	4	0.24

*****Based on the number of birds that presented lesion scores out of a total of 10 birds Lesion scores range from 0 to 4. Score 0 - intestine of healthy appearance; score 1 - gas-filled intestine with evidence of at least two necrotic lesions; score 2 - ballooned, friable, foul-smelling intestine with evidence of necrotic lesions; score 3 – intestine displaying all the above along with a yellow pseudomembrane (often described as "Turkish towel"); score 4 - prevalence of ruptures of the intestinal epithelial layer and blood-filled intestine. [1]Challenged birds were exposed to a subclinical necrotic enteritis challenge, induced by oral administration of *Eimeria* and *C.perfringens*. Unchallenged birds received oral administration of sterile thioglycolate broth.

From a production point of view, although there was no mortality, the subclinical NE challenge hampered ($P < 0.001$) bird performance. As shown in Table 4.3, for d10-16, during which the birds were challenged, the average feed intake was significantly lower in the challenged birds compared to the unchallenged birds (459 vs 357g), as was body weight (339 vs 206g), and corrected FCR figures were significantly higher in the challenged birds (1.36 vs. 1.74). Diet also had a significant effect ($P < 0.043$) on FCR at d10-16, where the diet containing AXOS produced the lowest FCR and AX produced the worst.

It is well known that AX, commonly known as pentosans, are anti-nutritive in their soluble, viscous form, depressing bird performance through increasing digesta viscosity, changing the gut microbiota and reducing nutrition digestion and absorption (Fafiolu *et al.*, 2015). Interestingly, xylanase supplementation in the AX diet did not ameliorate the negative affect of AX in the challenged birds, perhaps suggesting that *in situ* production of AXOS by the enzyme was either inadequate or was too distal to have an effect on NE. This speculation arises from the fact that AXOS added to the diet as an additive had a significant effect on FCR in the current study, possibly indicating that to have a preventative effect against NE, prebiotics needs to be available in the distal part of the gastrointestinal tract of broilers. Another week out from the challenge period, i.e., for d10-21, the negative impact of the challenge on feed intake and weight gain still remained ($P < 0.001$) although on FCR started to wane ($P < 0.068$). The FCR figures for this period were 1.37, 1.32 and 1.32, respectively, for AX, AX+E and AXOS. There was no diet x challenge interaction at any stage. This was probably a reflection of the small number of birds used in the experiment due to the extreme difficulty in amassing enough AXOS for a feeding study as well as the laborious nature of NE challenge work. Nevertheless, it produced performance levels there were comparable with the breed standard for the unchallenged (control) birds.

Table 4.3 Effect of diets containing either arabinoxylan (AX), arabinoxylo-oligosaccharides (AXOS) or arabinoxylan + xylanase (AX+E) and necrotic enteritis challenge[1] on broiler performance at d10-16 and d10-21

Treatments	d 10-16 Feed intake (g)	Weight gain (g)	FCR[1]	d 10-21 Feed intake (g)	Weight gain (g)	FCR[1]
Unchallenged						
AX	464	337	1.38	1016	747	1.37
AX+E	455	336	1.36	988	760	1.30
AXOS	459	346	1.33	967	766	1.27
Challenged						
AX	355	203	1.75	884	642	1.38
AX+E	358	197	1.82	886	665	1.34
AXOS	360	217	1.67	874	637	1.37
Diet						
AX	409	270	1.57[a]	950	695	1.37
AX+E	406	267	1.59[ab]	937	713	1.32
AXOS	409	281	1.50[b]	920	702	1.32
SEM	5.8	5.8	0.02	16.2	16.0	0.02
Necrotic Enteritis Challenge						
Unchallenged	459[a]	339[a]	1.36[b]	990[a]	758[a]	1.31
Challenged	357[b]	206[b]	1.74[a]	881[b]	648[b]	1.36
SEM	4.8	4.7	0.02	13.2	13.1	0.02
P-value						
Diet	0.920	0.195	0.043	0.441	0.726	0.202
Challenge	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.068
Diet*Challenge	0.736	0.856	0.213	0.689	0.747	0.343

[a,b] Means within the same column, within the same parameter, with no common subscript, differ significantly.

[1]Challenged birds were exposed to a subclinical necrotic enteritis challenge, induced by oral administration of *Eimeria* and *C.perfringens*. Unchallenged birds received oral administration of sterile thioglycolate broth.

[2]Calculated including the mortalities and sampled bird weights.

Table 4.4 Effect of diets containing either arabinoxylan (AX), arabinoxylo-oligosaccharides (AXOS) or arabinoxylan + xylanase (AX+E) and necrotic enteritis challenge[1] on short-chain fatty acid (SCFA) concentration and Log10 DNA enumeration of total anaerobic gut bacteria and lactobacilli using 16S-rDNA qPCR quantification in the in the caeca of broilers at d21

	SCFA (µmol/g)									Microbiota (log^{10} counts/g digesta)	
Treatments	Acetic	Propionic	Isobutyric	Isovaleric	Valeric	Butyric	Succinic	Total	Lactic Acid	Total Anaerobic	Lactobacilli
Unchallenged											
AX	30.88	1.80	0.60	0.05	0.58	11.47	1.62	46.42	0.30	10.51	8.69
AX+E	67.87	7.51	0.60	0.19	1.05	24.39	3.55	105.28	0.21	10.61	8.90
AXOS	65.47	5.05	0.39	0.21	1.32	22.48	5.44	100.75	0.66	10.53	8.83
Challenged											
AX	53.94	3.40	0.36	0.11	0.79	14.11	2.43	75.33	0.32	10.41	8.81
AX+E	77.73	3.83	0.53	0.24	1.60	20.27	11.58	116.02	0.38	10.39	8.93
AXOS	100.75	7.90	0.32	0.28	2.57	20.35	11.29	143.99	0.88	10.37	8.80
Diet											
AX	42.41^{b}	2.60	0.48	0.08^{b}	0.69^{b}	12.79	2.02^{b}	60.88^{b}	0.31^{b}	10.46	8.75
AX+E	72.80^{ab}	5.67	0.56	0.21^{a}	1.33^{ab}	22.33	7.57^{a}	110.65^{a}	0.29^{b}	10.50	8.92
AXOS	83.11^{a}	6.48	0.36	0.25^{a}	1.94^{a}	21.42	8.36^{a}	122.37^{a}	0.77^{a}	10.46	8.81
SEM	9.98	0.97	0.05	0.04	0.30	2.48	1.63	15.39	0.12	0.01	0.04
itis Challenge											
Unchallenged	54.74	4.78	0.53	0.15	0.99^{b}	19.45	3.54^{b}	84.12	0.39^{b}	10.55^{a}	8.81
Challenged	77.47	5.04	0.41	0.21	1.65^{a}	18.25	8.43^{a}	111.89	0.53^{a}	10.39^{b}	8.85
SEM	8.04	0.09	0.04	0.02	0.24	0.42	1.73	9.77	0.05	0.06	0.01
P-Value											
Diet	0.032	0.060	0.419	<0.001	0.003	0.114	0.045	0.003	<0.001	0.502	0.278
Challenge	0.076	0.842	0.351	0.053	0.020	0.763	0.027	0.060	0.043	<0.001	0.633
Diet*Challenge	0.702	0.109	0.818	0.944	0.294	0.774	0.377	0.642	0.424	0.406	0.797

[a,b,c] Means within the same column, within the same age group, with no common subscript differ significantly (P<0.05)

[1]Challenged birds were exposed to a subclinical necrotic enteritis challenge, induced by oral administration of *Eimeria* and *C.perfringens*. Unchallenged birds received oral administration of sterile thioglycolate broth.

Clinical NE models can produce mortality rates ranging from 10-60% (Ficken and Wages, 1997; Gholamiandehkordi et al., 2007), which renders a nutritional study meaningless. Subclinical NE compromises bird performance (Skinner et al., 2010; Wade and Keyburn, 2015) which provides a major economic driver for the broiler industry that can be examined using a nutritional study design. Necrotic enteritis drastically changes the intestinal environment (Stanley et al., 2012), resulting in a compromise in gut health which is often dealt with the use of antibiotics (Dahiya et al., 2006; Van Immerseel et al., 2009). Prebiotics are reported to selectively motivate the beneficial intestinal flora, thereby potentially inhibit enteric pathogens through antagonism or competitive exclusion (Patterson and Burkholder, 2003; Broekaert et al., 2011). The prebiotic properties of AXOS are evidenced in this study by heightened total SCFA concentration in the caeca of birds fed AXOS and AX+E compared to that fed AX ($P = 0.003$) (Table 4.4). This increase in SCFA concentration likely contributed towards the observed enhanced performance in birds fed these diets. In addition to presenting energy-yielding properties, SCFA formation in the caeca has been shown to reduce pH of the intestinal environment, which may inhibit acid-sensitive pathogenic bacteria (Rinttilä and Apajalahti, 2013). This is illustrated by numerically lower caeca pH observed in this study in birds fed AXOS (Table 4.6). Additionally, AXOS supplementation increased caecal lactic acid concentration ($P < 0.001$) and numerically increased butyric acid concentration, suggesting butyrate production may have been stimulated by cross-feeding between lactate-producing bacteria and lactate-utilising butyrate-producing bacteria; butyrate has been shown to improve performance, increase epithelial integrity and alter microbiota composition (De Maesschalck *et al.,* 2015; Guilloteau *et al.* 2010). The lack of significant dietary effect on butyrate and lactobacilli likely reflects the low number of replications used in the study, which was a result of the costly nature associated with amassing AX and AXOS. The NE challenged birds had greater caecal valeric, isovaleric, lactic and succinic acid concentrations compared to the unchallenged birds ($P < 0.050$). The gut microbiota of NE challenged birds is very different to that of unchallenged birds, as illustrated by the significant effect of a challenge on total anaerobic bacteria measured in the caeca ($P =< 0.001$) (Table 4.4). This has also been illustrated by Stanley *et al.* (2012), in which it was found that birds infected with *Clostridium perfringens* presented a reduced

abundance of bacterial species, namely butyrate producers, and phylotypes were more abundant in healthy birds. It is possible that one or all of these acids, such as succinic acid, may well act as a marker for bacterial metabolism in the gut that signals the onset of NE in broiler chickens. More work is required to investigate this lead further. Furthermore, these dissimilarities in SCFA concentration and microbiota likely also contributed to the significant effects of NE challenge on ileal pH at both d16 and d21 ($P < 0.001$ and $P = 0.031$, respectively). To the best of our knowledge, this is the first study that examined the prebiotic effect of AXOS against necrotic enteritis in chickens. The results obtained in this initial study are encouraging although a larger, more detailed experiment is needed to confirm the findings.

The present study measured the viscosity reading of the lower small intestine on the d 16 of age when the challenge effect was most drastic (Table 4.5). Challenge reduced the viscosity of intestinal content ($P < 0.001$), most possibly due to an increased water content of the digesta diluting the viscous NSP concentration. Diet also significantly ($P < 0.001$) affected digesta viscosity. As expected, the AX treatment markedly elevated viscosity whereas xylanase supplementation reduced it. It is well known that arabinoxylans extracted from wheat are viscous and when added to broiler diets, they increase the gut viscosity (Annison and Choct, 1991; Choct, 1999). Gizzard pH was lower in birds fed AX+E compared to those fed AXOS ($P=0.024$) (Table 4.6). This potentially indicates that partial degradation of AX, resulting in a prevalence of larger AXOS, stimulated bacterial fermentation or increased hydrochloric acid secretion. The AXOS in the AX diets may have escaped complete enzymatic digestion in the proximal part of the GIT, which increased reliance on caecal fermentation, but those in the AXOS diet were in a form that made them readily hydrolyzed into small AXOS and xylose.

Table 4.5 Effect of diets containing either arabinoxylan (AX), arabinoxylo-oligosaccharides (AXOS) or arabinoxylan + xylanase (AX+E) and necrotic enteritis challenge[1] on ileal digesta viscosity at d16

Treatment	Ileal Viscosity (mPas)
Unchallenged	
AX	19.51
AX+E	7.89
AXOS	13.75
Challenged	
AX	10.33
AX+E	4.70
AXOS	7.43
Diet	
AX	14.92[a]
AXOS	10.59[b]
AX+E	6.29[c]
SEM	2.03
Necrotic Enteritis Challenge	
Unchallenged	13.71[a]
Challenged	7.49[b]
SEM	2.20
P-Value	
Diet	< 0.001
NE Challenge	< 0.001
Diet x NE Challenge	0.083

a,b,c Means within the same column, within the same age group, with no common subscript differ significantly (P<0.05)

1Challenged birds were exposed to a subclinical necrotic enteritis challenge, induced by oral administration of *Eimeria* and *C.perfringens*. Unchallenged birds received oral administration of sterile thioglycolate broth.

Table 4.6 Effect of diets containing either arabinoxylan (AX), arabinoxylo-oligosaccharides (AXOS) or arabinoxylan + xylanase (AX+E) and necrotic enteritis challenge[1] on gizzard, ileum and caeca pH at d16 and d21

	d16			d21		
Treatments	Gizzard	Ileum	Caeca	Gizzard	Ileum	Caeca
Unchallenged						
AX	3.31	6.93	6.14	2.97	6.53	6.29
AX+E	2.71	6.84	5.97	3.02	6.46	6.27
AXOS	3.04	6.95	5.96	3.00	6.64	6.21
Challenged						
AX	2.88	5.78	6.27	3.59	6.10	6.21
AX+E	2.80	5.62	6.17	3.12	6.11	6.24
AXOS	3.43	5.36	6.06	3.19	6.20	6.12
Diet						
AX	3.10^{ab}	6.36	6.20	3.28	6.32	6.25
AX+E	2.75^{b}	6.23	6.07	3.07	6.28	6.26
AXOS	3.23^{a}	6.15	6.01	3.10	6.42	6.17
SEM	0.12	0.05	0.05	0.05	0.03	0.02
Necrotic Enteritis Challenge						
Unchallenged	3.01	6.91^{a}	6.02	2.99^{b}	6.54^{a}	6.26
Challenged	3.04	5.59^{b}	6.16	3.30^{a}	6.13^{b}	6.19
SEM	0.01	0.47	0.05	0.11	0.14	0.02
P-Value						
Diet	0.024	0.256	0.273	0.436	0.812	0.654
Challenge	0.897	<0.001	0.167	0.043	0.031	0.466
Diet*Challenge	0.064	0.169	0.904	0.305	0.972	0.948

^{a,b,c} Means within the same column, within the same age group, with no common subscript differ significantly (P<0.05)

[1]Challenged birds were exposed to a subclinical necrotic enteritis challenge, induced by oral administration of *Eimeria* and *C.perfringens*. Unchallenged birds received oral administration of sterile thioglycolate broth.

4.5 Conclusion

Prebiotics are used as an alternative to in-feed antibiotics for broiler chicken production (Choct, 2001; Patterson and Burkholder, 2003; Awad et al., 2009). The current study examined the effect of AXOS on bird performance, gastrointestinal pH and SCFA production with or without NE challenge. There is evidence that AXOS possesses prebiotic properties, as evidenced by its impact on increasing SCFA production and its potential for displaying preventative actions against NE, illustrated by reduced decline in bird performance and severity of intestinal lesions. Obviously, this was only an initial study with a limited number of birds and a short period due to a limitation in producing enough AXOS for the feeding study. Future studies will examine the chemical nature of AXOS in terms of types and structures, their effects in chickens with graded levels of inclusion on gut microflora, gut physiology (pH, microstructure) and digesta transit rate to elucidate their mechanisms of action and practical use.

4.6 References

ANNETT, C., VISTE, J., CHIRINO-TREJO, M., CLASSEN, H., MIDDLETON, D. & SIMKO, E. (2002) Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of Clostridium perfringens type A. Avian Pathology, 31:598-601.

ANNISON, G. & CHOCT, M. (1991) Anti-nutritive activities of cereal non-starch polysaccharides in broiler diets and strategies minimizing their effects. World's Poultry Science Journal, 47:232-242.

AO, Z., KOCHER A. & CHOCT, M. (2012) Effects of dietary additives and early feeding on the performance, gut development and immune status of broiler chickens challenged with Clostridium perfringens. Asian-Australasian Journal of Animal Science, 25:541-551.

AWAD, W., GHAREEB, K., ABDEL-RAHEEM, S. & BÖHM, J. (2009) Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poultry Science, 88:49-56.

BROEKAERT, W.F., COURTIN, C.M., VERBEKE, K., VAN DE WIELE, T., VERSTRAETE, W. & DELCOUR, J.A. (2011) Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. Critical Reviews in Food Science and Nutrition, 51:178-194.

CHOCT, M. (1996) Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non- starch polysaccharides in chickens. British Poultry Science, 37:609-621.

CHOCT, M. (1999) Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chickens fed wheat. British Poultry Science, 40:419-422.

COURTIN, C.M., BROEKAERT, W.F., SWENNEN, K., LESCROART, O., ONAGBESAN, O., BUYSE, J., DECUYPERE, E., VAN DE WIELE, T., MARZORATI, M., VERSTRAETE, W., HUYGHEBAERT, G. & DELCOUR, J.A. (2008) Dietary inclusion of wheat bran arabinoxylooligosaccharides induces beneficial nutritional effects in chickens. Cereal Chemistry, 85:607-613.

DAHIYA, J.P., WILKIE, D.C., VAND KESSEL, A.G. & DREW, M.D. (2006) Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Animal Feed Science and Technology, 129:60-88.

DE MAESSCHALCK, EECKHAUT, V., MAERTENS, L., DE LANGE, L., MARCHAL, L., NEZER, C., DE BAERE, S., CROUBELS, S., DAUBE, G., DEWULF, J., HAESEBROUCK, F., DUCATELLE, R., TAMINAU, B. & VAN IMMERSEEL, F. (2015) Effects of xylo-oligosaccharides on broiler chicken performance and microbiota. Applied and Environmental Microbiology, 81:5880-5888.

EBERSBACH T, ANDERSEN, J.B., BERGSTRÖM, A., HUTKINS, R.W. & LICHT, T.R. (2011) Xylo-oligosaccharides inhibit pathogen adhesion to enterocytes in vitro. Research in Microbiology, 163: 22-27.

FAFIOLU, A. O., ODUGUWA, O. O., JEGEDE, A. V., TUKURA, C. C., OLAROTIMI, I. D., TENIOLA, A. A. & ALABI, J. O. (2015) Assessment of enzyme supplementation on growth performance and apparent nutrient digestibility in diets containing undecorticated sunflower seed meal in layer chicks. Poultry Science, 94:1917-1922.

FEMIA, A.P., SALVADORI, M., BROEKAERT, W.F., FRANÇOIS, I.E., DELCOUR, J.A., COURTIN, C.M. & CADERNI G. (2010) Arabinoxylan-oligosaccharides (AXOS) reduce preneoplastic lesions in the colon of rats treated with 1, 2-dimethylhydrazine (DMH). European Journal of Nutrition, 49:127-132.

FICKEN, M. & WAGES, D. (1997) Necrotic enteritis. Diseases of Poultry, 10:261-264.

GERAYLOU, Z., SOUFFREAU, C., RURANGWA, E., DE MEESTER, L., COURTIN, C.M., DELCOUR, J.A., BUYSE, J. & OLLEVIER, F. (2013) Effects of dietary arabinoxylan-oligosaccharides (AXOS) and endogenous probiotics on the growth performance, non-specific immunity and gut microbiota of juvenile Siberian sturgeon (Acipenser baerii). Fish and Shellfish Immunology, 35:766-775.

GHOLAMIANDEHKORDI, A.R., TIMBERMONT, L., LANCKRIET, A., BROECK, W.V.D., PEDERSEN, K., DEWULF. J., PASMANS, F., HAESEBROUCK, F., DUCATELLE, R. & IMMERSEEL, F.V. (2007) Quantification of gut lesions in a subclinical necrotic enteritis model. Avian Pathology, 36:375-382.

GUILLOTEAU P., MARTIN, L., EECKHAUT, V., DUCATELLE, R., ZABIELSKI, R. & VAN IMMERSEEL, F. (2010) From the gut to the peripheral tissues: the multiple effects of butyrate. Nutrition Research Reviews, 23:366–384.

HELMBOLDT, C. & BRYANT, E. (1971) The pathology of necrotic enteritis in domestic fowl. Avian Diseases, 15: 775-780.

KALDHUSDAL, M., HOFSHAGEN, M., LØVLAND, A., LANGSTRAND, H. & REDHEAD, K. (1999) Necrotic enteritis challenge models with broiler chickens raised on litter:

evaluation of preconditions, Clostridium perfringens strains and outcome variables. FEMS Immunology & Medical Microbiology, 24:337-343.

KALDHUSDAL, M., SCHNEITZ, C., HOFSHAGEN, M. & SKJERVE, E. (2001) Reduced incidence of Clostridium perfringens-associated lesions and improved performance in broiler chickens treated with normal intestinal bacteria from adult fowl. Avian Diseases, 45:149-156.

MOORE, R.J. (2016) Necrotic enteritis predisposing factors in broiler chickens. Avian Pathology, 45:275-281.

NIEWOLD, T.A., SCHROYEN, M., GEENS, M.M., VERHELST, R.S. & COURTIN, C.M. (2012) Dietary inclusion of arabinoxylan oligosaccharides (AXOS) down regulates mucosal responses to a bacterial challenge in a piglet model. Journal of Functional Foods, 4:626-635.

PATTERSON, J. & BURKHOLDER, K. (2003) Application of prebiotics and probiotics in poultry production. Poultry Science, 82:627-631.

PRESCOTT, J. F. (1979) The prevention of experimentally induced necrotic enteritis in chickens by avoparcin. Avian Diseases, 23:1072-1074.

RIDDELL, C., & KONG, X.-M. (1992) The influence of diet on necrotic enteritis in broiler chickens. Avian Diseases, 36:499-503.

RINTTILÄ T. & APAJALAHTI, J. (2013) Intestinal microbiota and metabolites—Implications for broiler chicken health and performance. Journal of Applied Poultry Research, 22: 647-658.

RODGERS, N.J., SWICK, R.A., GEIER, M.S., MOORE, R.J., CHOCT, M. & WU, S.-B. (2014) A Multifactorial Analysis of the Extent to Which Eimeria and Fishmeal Predispose Broiler Chickens to Necrotic Enteritis. Avian Diseases, 59:38-45.

SKINNER, J.T., BAUER, S., YOUNG, V., PAULING, G. & WILSON, J. (2010) An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. Avian Diseases, 54:1237-1240.

STANLEY, D., KEYBURN, A.L., DENMAN, S.E. & MOORE, R.J. (2012) Changes in the caecal microflora of chickens following Clostridium perfringens challenge to induce necrotic enteritis. Veterinary Microbiology, 159:155-162.

TIMBERMONT, L., HAESEBROUCK, F., DUCATELLE, R. & VAN IMMERSEEL, F. (2011) Necrotic enteritis in broilers: An updated review on the pathogenesis. Avian Pathology, 40:341-347.

VAN IMMERSEEL, F., ROOD, J.I., MOORE, R.J. & TITBALL, R.W. (2009) Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in Microbiology, 17:32-36.

WU, S.-B., RODGERS, N. & CHOCT, M. (2010) Optimized necrotic enteritis model producing clinical and subclinical infection of Clostridium perfringens in broiler chickens. Avian Diseases, 54:1058-1065.

YANG,Y., IJI, P.A., KOCHER, A., MIKKELSEN, L. L. & CHOCT M. (2008) Effects of mannanoligosaccharide and fructooligosaccharide on the response of broilers to pathogenic Escherichia coli challenge. British Poultry Science, 49:550–559.

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

TYPE OF WORK	PAGE NUMBER
TABLE 4.1	86
TABLE 4.2	89
TABLE 4.3	91
TABLE 4.4	92
TABLE 4.5	95
TABLE 4.6	96

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CHAPTER 5

Dietary supplementation of xylanase, pectinase and protease in control of necrotic enteritis in broiler chickens

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5.1 Abstract

Dietary cereals contain soluble non-starch polysaccharides (soluble NSPs) that increase digesta viscosity and encourage the development of necrotic enteritis (NE) in poultry. High dietary protein levels and the presence of animal proteins also promote NE in broilers. Using enzymes to reduce indigestible carbohydrates and proteins that predispose birds to NE may, therefore, reduce the risk of NE outbreak. To explore this hypothesis, an experiment was conducted to evaluate the effects of supplementing diets with xylanase, pectinase and protease on bird performance, caeca volatile fatty acid (VFA) concentration and ileal pH and prevalence of intestinal lesions, in NE challenged birds. A 2 x 3 factorial arrangement study was conducted, where Ross 308 broilers (n=468) were fed wheat-soy commercial diets supplemented with either xylanase (WX), pectinase (VP) or protease (ProAct) and were either challenged or not with NE (challenged or unchallenged). A subclinical form of NE was induced, as highlighted by a marked increase in gross intestinal lesions ($P=0.003$) without notable mortalities. Protease enabled higher weight gain compared to those fed WX at d0-35 ($P=0.040$) and d0-24 in the unchallenged birds ($P<0.001$). Feed intake was higher at d0-35 ($P=0.001$), and d0-24 ($P=0.011$) in birds fed protease, resulting in better feed conversion ($P=0.009$) in birds fed WX compared to those fed protease. The effects of enzyme treatment and NE challenge on caecal VFA concentration were inconsistent, but interactions were observed and changes in the concentrations of formic, propionic, isobutyric, lactic and butyric acid, together with total VFA, were caused by the enzymes in the unchallenged birds but not in the challenged birds. Ileal pH was higher ($P=0.014$) in birds fed WX compared to those fed VP. Findings from this study suggest that enzyme application can be used as a tool to reduce the volume of nutrients reaching the hindgut and hence lessen the growth of pathogenic bacteria. Future studies are warranted to investigate the mechanisms by which these enzymes could aid towards combatting the effects of NE.

Key words: necrotic enteritis, xylanase, pectinase, protease, broiler

5.2 Introduction

Necrotic enteritis (NE) is a multifactorial, bacterial borne enteric disease in poultry. It causes devastating losses to productivity, resulting in mortality up to 30% in infected flocks, which costs the poultry industry as much as US$6 billion per annum worldwide (Dahiya, 2006; Van Immerseel et al., 2009; Wade and Keyburn, 2015). The subclinical form of the disease has a greater economic consequence compared to the clinical form, as the subclinical form shows less obvious signs, with initial symptoms of merely slightly reduced growth, meaning there is often delayed or even no onset of treatments and resulting in substantial loss to production (Skinner et al., 2010). NE infection is caused by Clostridium perfringens, a Gram-positive, anaerobic, rod spore-forming ubiquitous bacterium. It is an opportunistic enteric pathogen that is capable of producing a range of extracellular toxins and invasive enzymes, particularly netB toxin (Gibert et al., 1997; Keyburn et al., 2008). The proliferation of C. perfringens is usually initiated by predisposing factors in the intestine that have a negative influence on the intestinal flora or diminish the integrity of the intestinal epithelial layer. High amounts of crude protein, increased digesta viscosity and coccidiosis infection are key predisposing factors (Collier et al., 2008; Moore, 2016; Van Immerseel et al., 2009).

C. perfringens field strains are susceptible to some anti-microbial drugs (Devriese et al., 1993; Geier et al., 2010), meaning that NE can be effectively controlled and alleviated through the use of anticoccidials and antibiotics (Lanckriet et al., 2010). However, prolonged use of these broad-spectrum anti-microbial agents at low levels in poultry production has led to the fear that organisms are developing resistance, which will eventually render these antibiotics ineffective, exposing humans to infections that cannot be treated (Bedford, 2000; Choct, 2001; Lanckriet et al., 2010). Hence, there is a strong global move to phase out the use of these products in the feed (Casewell et al., 2003).

Alternatives to in-feed antibiotics as a tool to control NE, which are both cost-effective and able to be adapted to different farming systems, have been discussed extensively (Caly et al., 2015; Choct, 2001; Dahiya, 2006; Geier et al., 2010; Verstegen and Williams, 2002). A key factor for consideration is manipulation of conditions that trigger

proliferation of the causative agents of NE, namely C. perfringens (Dahiya et al., 2006; Kaldhusdal et al., 1999; Timbermont et al., 2011). In many countries, poultry diets are composed primarily of cereal grains, such as wheat, barley and rye, which contain non-starch polysaccharides (NSPs) that increase digesta viscosity, reduce nutrient availability due to impaired digestion and modify the microflora population, primarily in the small intestine (Annison and Choct, 1991; Bedford and Partridge, 2001). High levels of NSPs, particularly in the soluble form, cause C. perfringens populations to increase (Annett et al., 2002; McDevitt, 2006; Moore, 2016). Xylans are a major component of these NSPs, and they are catalysed by xylanases, from larger complex molecules into smaller chain xylans and even oligosaccharides (Collins et al., 2005). Xylanase application in poultry diets, therefore, reduces viscosity, shortens feed transit time and lowers the severity of NE by reducing the amount of nutrients that reach the hindgut (Choct, 1999; Liu et al., 2012). Another abundant soluble NSP compound in the plant cells is pectin. Pectin is primarily found in vegetable protein ingredients and has also been shown to increase digesta viscosity and water-holding capacity, and inflict changes to the microflora composition, resulting in reduced growth performance and metabolisable energy (Annison and Choct, 1991; Willats et al., 2001). Supplementing diets with pectinase hydrolyses pectin, reducing viscosity and eliminating the predisposing effect of pectin on NE.

Dietary protein sources and amino acid balance influence proliferation of C. perfringens; for example, animal proteins, especially meat and bone meal and fish meal, and consumption of diets containing lower energy: protein ratios and low CP digestibility enhance C. perfringens proliferation and toxin production, due to increased substrate availability for C. perfringens (Shojadoost et al., 2012; Palliyeguru et al., 2010). As a result, protease supplementation not only improves bird performance, due to enhanced amino acid digestibility which aids growth and improves immune response to inflammation (Oxenboll et al., 2011; Simbaya et al., 1996) but also potentially leads to reduced growth of C. perfringens.

The aim of this study was to examine the potential positive effects of xylanase, protease and pectinase in broilers challenged with necrotic enteritis. The hypothesis was that

application of these enzymes would reduce the amount of nutrients reaching the hindgut and hence alleviate the effects of NE, partly through reducing the proliferation of C. perfringens.

5.3 Materials and Methods

5.3.1 Birds and Husbandry

A total of 468 day-old male Ross308 broiler chickens were procured from a local hatchery and were immediately allocated into a total of 36 individual floor pens (approximate area of 120 x 75 cm2 per pen) with wood shavings as bedding material. All birds received vaccination against Marek's disease, infectious bronchitis and Newcastle disease at the hatchery under the Australian Code of practice for the distribution of broiler chickens. A 2 × 3 factorial arrangement was used, with 6 treatments of 6 pens per treatment and 13 birds per pen. The treatments were comprised of NE challenge (challenged or unchallenged) and 3 dietary treatments: a basal diet supplemented with either xylanase (Ronozyme WX, DSM) (WX), pectinase (Ronozyme VP, DSM) (VP) or protease (Ronozyme ProAct, DSM) (ProAct). Birds had ad libitum access to water and feed throughout the trial period. Temperature settings followed Ross 308 recommendations of 34-35°C (50-60% relative humidity) upon arrival up to d 3 of age; then the temperature was gradually decreased by 3°C until the temperature of 22-24°C was reached by d 21. The lighting regimen used was 24 h light on d1, with darkness increasing by one hour a day until 6 hours of darkness was reached, which was maintained throughout the remainder of the study. Total pen weight and feed intake (FI) was determined on d12, 20, 24 and 35 post-hatch and was used to calculate feed conversion ratio (FCR). Mortality was recorded daily, and any birds culled, or dead were weighed. All procedures used in this experiment were approved by the Animal Ethics Committee of the University of New England, Armidale, Australia.

5.3.2 Dietary Treatments

The basal diet was composed of wheat, tallow, soybean meal and canola solvent meal and was formulated to meet Ross 308 specifications (Aviagen, 2012), but with slightly lower

ME levels (Starter 2950kcal, Grower 3000kcal and Finisher 3050kcal). The WX, VP and protease were supplemented on top of the basal diet at levels of 150g/MT in the starter diet and 200g/MT in the grower and finisher diets. The diet was fed as a starter from d0-12, a grower from d13-24 and finisher from d25-35. The diet was cold pelleted and was fed as crumble (⌀0.1-0.2mm) from d 0 to d 8 and then pellet (⌀3mm pellet) for the remainder of the trial period.

5.3.2 Necrotic enteritis challenge

The NE challenge was initiated using a model previously described by Wu et al. (2010). In brief, the challenged birds (n=234) were orally administered 1 ml PBS suspension containing a vaccine strain of Eimeria (Bioproperties Pty Ltd., Werribee, VIC, Australia) at d 9 of age, followed by an oral infection of 1 ml freshly prepared Clostridium perfringens type A EHE-NE18 (approximately 108 CFU/ml)(CSIRO Animal, Australia) in a starch-thioglycollate broth at d 14. The C. perfringens count in the inoculant was quantified on perfringens tryptose-sulfite-cy-closerine (TSC) selective agar (Oxoid) following serial dilutions. The unchallenged birds orally received 1 ml of sterile PBS at d 9 and 1 ml sterile broth culture at d14. After the challenge, mortality was recorded, and a necropsy examination was performed to verify the cause of death.

Table 5.1 Feed formula and the nutrient composition

Ingredient, %	Starter d 0-12	Grower d 13-24	Finisher d 25-35
Wheat	59.9	64.4	65.6
Soy bean meal	23.7	19.8	16.8
Canola meal with solvent extracted	5.3	7.0	6.0
Meat and bone meal	4.0	1.6	3.0
Tallow	4.0	4.0	4.0
Limestone	1.0	1.0	0.9
Dical Phos 18P/21Ca	0.7	0.9	0.9
Salt	0.1	0.1	0.1
Na bicarb	0.2	0.2	0.2
UNE premix[1]	0.2	0.2	2.0
Choline Cl 70%	0.0	0.0	0.0
Phytase 5000 U/g	0.013	0.014	0.013
Nutrient Composition			
ME (kcal/g)	2950	2987	3050
Crude Protein (%)	22	22	20
Crude Fat (%)	5.9	5.7	5.8
Crude Fiber (%)	3.1	3.1	2.9
Insoluble NSP (%)	4.83	5.38	5.32
Calcium (%)	1.1	1.0	1.0
Available P (%)	0.6	0.6	0.6
Sodium (%)	0.2	0.2	0.2
Digestible Amino Acids (%)			
Iso-Leucine	1.0	1.0	0.9
Arginine	1.315	1.232	1.110
Lysine	1.242	1.150	1.020
Methionine	0.561	0.490	0.462
Methionine & Cystine	0.922	0.870	0.800
Trptophan	0.226	0.227	0.201
Threonine	0.810	0.770	0.680
Valine	0.943	0.941	0.849

[1]Premix per kg contains vitamins: vitamin A, 12 MIU; vitamin D, 5 MIU; vitamin E, 75 mg; vitamin K, 3 mg; nicotinic acid, 55 mg; Pantothenic acid, 13 mg; folic acid, 2 mg; Riboflavin, 8mg; cyanocobalamin, 0.016 mg; Biotin, 0.25 mg; pyridoxine, 5 mg; thiamine, 3 mg; antioxidant, 50 mg. And contains trace minerals per kg: Cu, 16 mg as copper sulphate; Mn, 60 mg as manganese sulphate; Mn, 60 mg as manganous oxide; I, 0.125 mg as potassium iodide; Se, 0.3 mg; Fe, 40 mg, as iron sulphate; Zn, 50 mg as zinc oxide; Zn, 50 mg as zinc sulphate.

5.3.3 Response Variables

On d16, 2 birds per pen (12 birds per treatment) were randomly selected and euthanized to examine the intestinal lesion score, collect caecal digesta for analysis of VFA content and measure pH in the ileum. Determination of lesion scores ranged from 0 to 4 as per Prescott (1979); no gross changes: score 0; mild friable small intestine or thin walled: score 1; moderate ulceration or focal necrosis: score 2; larger patches of necrosis: score 3; severe and extensive necrosis typical of field cases of necrotic enteritis: score 4. Scoring

was carried out by three experienced people who were trained for the procedure with no prior knowledge about the treatments, and lesion score was expressed as the mean value within the same treatment.

Ileal pH was measured by suspending 2 g of ileal digesta sample in 1 ml of ultra-pure water (pH 7.0 ± 0.1) and then measuring the pH using an EC-CON6-03PLUS pH meter (Eutech Instruments, Australia). The probe was rinsed with ultra-pure water after every bird and was recalibrated after every 6 samples.

VFA concentration of the caeca digesta was analysed. Briefly, 1 mL of internal standard (0.01 M ethyl butyric acid) was added to approximately 0.8 g of fresh homogenized digesta sample, and the solution was then mixed and centrifuged at 39,000 × g at 5°C for 20 minutes. Approximately 1 mL of the resulting supernatant, 0.5 mL of concentrated 36.5% HCl and 1 mL of diethyl-ether were then combined. An internal standard solution and a blank were also prepared using 1 mL of the standard acid mixture and 1 mL of water respectively in place of the supernatant. The mixture was then centrifuged at 2000 × g at 5°C for 15 minutes, and 400 µL of the resulting supernatant was combined with 40 µL of N-tert-butyldimethylsilyl-N-methyl trifluoroacetamide (MTBSTFA). The samples were then heated at 80°C for 20 minutes, left at room temperature for 48 hours and were then analysed on a Varian CP3400 CX Gas Chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA). Total VFA concentration was derived as the sum of all the VFAs measured in the sample, expressed as µmol/g digesta after log10+1 transformation.

5.3.4 Data Analysis

Statistical analysis was conducted using JMP® statistical software (2015 version 12 ed. SAS Inst. Inc., Cary, NC). When differences between treatments were statistically significant, comparisons of means were separated using Tukey's post-hoc test. Statistical significance was declared at $P \leq 0.05$.

5.4 Results

5.4.1 Performance and Ileal Lesion Scores

A subclinical form of NE challenge was induced in this study, as highlighted by a lack of significant impact of NE challenge on the livability of the birds but significantly higher ileal lesion scores (P=0.003) in the infected birds (Table 5.2). Enzyme treatment had no significant impact on livability or lesion score. Table 5.3 shows that an interaction between NE challenge and enzyme application was observed on bird weight gain (WG) from d0-24 (P<0.001); enzyme had no significant effect on the challenged birds, but the unchallenged birds fed protease had higher WG compared to those fed WX. The birds fed the diets with protease had higher WG at d0-35 (P=0.040) compared to those fed the diets with WX. Feed intake (FI) at d0-35 was higher (P=0.001) in birds fed protease compared to any other diet, and at d0-24 was higher (P=0.011) in birds fed protease compared to WX. As a result, feed conversion ratio (FCR) was better (P=0.009) at d0-35 in birds fed WX compared to those fed protease. The unchallenged birds had higher WG and FI and lowered FCR compared to the challenged birds at d0-24 (P<0.001) and d0-35 (P<0.001, P<0.001 and P=0.004, respectively). Performance at d12-20 was measured to assess the effects of the NE challenge on performance and determine whether the enzyme application showed an improvement effect (Table 5.4). It was shown that WG and FI were higher and FCR was worsened by the NE challenge at d12-20 (P<0.001), compared to the unchallenged birds. Birds fed the diets with protease had higher WG (P=0.001) at d12-20 than those fed VP or WX and had higher FI (P=0.009) than those fed WX. No significant interactions between challenge and enzymes were observed at d12-20.

Table 5.2 Effect of protease (), pectinase (VP) and xylanase (WX) and necrotic enteritis challenge on broiler livability at d0-35 and ileal lesion score at d16

Main effects	Livability %	Lesion Score
Challenge		
Unchallenged	92.6	0.06[b]
Challenged	89.5	1.11[a]
SEM	1.1	0.23
Enzyme		
ProAct	93.5	0.71
VP	87.0	0.50
WX	92.6	0.54
SEM	1.7	0.28
P-value		
Challenge	0.407	0.003
Enzyme	0.311	0.857
Challenge*Enzyme	0.590	0.909

[a, b,] Means within the same column, within the same parameter, with no common subscript differ significantly. The statistical significant difference was declared at $p \leq 0.05$.

Table 5.3 Effect of protease (ProAct), pectinase (VP) and xylanase (WX) and necrotic enteritis challenge on broiler performance at d0-12, d0-24 and d0-35

		d 0-12			d 0-24			d 0-35		
		WG (g)	FI (g)	FCR	WG (g)	FI (g)	FCR	WG (g)	FI (g)	FCR
*Unchal	ProAct	411.0 ± 4.2	447.6 ± 5.2	1.089 ± 0.005	1455.0 ± 19.5^{a}	1853.3 ± 40.5	1.274 ± 0.017	2653.6 ± 55.1	3704.8 ± 55.1	1.397 ± 0.015
	VP	412.1 ± 3.9	450.8 ± 5.7	1.094 ± 0.005	1439.7 ± 21.1^{ab}	1814.8 ± 39.9	1.260 ± 0.011	2666.5 ± 25.8	3651.2 ± 78.1	1.369 ± 0.020
	WX	399.8 ± 4.6	438.8 ± 6.2	1.086 ± 0.009	1361.0 ± 21.0^{b}	1698.3 ± 22.4	1.248 ± 0.008	2586.1 ± 18.0	3497.1 ± 24.4	1.352 ± 0.008
*Chal	ProAct	400.3 ± 6.2	444.3 ± 6.0	1.103 ± 0.004	1116.0 ± 21.1^{c}	1504.4 ± 32.0	1.348 ± 0.010	2342.1 ± 21.1	3358.3 ± 52.1	1.434 ± 0.011
	VP	402.8 ± 4.5	439.1 ± 5.2	1.090 ± 0.004	1053.5 ± 17.3^{c}	1412.2 ± 28.2	1.341 ± 0.013	2242.3 ± 58.1	3128.1 ± 59.7	1.397 ± 0.013
	WX	408.3 ± 5.3	439.9 ± 5.0	1.078 ± 0.006	1081.4 ± 22.7^{c}	1445.0 ± 33.3	1.336 ± 0.009	2201.5 ± 36.1	3062.8 ± 56.0	1.391 ± 0.011
Enzyme										
ProAct		405.6 ± 3.9	445.9 ± 3.8	1.096 ± 0.004	1285.5 ± 52.9	1678.9 ± 58.1^{a}	1.311 ± 0.015	2497.8 ± 54.8^{a}	3531.5 ± 63.5^{a}	1.416 ± 0.010^{a}
VP		407.5 ± 3.2	444.9 ± 4.1	1.092 ± 0.003	1246.6 ± 59.7	1613.5 ± 65.0^{ab}	1.300 ± 0.015	2454.4 ± 70.8^{ab}	3389.6 ± 91.7^{b}	1.383 ± 0.012^{ab}
WX		404.1 ± .6	439.4 ± 3.8	1.082 ± 0.005	1221.2 ± 44.7	1571.7 ± 42.7^{b}	1.292 ± 0.014	2393.8 ± 61.1^{b}	3279.9 ± 71.6^{b}	1.372 ± 0.009^{b}
Challenge										
Unchallenged		407.6 ± 2.7	445.7 ± 3.3	1.089 ± 0.003	1418.6 ± 15.0	1788.8 ± 24.9^{a}	1.261 ± 0.007^{b}	2635.4 ± 21.6^{a}	3617.7 ± 37.5^{a}	1.373 ± 0.009^{b}
Challenged		403.8 ± 3.0	441.1 ± 3.0	1.090 ± 0.004	1083.6 ± 12.7	1453.9 ± 19.3^{b}	1.342 ± 0.006^{a}	2261.9 ± 26.6^{b}	3183.0 ± 43.2^{b}	1.407 ± 0.008^{a}
P-value										
Challenge		0.338	0.318	0.846	<0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.004
Enzyme		0.78	0.457	0.053	0.014	0.011	0.294	0.040	0.001	0.009
Challenge*Enzyme		0.101	0.510	0.140	0.047	0.093	0.844	0.356	0.309	0.910

WG, FI, FCR represents mean individual weight gain, mean individual feed intake and feed conversion ratio, respectively.

$^{a, b, c}$ Means within the same column, within the same parameter, with no common subscript differ significantly. The statistical significant difference was declared at $P \leq 0.05$.

*Unchal = unchallenged; *Chal = challenged.

Table 5.4 Effect of protease (ProAct), pectinase (VP) and xylanase (WX) and necrotic enteritis challenge on broiler performance at d12-20

Treatments		WG (g)	FI (g)	FCR
Unchallenged	ProAct	581.9 ± 9.0	765.1 ± 12.5	1.315 ± 0.013
	VP	569.4 ± 11.6	741.6 ± 20.0	1.302 ± 0.014
	WX	542.4 ± 7.6	701.9 ± 6.6	1.295 ± 0.009
Challenged	ProAct	331.1 ± 10.8	534.2 ± 19.7	1.613 ± 0.018
	VP	291.4 ± 11.0	485.7 ± 20.8	1.667 ± 0.016
	WX	289.1 ± 9.4	489.6 ± 14.8	1.696 ± 0.022
Enzyme				
ProAct		456.5 ± 38.4[a]	649.7 ± 36.5[a]	1.464 ± 0.048
VP		430.4 ± 42.6[b]	613.7 ± 41.0[ab]	1.484 ± 0.057
WX		415.7 ± 38.6[b]	595.8 ± 32.9[b]	1.495± 0.061
Challenge				
Unchallenged		564.6 ± 6.5[a]	736.2 ± 9.9[a]	1.304 ± 0.007[b]
Challenged		303.9 ± 7.3[b]	503.2 ± 11.4[b]	1.658 ± 0.017[a]
P-values				
Challenge		< 0.001	< 0.001	< 0.001
Enzyme		0.001	0.009	0.354
Challenge*Enzyme		0.337	0.426	0.066

WG, FI, FCR represents mean individual weight gain, mean individual feed intake and feed conversion ratio, respectively.

[a, b, c] Means within the same column, within the same parameter, with no common subscript differ significantly. The statistical significant difference was declared at $P \leq 0.05$.

5.4.2 Caecal Volatile Fatty Acids and Ileal pH

The effects of enzyme treatment and NE challenge on caecal VFA concentration were inconsistent between the different fatty acids (Table 5.5). Challenge × enzyme interactions were observed on formic acid (p=0.014), propionic acid (p=0.003), isobutyric acid (p=0.004), butyric acid (p=0.014), lactic acid (p=0.006), and total VFA (P=0.024). Enzyme effects were observed only in the unchallenged birds; no significant differences between the enzymes treatments were observed in the NE challenged birds. Formic acid and total VFA were lower in the unchallenged birds fed VP compared to those fed WX. Propionic and isobutyric acid levels were lower in the unchallenged birds fed WX compared to those fed protease and VP, whereas lactic acid level was higher in the unchallenged birds fed WX compared to those fed protease or VP. Butyric acid level was lower in the unchallenged birds fed VP than birds fed protease and WX. Ileal pH was higher in birds fed WX compared to that fed VP (P=0.014) and in the unchallenged birds compared to the challenged birds (P=0.014) (Table 5.2). No challenge × enzyme interaction was observed on ileal pH (P > 005).

Table 5.5 Effect of protease (ProAct), pectinase (VP) and xylanase (WX) and necrotic enteritis challenge on caecal volatile fatty acid concentration (µmol/g) in broilers at d16

Treatments		Formic	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric	Lactic	Succinic	Total
Unchallenged	ProAct	5.42^{ab}	5.84	4.70^{a}	4.04^{ab}	5.52^{a}	3.74	4.42	3.14^{b}	4.48	6.16^{ab}
	VP	5.13^{b}	5.73	4.67^{a}	4.08^{a}	5.25^{b}	3.75	4.28	3.13^{b}	4.30	6.02^{b}
	WX	5.68^{a}	5.87	4.52^{bc}	3.83^{c}	5.48^{a}	3.60	4.32	3.24^{a}	4.72	6.23^{a}
Challenge	ProAct	5.48^{ab}	5.85	4.65^{ab}	4.02^{ab}	5.33^{ab}	3.77	4.33	3.18^{ab}	4.52	6.13^{ab}
	VP	5.73^{a}	5.88	4.48^{bc}	3.90^{bc}	5.50^{a}	3.65	4.33	3.27^{a}	4.65	6.25^{a}
	WX	5.28^{ab}	5.82	4.72^{ab}	4.06^{ab}	5.52^{a}	3.72	4.42	3.14^{b}	4.56	6.16^{ab}
SEM		*0.07*	*0.02*	*0.03*	*0.03*	*0.03*	*0.02*	*0.02*	*0.02*	*0.06*	*0.02*
Dietary Enzymes											
ProAct		5.45	5.85	4.68	4.03	5.43	3.75	4.38	3.16	4.50	6.15
VP		5.43	5.80	4.57	3.99	5.38	3.70	4.30	3.20	4.48	6.13
WX		5.48	5.84	4.62	3.95	5.50	3.66	4.37	3.19	4.64	6.20
SEM		*0.12*	*0.03*	*0.04*	*0.04*	*0.05*	*0.03*	*0.03*	*0.03*	*0.10*	*0.04*
Unchallenged		5.50	5.80	4.60	4.00	5.50	3.70	4.40	3.20	4.60	6.20
Challenge		5.40	5.80	4.60	4.00	5.40	3.70	4.30	3.20	4.50	6.10
SEM		*0.10*	*0.03*	*0.04*	*0.04*	*0.05*	*0.03*	*0.03*	*0.02*	*0.08*	*0.03*
P-Values											
Challenge		0.513	0.392	0.768	0.884	0.555	0.666	0.600	0.321	0.517	0.331
Enzyme		0.945	0.660	0.162	0.334	0.199	0.110	0.242	0.499	0.471	0.485
Challenge × Enzyme		0.014	0.176	0.003	0.004	0.014	0.061	0.108	0.006	0.230	0.024

[a, b, c] Means within the same column, within the same parameter, with no common subscript differ significantly. The statistical significant difference was declared at $P \leq 0.05$.

Table 5.6 Effect of protease (ProAct), pectinase (VP) and xylanase (WX) and necrotic enteritis challenge on ileal pH in broilers at d16

Treatments	pH
Enzyme	
ProAct	6.22[ab]
VP	6.07[b]
WX	6.54[a]
SEM	0.11
Challenge	
Unchallenged	6.90[a]
Challenged	5.65[b]
SEM	0.44
P-value	
Challenge	< 0.001
Enzyme	0.014
Challenge × Enzyme	0.664

[a, b,] Means within the same column, within the same parameter, with no common subscript differ significantly. The statistical significant difference was declared at $p \leq 0.05$.

5.5 Discussion

The NE challenge model (Rodgers et al., 2015; Wu et al., 2010) used in this study produced a successful subclinical form of the disease, as illustrated by a lack of a notable increase in mortality but a marked reduction in bird performance and increased prevalence of lesions in the ileum. As expected, the NE challenge resulted in reduced weight gain and feed intake at d12-24, immediately after delivery of the Eimeria species at d 9 and C. perfringens d 14. It is interesting to observe that the negative effects of the challenge in the younger birds were still apparent by d35, as highlighted by significant challenge effects on bird performance observed at d0-35. This again highlights the devastating impact that the subclinical form of NE has, namely as it is morbidity as opposed to mortality that depletes feed efficiency without any visible signs that could otherwise prompt immediate treatment.

The strong global move to phase out prophylactic use of antibiotics from animal industries means that a change of focus is required. Cereals such as barley, rye and wheat contain considerable amounts of arabinoxylans, β glucans, mannans, cellulose and lignin, which are not readily digested by monogastric (Choct, 2003; Collins et al., 2005). The presence of these non-digestible feed carbohydrates predisposes birds to NE (Dahiya et al., 2006).

The principal behind the use of xylanases in this study was that xylanase would potentially reduce digesta viscosity and the amount of nutrients reaching the hindgut, thus lessening the amount of nutrients available in the microflora and hence the pathogen population in the gastrointestinal tract, resulting in increased bird health (Bedford and Morgan, 1996). The findings from this study are in agreement with this hypothesis, as reflected by the best feed conversion at d0-24 and d0-35 in the birds being observed in those fed the diets with xylanase. Bird weight gain was, however, the worst in birds fed xylanase. A possible explanation is that ileal pH was the highest in birds fed this diet, which potentially reduced apparent ileal digestibility of the dietary crude protein, calcium and phosphorus, among other nutrients. This may be because viscosity was reduced in the presence of the xylanase; Smits et al. (1998) have previously shown that chickens fed a high-viscosity diet have a lower ileal digesta pH. Furthermore, there is evidence that xylanases can degrade insoluble NSP into soluble NSP (Choct et al., 2003), which can also cause an increase in pH (Wellock et al. 2008). The Ronozyme WX used in the current study contains multiple families of xylanase compounds which claim to have an affinity for both the soluble and insoluble xylans, suggesting that it may be simply that a greater dosage of the xylanase was required in order to manifest into positive effects on bird weight gain as well as FCR. It was also proposed that xylanase would reduce the number of C. perfringens (Engberg et al., 2004) and potentially the severity of the effects of the Eimeria on intestinal lesion scores (M'Sadeq et al., 2015). In this study, however, xylanase had no impact on lesion score, again suggesting that maybe higher levels of xylanase were required.

A potential additional benefit of supplementation with xylanase, alongside improvements in viscosity and nutrient availability, is that arabinoxylo-oligosaccharides (AXOS) were produced as a result of degradation of the arabinoxylans in the dietary cereals. These AXOS potentially aided towards alleviating the effects of NE by acting as a prebiotic; selectively stimulating beneficial intestinal flora, thereby potentially inhibiting enteric pathogens by competitive exclusion of antagonism (Broekaert et al., 2011). This is evidenced in this study by significantly higher formic, butyric and lactic acid and total VFA in the unchallenged birds fed WX and butyric acid in the challenged birds compared to the protease diet. The increased presence of butyric and lactic acid suggests butyrate

production may have been stimulated by cross-feeding between lactate-utilising butyrate-producing bacteria and lactate-producing bacteria.The increase in lactic acid production from the fermented carbohydrates may also have resulted in reduced pH which inhibited the growth of acid sensitive pathogens and hence enabled beneficial bacteria to flourish (Rinttilä and Apajalahti, 2013). It has previously been reported by Stanley (2012) that NE infected birds have a reduced abundance of bacterial species, especially butyrate producers and phylotypes, suggesting that the production of AXOS as a result of xylanase presence was able to compensate for this. This may partly explain why birds fed xylanase had the best feed conversion at both d0-24 and d0-35. The findings from this study suggest that xylanase may generate a protective effect against NE challenge via the action of AXOS. However, more research is warranted into the use of xylanase supplement as an alternative approach to counter NE in a post-antibiotic era, particularly focussing on the benefits xylanase has on intestinal flora (Choct, 2006; Huyghebaert et al., 2011).

Poultry diets with high protein content, or those rich in animal proteins such as meat and bone meal or fishmeal, predispose birds to NE (Wu et al., 2014). The impact of protease and pectinase supplementation was therefore investigated in this study to determine if using them to enhance the efficiency of protein digestion from both vegetable and animal based protein sources could possibly lead to reduced growth of C. perfringens. In this study, body weight gain at d0-24, d12-24 and d0-35 were the highest in birds fed protease, likely due to enhanced amino acid digestibility. However, birds fed protease also had higher feed intake, and feed conversion was the worst, likely because the contribution of the enzyme on the AME values was not sufficient to maintain an appropriate amino acid to energy ratio, particularly as the diets fed were low in energy. Proteases reduce the quantity and likelihood of undigested protein entering the hind gut, which reduces protein fermentation in the large intestine and improves gut health. In the unchallenged birds, protease resulted in heightened caecal propionic acid content compared to those fed WX, and butyric acid compared to that fed VP. These fatty acids play a direct role in the development of the intestinal epithelium, namely increasing proliferation of crypt cells and improving permeability of the intestinal mucosa, resulting in increased rate of absorption and utilisation of amino acids which may reflect changes in the microflora (Panda et al., 2009). This suggests the increased weight gain observed in birds fed

protease may be partly because of enzymatic effect on VFA production and suggests it would be advantageous to investigate if VFA production could be manipulated further by supplementing higher levels of protease or pectinase in combination with other enzymes.

As predicted, pectinase supplementation resulted in weight gain and feed conversion outputs that were similar to those observed with both the xylanase and the protease. Soybean meal contains an appreciable level of pectic polysaccharides in the form of rhamnoglacturonan and arabinogalactans (Choct et al., 2010), which can damage the integrity of epithelial tissue in the intestine and inflict notable changes to intestinal microbial populations (Kleessen et al., 2003; Pusztai and Bardocz, 1996). Thus, VP application likely alleviated these issues, instigating positive effects on the microflora. Total caecal VFA and formic acid concentrations in birds fed VP were higher in the challenged birds compared to the unchallenged birds, perhaps because in the unchallenged birds the VFAs in the diet were readily metabolised and absorbed in the upper gastrointestinal tract, so they played a limited role in modifying the host microflora populations, but the damage and hence poor integrity of the intestinal tract in the challenged birds meant little was absorbed so more was prevalent in the caeca. Formic acid has been shown to increase both villus height and crypt depth, suggesting it likely contributed towards the positive effects seen on performance by enhancing tissue turnover and maintenance (Khan and Iqbal, 2016). The lower ileal pH seen in birds fed VP probably coincides with this as VFA formation in the caeca has been shown to reduce pH of the entire intestinal environment (Rinttilä and Apajalahti, 2013). Pathogens grow in the gut where pH is near neutral or higher whereas beneficial microorganisms live in pH 5.8-6.2 and compete with pathogens, so lowering the pH by organic acids reduces the amount of pathogenic microbes and improves nutrient absorption (Boling-Frankenbach et al., 2001). This suggests it would be of interest to observe the extent to which pectinase could be used to influence pH to make it favourable for beneficial bacteria and detrimental for harmful pathogens.

In conclusion, this study has shown that it may be beneficial to use xylanase, protease and pectinase supplementation to reduce the amount of nutrients that reaches the hindgut and diminish indigestible carbohydrates and proteins that predispose birds to NE, thus

reducing the risk of NE outbreaks. The findings from this study suggest enzyme application can be used as an effective tool for manipulating microflora and could hence act as an alternative to the antibiotic application. Further investigation is needed into the effects of different enzyme concentrations and combinations on microflora composition in NE challenged birds to identify their potential positive effects on protecting birds from, or at least alleviating the damage of, NE infection.

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5.7 References

Annett, C., J. Viste, M. Chirino-Trejo, H. Classen, D. Middleton, and E. Simko. 2002. Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of Clostridium perfringens type A. Avian Path. 31:598-601.

Annison, G., and M. Choct. 1991. Anti-nutritive activities of cereal non-starch polysaccharides in broiler diets and strategies minimizing their effects. World. Poult. Sci. J. 47:232-242.

Aviagen, 2012. Ross 308 Broiler Nutrient Specification. www.aviagen.com, 1-8.

Bedford, M. 2000. Removal of antibiotic growth promoters from poultry diets: implications and strategies to minimise subsequent problems. World. Poult. Sci. J. 56:347-365.

Bedford, M., and A. Morgan. 1996. The use of enzymes in poultry diets. World. Poult. Sci. J. 52:61-68.

Bedford, M. R., and G. G. Partridge. 2001. Enzymes in farm animal nutrition. CABI.

Broekaert, W. F., C. M. Courtin, K. Verbeke, T. Van de Wiele, W. Verstraete, and J. A. Delcour. 2011. Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. Crit. rev. food sci. nutr. 51:178-194.

Boling-Frankenbach, S. D., J. L. Snow, C. M. Parsons, and D. H. Baker. 2001. The effect of citric acid on the calcium and phosphorus requirements of chicks fed corn-soybean meal diets. Poult. Sci. 80:783-788.

Caly, D. L., R. D'Inca, E. Auclair, and D. Drider. 2015. Alternatives to Antibiotics to Prevent Necrotic Enteritis in Broiler Chickens: A Microbiologist's Perspective. Front. Micro. 6:1336-1336.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Anti. Chem. 52:159-161.

Choct. 1999. Effects of a xylanase on individual bird variation, starch digestion throughout the intestine, and ileal and caecal volatile fatty acid production in chickens fed wheat. Br. Poult. Sci. 40:419-422.

Choct. 2001. Alternatives to in-feed antibiotics in monogastric animal industry. ASA Tech. Bull. AN 30.

Choct. 2003. Xylanase with different substrate affinities in broiler diets. Proc. Aust. Poult. Sci. Sym. 15.

Choct, M. 2006. Enzymes for the feed industry: past, present and future. World. Poult. Sci. J. 62:5-16.

Choct, M., Y. Dersjant-Li, J. McLeish, and M. Peisker. 2010. Soy Oligosaccharides and Soluble Non-starch Polysaccharides: A Review of Digestion, Nutritive and Anti-nutritive Effects in Pigs and Poultry. Asian Austral J Anim 23: 1386-1398.

Collier, C., C. Hofacre, A. Payne, D. Anderson, P. Kaiser, R. Mackie, and H. Gaskins. 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth. Vet. Immunol. Immuopathol. 122:104-115.

Collins, T., C. Gerday, and G. Feller. 2005. Xylanases, xylanase families and extremophilic xylanases. FEMS Micro. Rev. 29:3-23.

Dahiya. 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Anim. Feed. Sci. Tech. 129:60-88. doi http://dx.doi.org/10.1016/j.anifeedsci.2005.12.003

Dahiya, J., D. Wilkie, A. Van Kessel, and M. Drew. 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Anim. Feed. Sci. Tech. 129:60-88.

Devriese, L., G. Daube, J. Hommez, and F. Haesebrouck. 1993. In vitro susceptibility of Clostridium perfringens isolated from farm animals to growth- enhancing antibiotics. J. Appl. Micro. 75:55-57.

Engberg, R. M., M. S. Hedemann, S. Steenfeldt, and B. B. Jensen. 2004. Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poult. Sci.83:925-938.

Geier, M. S., L. L. Mikkelsen, V. A. Torok, G. E. Allison, C. Olnood, M. Boulianne, R. J. Hughes, and M. Choct. 2010. Comparison of alternatives to in- feed antimicrobials for the prevention of clinical necrotic enteritis. J. Appl. Micro. 109:1329-1338.

Gibert, M., C. Jolivet-Renaud, and M. R. Popoff. 1997. Beta2 toxin, a novel toxin produced by Clostridium perfringens. Gene. 203:65-73.

Huyghebaert, G., R. Ducatelle, and F. Van Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. Vet. J. 187:182-188.

Kaldhusdal, M., M. Hofshagen, A. Løvland, H. Langstrand, and K. Redhead. 1999. Necrotic enteritis challenge models with broiler chickens raised on litter: evaluation of preconditions, Clostridium perfringens strains and outcome variables. FEMS Immu. Med. Micro. 24:337-343.

Keyburn, A. L., J. D. Boyce, P. Vaz, T. L. Bannam, M. E. Ford, D. Parker, A. Di Rubbo, J. I. Rood, and R. J. Moore. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. PLoS pathog. 4:e26.

Khan, S. H., and J. Iqbal. 2016. Recent advances in the role of organic acids in poultry nutrition. J. Appl. Anim. Res. 44:359-369.

Kleessen, B., L. Hartmann, and M. Blaut. 2003. Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. Br. J. Nutr. 89:597-606.

Lanckriet, A., L. Timbermont, M. De Gussem, M. Marien, D. Vancraeynest, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2010. The effect of commonly used anticoccidials and antibiotics in a subclinical necrotic enteritis model. Avian Path. 39:63-68.

Liu, D., S. Guo, and Y. Guo. 2012. Xylanase supplementation to a wheat-based diet alleviated the intestinal mucosal barrier impairment of broiler chickens challenged by Clostridium perfringens. Avian Path. 41:291-298.

McDevitt, R. M., J. D. Brooker, T. Acamovic, and N. H. C. Sparks. 2006. Necrotic enteritis; a continuing challenge for the poultry industry. World. Poult. Sci. J. 62:221-247.

Moore, R. J. 2016. Necrotic enteritis predisposing factors in broiler chickens. Avian Path. 45:275-281.

M`Sadeq, S. A., S-B. Wu, R. A. Swick, and M. Choct. 2015. Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. Anim. Nutr. 1:1-11

Oxenboll, K., K. Pontoppidan, and F. Fru-Nji. 2011. Use of a protease in poultry feed offers promising environmental benefits. Int. J. Poult. Sci.10:842-848.

Panda, A. K., S. R. Rao, M. V. L. N. Raju, and G. S. Sunder. 2009. Effect of butyric acid on performance, gastrointestinal tract health and carcass characteristics in broiler chickens. Asia. J. Anim. Sci. 22:1026-1031.

Pusztai, A., and S. Bardocz. 1996. Biological effects of plant lectins on the gastrointestinal tract: metabolic consequences and applications. Trend. Glycosci. Glycotech8:149-166.

Rodgers, N. J., R. A. Swick, M. S. Geier, R. J. Moore, M. Choct, and S.-B. Wu. 2015. A Multifactorial Analysis of the Extent to Which Eimeria and Fishmeal Predispose Broiler Chickens to Necrotic Enteritis. Avian Dis.59:38-45. doi:10.1637/10774-011614-Reg.1

Rinttilä, T., and J. Apajalahti. 2013. Intestinal microbiota and metabolites—Implications for broiler chicken health and performance1. J. Appl. Poult. Res. 22:647-658.

Simbaya, J., B. Slominski, W. Guenter, A. Morgan, and L. Campbell. 1996. The effects of protease and carbohydrase supplementation on the nutritive value of canola meal for poultry: In vitro and in vivo studies. Anim. Feed. Sci. Tech. 61:219-234.

Skinner, J. T., S. Bauer, V. Young, G. Pauling, and J. Wilson. 2010. An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. Avian Dis. 54:1237-1240. doi 10.1637/9399-052110-Reg.1

Stanley, D., A. L. Keyburn, S. E. Denman, and R. J. Moore. 2012. Changes in the caecal microflora of chickens following Clostridium perfringens challenge to induce necrotic enteritis. Vet. Micro. 159:155-162.

Smits, C. H., A. Veldman, H. J. Verkade, and A. C. Beynen. 1998. The inhibitory effect of carboxymethylcellulose with high viscosity on lipid absorption in broiler chickens coincides with reduced bile salt concentration and raised microbial numbers in the small intestine. Poult. Sci. 77:1534-1539.

Timbermont, L., F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Path. 40:341-347.

Van Immerseel, F., J. I. Rood, R. J. Moore, and R. W. Titball. 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trend. Micro. 17:32-36.

Verstegen, M. W., and B. A. Williams. 2002. Alternatives to the use of antibiotics as growth promoters for monogastric animals. Anim. Biotech. 13:113-127.

Wade, B., and A. Keyburn. 2015. The true cost of necrotic enteritis. World Poult. 31:16-17.

Wellock, I. J., P. D. Fortomaris, J. G. M. Houdijk, and I. Kyriazakis. 2008. Effects of dietary protein supply, weaning age and experimental enterotoxigenic Escherichia coli infection on newly weaned pigs: health. 834-842.

Willats, W. G., L. McCartney, W. Mackie, and J. P. Knox. 2001. Pectin: cell biology and prospects for functional analysis. Plant Cell Wall. 9-27. Springer Netherland.

Wu, S.-B., N. Rodgers, and M. Choct. 2010. Optimized necrotic enteritis model producing clinical and subclinical infection of Clostridium perfringens in broiler chickens. Avian Dis.54:1058-1065.

Wu, S.-B., D. Stanley, N. Rodgers, R. A. Swick, and R. J. Moore. 2014. Two necrotic enteritis predisposing factors, dietary fishmeal and Eimeria infection, induce large changes in the caecal microbiota of broiler chickens. Vet. Micro. 169:188-197.

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TYPE OF WORK	PAGE NUMBER
TABLE 5.1	110
TABLE 5.2	113
TABLE 5.3	114
TABLE 5.4	115
TABLE 5.5	116
TABLE 5.6	117

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CHAPTER 6

Effect of arabinoxylans and xylo-oligosaccharides on net energy and nutrient utilization in broilers

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6.1 Abstract

Arabinoxylo-oligosaccharides (AXOS) are hydrolytic degradation products of arabinoxylans (AX) that can be fermented by the gut microbiota, thus potentially displaying prebiotic properties. This study examined the effects of AX and AXOS on net energy and nutrient utilisation in broilers. This was assessed by feeding Ross 308 broilers (n=90) wheat-soybean diets supplemented with either pure AX, AXOS produced by exposing the AX to xylanase *in vitro* (AXOS), or AX with xylanase (AX+E) from d10 to d21. Performance parameters were measured from d10-21. On d15, 10 birds per treatment were allocated to closed-circuit net energy chambers to assess the impact of AX and AXOS on dietary energy utilisation, through assessment of both metabolisable energy (ME) and net energy (NE). Ileal and caecal digesta samples were collected on d21 to determine the effect of AX and AXOS on ileal and total tract dry matter digestibility, ileal digestible energy, digesta pH and short chain fatty acid (SCFA) and microbiota concentration. FCR was numerically lowest in birds fed the diet supplemented with AXOS, at 1.26 compared to 1.37 and 1.30 for AX and AX+E, respectively. Ileal dry matter digestibility was higher in birds fed AXOS compared to that fed AX (P=0.047). Ileal digestible energy and total tract dry matter digestibility was higher in birds fed AXOS compared to that fed AX or AX+E (P=0.004 and P=0.001, respectively). Birds fed AXOS had higher ME intake (P=0.049) and nitrogen retention (P=0.001) and a strong trend of higher NE (P=0.056), NE intake (P=0.057) and retained energy (P=0.054) compared to that fed AX. Total ileal SCFA concentration, including lactic and formic acid, was higher in birds fed AXOS compared to that fed AX (P=0.011, P=0.012 and P=0.023, respectively). Birds fed AXOS, or AX+E had higher caecal total SCFA concentration, including acetic, butyric and isovaleric acid, compared to that fed AX (P=0.001, P=0.004, P=0.016 and P=0.008), and caecal propionic acid concentration was higher in birds fed AX+E than that fed AX (P=0.050). Generally, ileal and caecal microbiota concentration was numerically higher and pH lower in birds fed AXOS and AX+E compared to those fed AX. These findings indicate that AXOS generation in the gastrointestinal tract via the use of enzymes is not as efficient as feeding AXOS directly.

Results from this study suggest that AXOS has potential to be an efficacious prebiotic in broiler diets, based on its ability to stimulate SCFA production and improve utilization of dietary energy.

Key Words: Arabinoxylan; AXOS; Xylanase; Broiler chicken

6.2 Introduction

Xylans, also known as arabinoxylans and pentosans, are the most abundant hemicelluloses in the cell walls of monocotyledonous plants, such as cereals. The presence of these polysaccharides has a direct negative impact on energy availability of monogastric diets (Choct and Annison, 1990), largely due to the direct effect of soluble arabinoxylans on increasing digesta viscosity. These negative effects can be combatted by supplementing the diet with endo-β1,4-xylanases, which hydrolyses the xylan backbone. These enzymes cleave the internal β-xylosidic glycosidic linkages uninterrupted by side chains to short-chain xylans or xylo-oligosaccharides (Jommuengbout et al., 2009); resulting in a mixture of low-molecular weight xylans, arabinose-substituted xylo-oligosaccharides (arabinoxylan-oligosaccharides) (AXOS) and non-substituted xylo-oligosaccharides (XOS). Partial depolymerisation of arabinoxylans by enzymes reduces molecular chains containing more than 5,000 sugars to just over 1,000 sugars. The resulting short-chain xylans and xylo-oligosaccharides can be utilized more efficiently by organisms, which have a direct positive impact on the overall energy utilization of the cereals. Access of endogenous digestive enzymes to cell contents is also improved, and there is reduced the loss of endogenous amino acids, namely through modifications to pancreatic amylase and mucin secretion (Cowieson and Bedford, 2009; Meng et al., 2005).

Caecal and colonic bacteria generate energy from the fermentation products manufactured by hydrolysis of polysaccharides. Selective fermentation of AXOS and XOS by intestinal bacteria instigates positive effects on the composition and activity of gastrointestinal microbiota, improving health and performance in the host. XOS, therefore, fulfils the definition of a prebiotic. The potential prebiotic effects of XOS include optimizing colon function, increasing or changing the composition of short-chain fatty acids (SCFAs),

increasing mineral absorption, immune stimulation and increased ileal villus length (Kim et al., 2011). Fermentation of XOS results in the production of SCFAs, including butyrate, and lactate, which stimulates butyrate production. Butyrate fuels epithelial cells and increases intestinal epithelial integrity, which results in improved growth performance and positive changes to intestinal microbiota composition and metabolic activity (De Maesschalck et al., 2015). Indeed, Mäkeläinen et al. (2010a,b) reported that XOS were fermented with high specificity by strains of Bifidobacteria. This suggests that feeding poultry AXOS could potentially result in improved gut heath as well as heightened energy and nutrient utilization. This study examines whether it is more advantageous for arabinoxylans to be hydrolyzed into AXOS *in situ* via supplemental enzymes, or to feed diets with AXOS that has been prepared *in vitro*.

The anti-nutritive effects of soluble non-starch polysaccharides (NSP) on energy utilization are evidenced by the negative relationship between soluble arabinoxylans and apparent metaboliseable energy (AME) and that *in situ* degradation of cell wall NSP by enzymes increases AME (Hughes and Choct, 1999). The accuracy of methods used to measure metabolisable energy may be questionable as they do not take into consideration the efficiency of nutrient utilization. Additionally, AME is often corrected for nitrogen, but this system is unable to fully take into account the energy value of high protein ingredients and it partitions energy use into meat production, waste and heat production (Swick et al., 2013). As a result, net energy (NE) was used in this study to determine the true energy value of the diets, as this method takes into account energy lost as heat and differences in metabolic utilization of ME of nutrients for maintenance and production requirements (Noblet et al., 2010). It was predicted by van der Klis et al. (2010) that when an NE system is used instead of an ME system cost savings as high as €4.00–4.50/t could be achieved without any detrimental impact on production performance. This suggests that using an NE system to accurately assess the amount of energy provided from polysaccharides and oligosaccharides in feed ingredients could have significant economic value. The aim of this study was to examine the effects of arabinoxylans and AXOS on net energy and nutrient utilisation in broilers. This was assessed by feeding broilers diets containing either pure arabinoxylans, AXOS produced by exposing arabinoxylans to xylanase *in vitro* or arabinoxylans in combination with xylanase from d10 to d21.

6.3 Materials and Methods

6.3.1 Extraction of Arabinoxylans and AXOS

This study examined whether it was more efficacious to feed broilers a dietary supplement of AXOS produced in the laboratory or to feed intact arabinoxylans with xylanase. Arabinoxylan was isolated from a starch milling by-product that contained 191g arabinoxylans, 681g starch and 43g crude protein per kg dry matter. The arabinoxylans were extracted by adjusting the milling by-product to 60-70% ethanol (according to the water content), leaving the mixture at room temperature for a minimum of 24 hours, centrifuging it at 13,000 x g for 15 minutes and then freeze-drying the residue. AXOS was prepared from the resulting arabinoxylans by hydrolysing it with 16,000 BXU/g xylanase (Econase® XT 25, AB Vista Feed Ingredients, Marlborough, UK); the arabinoxylan was suspended in citrate buffer (50mM, pH 5.4) containing 0.02% azide, the pH was adjusted to pH 2.5 with HCl, and then the xylanase was added. The solution was then shaken at 50°C for 24 hours, centrifuged at 13,000 x g for 15 minutes and the residue was freeze-dried.

6.3.2 Birds and Husbandry

Ross 308, male broilers (n=90) were obtained from a commercial hatchery at the day of hatch. Chicks were randomized by weight and placed in 120 x 75 cm^2 floor pens in groups of 6, bedded on clean wood shavings. All birds received vaccination against Marek`s disease, infectious bronchitis and Newcastle disease at the hatchery under Australian code of practice for the distribution of broiler chickens. On d10 birds were individually weighed and allocated to a pen. Pen allocation was randomised across the room. Total pen weight and mean chick body weight (BW) were calculated and diet allocation was arranged to ensure there was no significant difference in BW by pen across diets. Birds were allowed *ad libitum* access to the treatment diets and water for the duration of the trial. The room was thermostatically controlled to produce an initial temperature of 34-35°C (50-60% relative humidity) upon arrival and reduced in steps of approximately 0.5°C per day, reaching 22-24°C by d21. The lighting regimen used was 24 h light on d1, with darkness increasing by one hour a day until 6 hours of darkness was reached, which

was maintained throughout the remainder of the study. All birds sampled were euthanised by cervical dislocation on d21 post hatch. This occurred after at least 6 hours of light, to ensure maximum gut fill. Total pen weight and feed intake (FI) were determined on d21 post-hatch and were used to calculate feed conversion ratio (FCR). Mortality was recorded daily and any birds culled, or dead were weighed. Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the university's animal ethics review committee.

6.3.3 Dietary Treatments

All birds were fed a standard wheat-soybean starter crumble diet from arrival to d10 (Table 6.1). From d10-21, birds were allocated to one of 3 dietary treatments; a standard wheat-soybean meal based grower diet supplemented with either an additional 2% arabinoxylan (AX), 2% AXOS or 2% arabinoxylan with 16,000 BXU xylanase (Econase® XT 25, AB Vista Feed Ingredients, Marlborough, UK) (AX+E). Diets were composed of wheat, sorghum and soybean meal and were formulated to be adequate in all nutrients, based on the Ross 308 nutrient specifications. The diets were mixed in house using a ribbon mixer and cold-pelleted (3-3.5mm, 50-70°C).

The analysed nutrient values of the diets are presented in Table 6.2. The nitrogen content of the diets was determined with a combustion analyser (Leco model FP-2000N analyser, Leco Corp., St. Joseph, MI), using EDTA as a calibration standard, and was multiplied by 6.25 to determine the protein content of the diet. Calcium and phosphorus content were analysed using inductively coupled plasma optical emission spectrometer (ICP-OES, Model- 725 radial viewed) and gross energy was determined using an adiabatic bomb calorimeter (IKA Werke, C7000, GMBH and Co., Staufen, Germany), with benzoic acid as a calibration standard. The extractable fat content was analysed by the Soxhlet method (AOAC official method 2003.05), and dry matter and ash were determined by the AOAC standard methods (930.15 and 942.05, respectively). Titanium dioxide was added at a rate of 0.5% to act as an inert marker for nutrient digestibility evaluation and the dietary content quantified by UV-spectroscopy, by the method of Short *et al.* (1996). The soluble and insoluble NSP contents and oligosaccharide concentration were analysed in each diet. Briefly, the sample was fat extracted, and the oligosaccharides were removed for further

analysis. The starch in the resulting residue was gelatinised and α-amylase and amyloglucosidase added. The prepared sample was then incubated and centrifuged and the resulting supernatant and residue used for the analysis of soluble and insoluble NSP respectively. For the soluble NSP analysis, the sugars released were removed using ethanol, the residue was dried and trifluoroacetic acid added. The supernatant from the soluble NSP analysis was used to analyse the total starch content of the diets by Megazyme Total Starch Assay (AA/AMG) kit (Megazyme, Wicklow, Ireland, UK). For the insoluble NSP analysis, the glucose released from starch digestion was removed and then acetone was added. The sample was centrifuged, the resulting supernatant was removed and the residue was dried. Dilute H_2SO_4 was added and the sample was heated, cooled and then centrifuged to sediment the insoluble materials. Ammonium (28%) was added to an aliquot of the resulting supernatant. For the oligosaccharide analysis, the sample was dried and the residue hydrolysed with H_2SO_4. It was then centrifuged to sediment the insoluble material, and 28% ammonium was added to an aliquot of the supernatant. For all the resulting samples, an internal standard was added (allose, 4mg/ml) and the sample was evaporated to dryness and re-dissolved in water with slight alkalinity. $NaBH_4$ was then added and, after incubation, excess decomposed with $C_2H_4O_2$. 1-methylimidazole and 5ml of $C_4H_6O_3$ were added followed by water, after which, the pellet was dried and reconstituted before a final centrifugation step. The supernatant was then analysed by gas chromatography.

Table 6.1 Composition of basal diet

Ingredient (%)	Starter	Grower
Wheat	56.8	63.1
Soybean meal 45.2%	26.1	19.9
Canola meal 37%	8.0	10.0
Meat and Bone Meal 53%	3.5	2.5
Tallow	3.5	2.4
Limestone	0.732	0.766
Dicalcium phosphate (18P:21Ca)	0.039	0.059
Salt	0.199	0.159
Sodium Bicarbonate	0.150	0.150
Premix[1]	0.200	0.200
Choline Cl 60%	0.074	0.068
L- Lysine HCl 78.4%	0.257	0.245
DL- Methionine	0.292	0.232
L- Threonine	0.174	0.138
Phytase 5000 U/g[2]	0.010	0.010
TiO_2	0.50	0.50

[1] Vitamin-Mineral concentrate supplied per kilogram of diet: retinol, 12000 IU; cholecalcifer 5000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niac 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamine, 16 µg; biotin, 2 µg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg; Cu (sulphate), 16 mg; Fe (sulphate), 40 m I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate a oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg [2]Phyzyme XP (Feedwor Australia)

Table 6.2 Analysed proximate composition of the experimental diets containing either 2% arabinoxylan (AX), AXOS or arabinoxylan + xylanase (AX+E)

	Starter	AX	AXOS	AX+E
Dry Matter (g/kg)	919.13	924.27	921.13	923.41
Ash (g/kg)	59.58	60.06	60.12	59.58
Protein (g/kg DM)	299.13	276.63	276.29	265.67
Total P (g/kg DM)	7.38	6.52	6.54	6.53
Total Ca (g/kg DM)	11.01	10.05	9.96	10.07
Gross Energy (MJ/kg DM)	20.24	19.97	20.01	19.80
Fat (g/kg DM)	67.66	65.25	65.73	65.87
Starch (g/kg DM)	340.40	367.09	375.27	381.88
Soluble NSP (g/kg DM)	16.13	16.37	17.52	20.30
Insoluble NSP (g/kg DM)	73.38	77.57	74.68	74.26
Oligosacchrides (g/kg DM)	38.14	34.83	33.04	36.00

6.3.4 Response Variables

Net Energy

On d15, 2 birds per pen were allocated to one of fifteen closed-circuit calorimeter chambers, 5 replicate chambers per dietary treatment. Birds were acclimatised to the calorimeter chambers for four days before collecting data and calculation of heat production (HP). All birds had *ad libitum* access to feed and water throughout the net energy trial period. Feed intake and total excreta output during the 3-day period (d19, 20 and 21) were measured. The total excreta collected was weighed and was then thoroughly homogenized and subsamples were taken for analysis of dry matter and gross energy, as described above for the diet analysis. As described by Swick *et al.* (2013), chambers were approximately 100cm long, 76cm high and 70cm wide and were made of stainless steel. Each chamber housed a wire mesh cage that was approximately 89cm long, 60cm high and 61cm wide. Water was used to seal the chambers, as highlighted by Farrell (1972). The pressure in the chamber was controlled using a barometric sensor connected to an electronic switch that activated a solenoid valve. Temperature and humidity in each chamber were monitored continuously using temperature and humidity sensors. Humidity was maintained at less than 70% for the run. Chamber air was circulated by a 28L/min diaphragm pump through a bottle containing 2L of 320g/kg KOH solution and a bubbler assembly, to absorb the CO_2 expired by the birds. The air was then passed through a trap containing 3kg of dried silica, to absorb the humidity, and was then returned to the chamber. CO_2 concentrations were maintained at less than 4ml/L. Each chamber was equipped with a 490L cylinder of medical grade O_2 fitted with a regulator and a reducing valve to replenish the O_2 as it was consumed. O_2 consumption was calculated by subtracting the weight of the O_2 cylinder at the end of each daily run from its weight at the beginning of the run. The conversion of weight to volume was based on the density of the O_2, which was approximately 1.331g/L at 20°C and 101.325 kN/m^2. Subsamples of the KOH from each chamber were collected at the end of each daily run to analyse CO_2 production by the birds. Recovery of the CO_2 was determined based on a $BaCl_2$ precipitation technique, as described by Annison and White (1961) and Swick et al., (2013). The apparent metabolisable energy was determined by the total excreta collection

method. Total heat production was measured during the whole 3 day trial period and was estimated from the O_2 consumed and CO_2 produced by the birds, using the equation total heat (kcal) = 3.866 x O_2 consumed (L) + 1.200 x CO_2 produced. The respiratory quotient (RQ) of the trial period was calculated as the ratio of CO_2 produced to the volume of O_2 consumed. Heat increment (HI) was calculated by subtracting fasting heat production from the total heat production. To correct for zero activity, a value of 450 kJ/kg $BW^{0.70}$ per bird per day was used, which corresponds to the asymptotic heat production (at zero activity) during a 24-h fast, as proposed by Noblet *et al.* (2010). Net energy (NE) was calculated as metabolisable energy (ME) intake minus HI divided by feed consumed on an as-is basis.

Ileal and Caecal pH

On d21, two birds per pen were randomly selected and euthanized by cervical dislocation to measure ileum and caeca pH and collect ileum and caeca digesta for analysis of short-chain fatty acid (SCFA) and microbiota concentration and dry matter and energy digestibility. Immediately post-euthanasia the ileum and caeca were removed intact, and a digital pH meter (Ecoscan, Eutech Instruments, Singapore) with a spear tip piercing pH electrode (Sensorex S175CD) was directly inserted into the digesta in the lumen, whilst ensuring the pH electrode did not touch the intestinal wall, and pH was recorded. This was repeated 3 times, putting the probe in different areas of the section of tract each time. The probe was then rinsed with ultra-pure water.

Ileal and Total Tract Digestibility

Following the pH measurements, the digesta was collected on an individual bird basis and was weighed. It was then freeze-dried and re-weighed to determine the dry matter content. The dried digesta was then ground into a fine powder, and the energy and TiO_2 content were measured, using the same methods as described earlier for the diet and excreta analysis. Ileal dry matter digestibility and total tract dry matter digestibility was determined using the equation: Digestibility (%) = [1 − (TiO_2 diet/TiO_2 ileal digesta or excreta) × (DM ileal digesta or excreta/DM diet)] × 100. Ileal digestible energy was calculated by multiplying the percent digestibility by the diet energy content.

Ileal and Caecal Short Chain Fatty Acid Concentration

To determine the SCFA concentration in the ileal and caecal digesta, briefly, 1 mL of internal standard (0.01 M ethyl butyric acid) was added to approximately 2 g of fresh homogenized digesta sample and the solution was then mixed and centrifuged at 38625 × g at 5°C for 20 minutes. Approximately 1 mL of the resulting supernatant, 0.5 mL of concentrated HCl and 2.5 mL of ether were then combined. An internal standard solution and a blank were also prepared using 1 mL of the standard acid mixture and 1 mL of water respectively in place of the supernatant. The mixture was then centrifuged at 2000 ×g at 5°C for 15 minutes, and 400 µL of the resulting supernatant was combined with 40 µL of N-tert-butyldimethylsilyl-N-methyl trifluoroacetamide (MTBSTFA). The samples were then heated at 80 °C for 20 minutes, left at room temperature for 48 hours and were then analysed on a Varian CP3400 CX Gas Chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA). Total SCFA concentration was derived as the sum of all the SCFAs measured in the sample, expressed as µmol/g digesta.

Ileal and Caecal Microbiota

Immediately post-collection, approximately 1g of digesta was snap-frozen in liquid nitrogen and stored at -20°C for DNA extraction. PCR amplification of 16S ribosomal DNA was used to quantify the chromosomal DNA counts of the total microflora, *Lactobacillus spp* and *Enterobacteria spp*. Template DNA samples were prepared from the digesta using Bioline Isolate II Plant DNA Kit (Bioline, Alexandria, NSW, Australia). For DNA preparation, approximately 200mg of ileal digesta was accurately weighed and vigorously shaken with 0.2 g of ⌀0.1mm glass beads before the extraction step. For the caecal samples, 60 mg of digesta was processed by a Qiaxtractor automated DNA extractor robot (Qiagen, Australia). A NanoDrop ND-8000 UV spectrophotometer was used to assess the DNA purity in all the samples (Thermo Fisher Scientific, Waltham, USA). Only DNA elutions that emitted ratios of between 1.6 and 1.8 in 260/280 nm wavelength were used for PCR analysis. The quantitative PCR analysis was performed on a Rotorgene-6500 real-time PCR machine (Corbett, Sydney, Australia). Duplicate samples of 10 µl were used in each PCR reaction. SensiMix™ SYBR® No-ROX (Bioline, Meridian Life Science, Memphis, USA) was used to amplify the 16S ribosomal DNA for

analysis. A SensiMix™ SYBR® No-ROX Kit was used to quantify the total bacteria, Enterobacteriaceae, and lactobacilli. Species-specific 16 rRNA annealing primers, which were maintained at 300Nm, were used as follows: Enterobacteriaceae F: CATTGACGTTACCCGCAGAAGAAGC and R: CTCTACGAGACTCAAGCTTGC, Lactobacillus spp. F: CACCGCTACACATGGAG and R: AGCAGTAGGGAATCTTCCA and total bacteria F: CGGYCCAGACTCCTACGGG and R: TTACCGCGGCTGCTGGCAC. Serial dilutions of linearised plasmid DNA (pCR®4-TOPO Vector, Life Technologies, Carlsbad, USA) inserted with respective amplicons were used to construct a standard curve. A threshold cycle average from the replicate samples was assigned for quantification analysis. The number of target DNA copies was calculated from the mass of the DNA, taking into account the size of the amplicon insert in the plasmid. Bacteria numbers were expressed as $\log^{10}$ (genomic DNA copy number)/g digesta.

6.3.5 Statistical Analysis

All data were analysed using IBM SPSS Statistics version 23. After Kolmogorov-Smirnov testing to confirm normality, one-way ANOVA was used to determine the equality of the means, with diet as the factor. Treatment means were separated using Tukey post-hoc test where appropriate. Statistical significance was declared at $P < 0.05$.

6.4 Results

6.4.1 Performance

The effect of diets containing either AX, AXOS or AX+E on broiler performance from d10-21 is illustrated in Table 6.3. Dietary treatment had no significant effect on bird performance. However, FCR was lowest numerically in birds fed the diet with AXOS and feed intake and FCR were numerically highest, and body weight gain lowest in birds fed the diet with AX.

Table 6.3 Effect of diets containing either 2% arabinoxylan (AX), AXOS or arabinoxylan + xylanase (AX+E) on individual bird performance from d10-21

Diet	FI (g)	BWG (g)	FCR
AX	1015.69	746.67	1.37
AXOS	967.00	766.43	1.26
AX+E	987.55	760.36	1.30
SEM	11.52	4.77	0.02
P-Value	0.267	0.818	0.167

6.4.2 Ileal and Total Tract Digestibility

As illustrated in Table 6.4, dietary treatment had no significant effect on the dry matter content of the ileal digesta or excreta. Ileal dry matter digestibility was higher (P=0.047) in birds fed AXOS compared to those fed AX. Ileal digestible energy and total tract dry matter digestibility was higher (P=0.004 and P=0.001, respectively) in birds fed AXOS compared to those fed AX or AX+E.

Table 6.4 Effect of diets containing either 2% arabinoxylan (AX), AXOS or arabinoxylan + xylanase (AX+E) on ileal and total tract digestibility in broilers from d10-21

Treatment	DM (%)		Ileal Digestibility		Total Tract Digestibility
	Ileal Digesta	Excreta	DM (%)	IDE (MJ/Kg)	DM (%)
AX	18.59	25.81	85.89^{b}	17.16^{b}	91.15^{b}
AXOS	17.55	24.56	86.84^{a}	17.38^{a}	91.58^{a}
AX+E	18.31	26.60	86.18^{ab}	17.06^{b}	91.32^{b}
SEM	0.25	0.48	0.23	0.08	0.10
P-Value	0.169	0.313	0.047	0.004	0.001

IDE = ileal digestible energy
$^{a-b}$ Means within the same column with no common superscript differ significantly ($P \leq 0.05$)

6.4.3 Net Energy

The dietary treatment effects on energy balance and efficiency of energy utilisation are shown in Table 6.5. Metabolisable energy intake (kJ/kg $BW^{0.7}$/d) was higher (P=0.049) in birds fed the diet with AXOS compared to those fed the diet with AX. Retained energy (g/b/d) was lower (P=0.001) in birds fed AX compared to those fed AXOS or AX+E. Retained energy and NE intake (kJ/kg $BW^{0.7}$/d) and net energy (kJ/b/d) presented a strong trend of being higher in birds fed AXOS or AX+E compared to those fed AX (P=0.054, P=0.057 and P=0.056, respectively).

Table 6.5 Effect of diets containing either 2% arabinoxylan (AX), AXOS or arabinoxylan + xylanase (AX+E) on energy balance and efficiency of energy utilization in broilers from d10-21

Diet	AX	AXOS	AX+E	SEM	*P*-Value
Energy Value (DM basis)					
ME feed (kJ/g)	13.51	13.48	13.47	0.15	0.995
MEn feed (kJ/g)	12.73	12.64	12.49	0.15	0.825
NE feed (kJ/g)	10.50	10.88	10.63	0.16	0.662
NE:ME	0.77	0.81	0.79	0.01	0.143
Energy Partition (kJ/b/d)					
ME	1547	1631	1631	32.02	0.532
NE	1163	1358	1289	21.56	0.056
HI	431	423	449	7.59	0.410
Energy/nitrogen balance (kJ/kg $BW^{0.70}$/d)					
ME intake	1497[b]	1682[a]	1631[ab]	32.03	0.049
NE intake	1090	1240	1186	25.92	0.057
HP	814	817	831	3.55	0.122
HI	388	402	413	4.59	0.081
RE	683	865	801	31.27	0.054
Retained N (g/d/b)	2.50[b]	3.06[a]	3.46[a]	0.12	0.001
Respiratory quotient					
RQ	1.03	1.02	1.02	0.00	0.363

ME Intake= Metabolisable energy intake (kj/kg $BW^{0.7}$/d); NE intake= Net energy intake (kj/kg $BW^{0.7}$/d); HP= Heat production (HP/$BW^{0.7}$/d); FHP= Fasting heat production ($FHP^{0.7}$= 450); HI=heat increment of feeding calculated as HP-$FHP^{0.7}$ x $BW^{0.7}_{fed}$; RE = Retained energy (MEI-HP); RQ=respiratory quotient calculated as the volume of CO_2 expired/the volume of O_2 consumed; ME= Metabolisable energy measured by total excreta collection for indirect calorimetry (3 d); NE= Net energy (RE+FHP/per g of DM intake); NE/b/d = Net energy /bird/day (RE + $FHP^{0.7}$ x $BW^{0.7}_{fed}$)

[a-b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$)

Table 6.6 Effect of diets containing either 2% arabinoxylan (AX), AXOS or arabinoxylan + xylanase (AX+E) on pH, short chain fatty acid (SCFA) concentration and Log^{10} DNA enumeration of gut bacteria using 16S-rDNA qPCR quantification in the ileum in broilers at d21

		SCFA (µmol/g)			Microbiota (log^{10} counts/g digesta)		
	pH	Total	Lactic	Formic	Total anaerobic	Lactobacillus	Enterobacteria
AX	6.64	21.25[b]	18.61[b]	0.51[b]	9.61	8.15	6.01
AXOS	6.46	49.18[a]	43.58[a]	1.42[a]	9.98	8.47	6.14
AX+E	6.53	38.79[ab]	35.59[ab]	0.70[ab]	10.03	8.51	6.19
SEM	0.04	6.65	6.01	0.23	0.11	0.09	0.04
P-Value	0.831	0.011	0.012	0.023	0.088	0.375	0.499

[a-b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$)

Table 6.7 Effect of diets containing either 2% arabinoxylan, AXOS or arabinoxylan + xylanase on pH, short chain fatty acid (SCFA) concentration and Log^{10} DNA enumeration of gut bacteria using 16S-rDNA qPCR quantification in the caeca in broilers at d21

	pH	SCFA (µmol/g)						Microbiota (log^{10} counts/g digesta)		
		Total	Acetic	Propionic	Butyric	Isovaleric	Lactic	Total anaerobic	Lactobacillus	Enterobacteria
AX	6.29	46.42[b]	30.88[b]	1.79[b]	11.47[b]	0.05[b]	0.21[b]	10.51	8.69	7.70
AXOS	6.21	100.75[a]	65.47[a]	5.05[ab]	22.48[a]	0.21[a]	0.66[a]	10.53	8.90	7.80
AX+E	6.27	105.28[a]	67.87[a]	7.51[a]	24.39[a]	0.19[a]	0.30[b]	10.61	8.83	7.92
SEM	0.02	15.44	9.76	1.35	3.29	0.04	0.11	0.02	0.05	0.05
P-Value	0.869	0.001	0.004	0.050	0.016	0.008	0.005	0.411	0.290	0.881

[a-b] Means within the same column with no common superscript differ significantly ($P \leq 0.05$)

6.4.4 Ileal and Caecal SCFA and Microflora Concentration

Dietary treatment had no significant effect on the pH or microbiota content of the ileum or caeca. Table 6.6 shows that total SCFA acid concentration, along with lactic and formic acid concentration, was higher in the ileum of birds fed the diets with AXOS compared to those fed AX (P=0.011, P=0.012 and P=0.023, respectively). Table 6.7 shows that total SCFA, including acetic, butyric and isovaleric acid, was lower in the caeca of birds fed AX compared to those fed AXOS or AX+E (P=0.001, P=0.004, P=0.016 and P=0.008, respectively). Propionic acid concentration in the ceaca was higher in birds fed AX+E compared to those fed AX (P=0.050), and lactic acid concentration was higher in birds fed AXOS compared to those fed AX or AX+E (P=0.005). Generally, microbiota content was numerically higher in birds fed AXOS and AX+E compared to those fed AX.

6.2 Discussion

Results from this study suggest that AXOS has the capacity to be an efficacious prebiotic in broiler diets, as highlighted by its positive effects on broiler performance, intestinal SCFA production and energy utilisation. An interesting observation from this study was that feeding AXOS prepared *in vitro* was generally more advantageous than feeding AX+E, particularly when observing ileal and total tract digestibility. This was probably because depolymerisation of NSP *in situ* is not instantaneous, and hence AXOS generation in the gut via the use of enzymes is not as efficient as feeding AXOS directly. The concept of using AXOS as a feed additive to reduce the reliance on in-feed antibiotics is noteworthy. However, in order to develop further in this research area, technologies will need to be developed that can amass large volumes of AXOS as it is highly costly to produce on a laboratory scale to conduct a feeding experiment as we have done here. Additionally, it may be even more profitable to produce specific sized XOS *in situ*, resulting in customized prebiotic activities in broiler diets. In order to do this, a deeper understanding of the gastrointestinal microbiota is required to determine the substrate requirements and hence tailor the prebiotic capabilities to reflect the specific function and activity of the microbiota.

The observed numerical positive effect of xylanase on energy efficiency and ileal and total tract digestibility in this study is likely because xylanase reduces the integrity of plant cell walls and gastrointestinal viscosity and releases previously encapsulated nutrients (primarily starch and protein). Depolymerisation and solubilisation of AX by xylanase reduces the number of sugars in the xylan molecule, resulting in increased nutritive value by both reducing the anti-nutritional effects of arabinoxylans and increasing its competence as a prebiotic via oligosaccharide production. Performance and energy utilisation was lower in birds fed the diet containing AX, presumably because more digestive and metabolic effort was required for the birds to utilise this diet, meaning it was less efficient at providing energy for maintenance and production. This may be partly because the weight and relative proportion of energetically active organs, such as the gastrointestinal tract and pancreas, was greater in birds fed this diet (Wu et al., 2004), which increased the total cost of maintenance. This was illustrated by Gao et al. (2008) who showed that supplementing a wheat based-diet with xylanase resulted in reduced relative weights of the duodenum, jejunum, colon and pancreas in 21 day old broilers. AX contributes towards heat production and this study showed that if AX is hydrolysed *in vitro* there was numerically reduced heat increment and total cost of maintenance. It is also likely that the reduced viscosity as a result of hydrolysing the AX meant there was reduced secretion of endogenous protein, minerals and fatty acid and hence improved energy efficiency (Angkanaporn et al., 1994; Wang, 2003; Nian et al., 2011). Apparent metaboliseable energy systems are traditionally used to evaluate dietary energy utilisation in broilers, but this system does not take into consideration the efficiency of nutritent utilisation and partitioning into meat, waste (namely depot fat), losses of chemical energy in the solid, liquid and gaseous excreta and energy and chemical losses due to heat production during digestion and absorption (Swick et al., 2013). The NE:ME ratio presented in this study suggests that the net energy system may provide a more sensitive measure of energy utilisation compared to the metaboliseable energy system, but the low number of replicates used in this study means that this cannot be confirmed and requires further investigation. In order to improve energy utilization from carbohydrate fractions, further knowledge on the NSP substrates that are prevalent in different diets needs to be

improved, which is currently constrained by a lack of rapid methods available to detect substrates in real time.

Supplemental xylanase and degradation of AX *in vitro* not only enhanced nutrient digestibility but also increased the concentration of ileal and caecal SCFA, associated with increased flow of xylo-oligomers. The results presented in this study provide evidence that AXOS is readily fermented in the ileum and caeca, producing SCFA that can be absorbed and used as an energy source; namely acetic, propionic and butyric acid which are recognized for their role in enhancing energy metabolism (den Besten et al., 2013). Thus heightened SCFA production may partly explain the observed improvements in energy utilisation observed in birds fed AXOS and AX+E compared to those fed AX. Butyric acid has anti-inflammatory properties, fuels epithelial cells and increases intestinal epithelial integrity (Guilloteau et al., 2010), and hence may also have contributed to the observed numerical improvements in performance. Additionally, De Maesschalck et al. (2015) observed longer villi in the ileums of chickens fed XOS, which may be due to butyrate stimulating glucagon-like peptide 2 (GLP-2) production. This warrants further investigation. Additionally, acetic acid in the form of acetyl-coenzyme A in cells is particularly integral in energy production and is vital for ATP production and biosynthesis of long chain fatty acid, as well as playing a role in improving growth performance and increasing intestinal epithelial cell division and villus width, height and area (Hudha et al., 2010). According to Cuche et al. (2000), fermentation of oligosaccharides into short-chain fatty acids potentially triggers a neuro-hormonal response, through stimulating peptide YY, resulting in delayed gastric emptying and duodenal transit time rates and hence heightened diet digestion and nutrient absorption in the small intestine. Findings from this study suggest that microbial metabolites such SCFA have the potential to be indicators of generation and prevalence of fermentative oligosaccharides and could hence be used to measure the effects of xylanase on nutrient digestibility and retention.

Microbiota hydrolyse indigestible carbohydrates into oligosaccharides and then monosaccharides, which they then ferment in the anaerobic environment of the gut. AXOS selectively stimulates beneficial bacteria, namely bifidobacteria, and non-

digestible carbohydrates act as the main source of energy during microbial proliferation in the hindgut (Eeckhaut et al., 2008; Mäkeläinen et al., 2010a, b). The impact of diet on microbiota was not significant in this study, likely due to the low number of replicates. Microbiota concentration was however numerically higher in birds fed AXOS and AX+E compared to those fed AX, particularly lactobacillus concentration, highlighting that XOS alters microbiota composition. Lactobacilli are able to aid towards protecting the intestinal wall via antagonistic activities against pathogens and can cause immunomodulation (Servin, 2004). The heightened abundance of both lactobacilli and butyric acid can be partly explained by cross-feeding mechanisms between lactate-producing bacteria and lactate-utilising butyrate-producing bacteria. This act of microbial cross-feeding likely stimulated gut health and consequently performance through the beneficial effects of butyrate. Lactobacillus also readily ferment AXOS into lactic acid which lowers pH. This is highlighted in this study by the comparatively higher ileal and caecal lactic acid levels and lower pH in birds fed the diets with AXOS. This reduction in pH potentially inhibited the growth of acid-sensitive pathogenic bacteria, hence why performance and energy utilization was better in birds fed this diet. The lack of significant effect of dietary treatment on pH may be partly because the lactic acid was absorbed in the intestine or used as a substrate for the lactate- utilizing bacteria. The SCFA produced by lactobacilli can also directly stop harmful bacteria from reproducing, by acting as bacteriostatic agents, producing bacteriocins with microbicidal or microbiostatic properties and modifying the receptors used by pathogenic bacteria, which increases resistance against pathogenic microbes (Adil and Magray, 2012). Also, propionic and formic acid, which were stimulated by AXOS and AX+E, have high bacteriostatic properties due to their pH reduction activity both in feed and in the gastrointestinal tract, through pharmacogenic actions on microflora (Haque et al., 2009; Hernández et al., 2005). Reduced digesta viscosity in birds fed AXOS and AX+E compared to those AX likely increased the rate of digesta passage and hence stomach emptying which decreased fermentation of the pathogenic microbial populations, enabling the beneficial bacteria to flourish. Bacteria compete with the host for nutrients within the tract, eliciting an immune response that can dictate appetite, muscle catabolism, disease prevalence and nutrient absorption (Bedford, 2000).It is important therefore to promote the growth of bacteria that can provide nutrients

for the host and reduce the growth of bacteria that are detrimental to the host. Findings from this study suggest there is potential to use AXOS to aid and control this, but further investigation in this research area is required.

6.3 Conclusion

In conclusion, AXOS appear to be efficacious prebiotics that have positive effects on net utilization of dietary energy and bird performance. This appears to be largely due to the ability of AXOS to stimulate beneficial bacteria and SCFA production. In this study it was found feeding broilers AXOS that had been prepared *in vitro* was more effective than *in situ* AXOS production, particularly with regards to enhancing diet digestibility, highlighting the potential for using AXOS as an in feed additive in the future. Further studies are required to examine the effect of the source and structure of AXOS on their effects in broilers, and to investigate the response and impact of AXOS in different environmental conditions, namely focusing on pH, transit time and microflora composition.

6.4 References

Adil, S., Magray, S.N., 2012. Impact and manipulation of gut microflora in poultry: A review. J. Anim. Vet. Adv. 11, 873-877.

Angkanaporn, K., M. Choct, W. L. Bryden, E. F. Annison, and G. Annison. 1994. Effects of wheat pentosans on endogenous amino acid losses in chickens. Journal of the Science of Food and Agriculture 66: 399-404.

Annison, E., White, R., 1961. Glucose utilization in sheep. Biochem. J. 80, 162.

Bedford, M., 2000. Removal of antibiotic growth promoters from poultry diets: Implications and strategies to minimize subsequent problems. World's Poult. Sci. J. 56, 347-365.

Cowieson, A. J., Bedford M.R., 2009. The effect of phytase and carbohydrase on ileal amino acid digestibility in monogastricdiets: Complementary mode of action? Worlds Poult. Sci. J. 65, 609–624.

Cuche, G., Cuber, J. C., Malbert, H., 2000. Ileal short-chain fatty acids inhibit gastric motility by a humoral pathway. Am. J. Physiol. Gastro. Liver Physiol. 279, G925-G930.

De Maesschalck, C.D., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., De Baere, S., Croubels, S., Daube, G., Dewulf, J., Hasebrouck, F., Ducatelle, R.,

Taminau, B., Immerseel, F.V., 2015. Effects of xylo-oligosaccharides on broiler chicken performance and microbiota. Appl. Environ. Micrbiol. 81, 5880-5888.

Den Besen, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D-J., Bakker, B.M. 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota and host energy metabolism. J. Lipid. Res. 54, 2325-2340.

Farrell, D., 1972. An indirect closed circuit respiration chamber suitable for fowl. Poult. Sci. 51, 683–688.

Gao, F., Y. Jiang, G. H. Zhou and Z. K. Han., 2008. The effects of xylanase supplementation on performance, characteristics of the gastrointestinal tract, blood parameters and gut microflora in broilers fed on wheat-based diets. Anim. Feed Sci. Technol.142, 173-184.

Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R., Van Immerseel, F.V., 2010. From the gut to the peripheral tissues: The multiple effects of butyrate. Nutr. Res. Rev. 23, 366-384.

Hernández, F., García, V., Madrid, J., Orengo, J., Catalá., Megías, M.D., 2005. Effect of formic acid on performance, digestibility, intestinal histomorphology and plasma metabolite levels of broiler chickens. Br. Poult. Sci. 47, 50-56.

Haque, M.N., Chowdhury, R., Islam, K.M.S.,Akbar, M.A., 2009. Propionic acid is an alternative to antibiotics in poultry diet. Bang. J. Anim. Sci. 38,115-122.

Hudha, M.N., Ali, M.S., Azad, M.A.A., Hossain, M.M., Tanjim M 2010. Effect of acetic acid on growth and meat yield in broilers. Int. J. BioRes. 1, 31-35.

Hughes, R.J., Choct, M., 1999. Chemical and physical characterisitcs of grains related to variability in energy and amino acid availability on poultry. Aust. J. Agric. Res. 50, 689-701.

Jommuengbout, P., Pinitglang, S., Kyu, K.L., Ratanakhanokchai, K., 2009. Substrate-binding site of Family 11 xylanase from Bacillus firmus K-1 by molecular docking. Biosci. Biotechnol. Biochem. 73, 833-839.

Kim, G-B., Seo, Y.M., Kim, C.H., Paik, I.K., 2011. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult. Sci. 90, 75-82.

Meng, X., Slominski, B. A. C. M. Nyachoti, L. D. Campbell, Guenter, W., 2005. Degradation of cell wall polysaccharides by combinations of carbohydrase enzymes and their effect on nutrient utilization and broiler chicken performance. Poult. Sci. 84, 37-47.

Mäkeläinen, H. et al. 2010. Xylo-oligosaccharides enhance the growth of bifidobacteria and Bifidobacterium lactis in a simulated colon model. Benef Microbes 1: 81-91.

Mäkeläinen, H., M. Saarinen, J. Stowell, N. Rautonen, and A. C. Ouwehand. 2010. Xylo-oligosaccharides and lactitol promote the growth of Bifidobacterium lactis and Lactobacillus species in pure cultures. Benef Microbes 1: 139-148.

Nian, F., Guo, Y.M., Ru, Y.J., Li, F.D. and Péron, A., 2011. Effect of exogenous xylanase supplementation on the performance, net energy and gut microflora of broiler chickens fed wheat-based diets. Asian-Aust. J. Anim. Sci. 24, 400-406.

Noblet, J., van Milgen, J., Dubois, S., 2010. Utilisation of metabolisable energy of feeds in pigs and poultry: interest of net energy systems? In 'Proceedings of the 21st annual Australian poultry science symposium'. (Ed. P Selle) pp. 26–35. (Poultry Research Foundation: Sydney)

Sanchez, J.I., Marzorati, M., Grootaert, C., Baran, M., Craeyveld, V.V., Courtin, C.M., Broekaert, W.F., Delcour, J.A., Verstraete, W., Van de Wiele, T., 2008. Arabinoxylan-oligosaccharides (AXOS) affect the protein/carbohydrate fermentation balance and microbial population dynamics of the simulator of human intestinal microbial ecosystem. Microb. Biotechnol. 2, 101-113.

Short, F. J., Gorton, P., Wiseman J., Boorman. K. N., 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Anim. Feed Sci. Tech. 59, 215-221.

Swick, R.A., Wu, S-B., Zuo, J., Rodgers, N., Barekatain, M.R., Choct, M., 2013. Implications and development of a net energy system for broilers. Anim. Prod. Sci. 1231-1237.

van der Klis, J.D., Kwakernaak. C., Jansman, A., Blok, M., 2010. Energy in poultry diets: adjusted AME or net energy? In Proceedings of the 21st Annual Australian Poultry Science Symposium'. (Ed. P Selle) pp. 44–49. (Poultry Research Foundation: Sydney).

Wang, H. Y., Guo, Y.U., Yuan, J.M. 2003.The effect of xylanase on performance in broilers fed on wheat diets. Feed Study. 12,1-5

Wu, Y. B., Ravindran, V., Thomas, D.G., Birtles, M.J., Hendriks, W.H., 2004. Influence of phytase and xylanase, individually or in combination, on performance, apparent metabolisable energy, digestive tract measurements and gut morphology in broilers fed wheat-based diets containing adequate level of phosphorus. Br. Poult. Sci. 45, 76-84.

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STATEMENT OF ORIGINALITY

We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

TYPE OF WORK	PAGE NUMBER
TABLE 6.1	136
TABLE 6.2	136
TABLE 6.3	141
TABLE 6.4	141
TABLE 6.5	142
TABLE 6.6	143
TABLE 6.7	143

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STATEMENT OF AUTHORS' CONTRIBUTION

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the Statement of Originality.

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CHAPTER 7

General Conclusion

This thesis has demonstrated that there is a great deal of scope to alleviate the impact of NE in broiler chicken production by using nutritional tools. For instance, adding 10% extra amino acids reduced the severity of NE-associated performance losses in broilers, showing that a high plane of nutrition is one strategy to use in broiler production without the reliance on antimicrobials.

When birds are challenged with NE, be it clinical or subclinical, a large number of them will normally perform without any sign of sickness. These "NE-resistant" birds may have a gut microbiome completely different to birds that are susceptible to NE. Indeed, when the crude contents of the caeca and ileum from the NE-resistant birds were collected and then administered cloacally to another batch of NE challenged chicks, these crude inoculants offered significant protection against NE. This novel finding indicates that the susceptibility of birds to NE, to an extent, depends on the gut microflora of the birds.

Enzyme supplementation of poultry feed is ubiquitous throughout the world. One of the key enzymes is xylanase. Xylanase breaks down arabinoxylans in feed, producing low-molecular-weight fragments of xylans, including oligomers known as arabinoxylo-oligosaccharides or AXOS. Experimental results suggest that AXOS produced both *in situ* by supplementing an arabinoxylan-rich diet with a xylanase and *in vitro* exhibited positive effects against the onset of NE in broiler chickens. In particular, AXOS provided as a supplement in the diet appear to be effective in preventing performance losses, suggesting perhaps that a large part of the effect of xylanase supplementation in poultry feed may be due to the effects of its hydrolysis products, i.e., AXOS and other low-molecular weight xylans. Obviously, such a conclusion needs to be supported by more evidence in the future.

There is well conceded that there are a number of predisposing factors for the onset of NE. One of the factors is feed constituents, such as soluble non-starch polysaccharides and undigested proteins. Thus, this thesis investigated whether supplemental xylanase, pectinase and protease would have an effect on the severity of NE regarding performance losses. Although the findings of the study showed that enzyme application reduces the

volume of nutrients reaching the hindgut and hence lessen the growth of pathogenic bacteria, future studies are warranted to investigate the mechanisms by which these enzymes could aid towards combatting the effects of NE.

A series of studies reported in this thesis suggest that nutrition has an important role to pay in managing and mitigating the impact of NE in broiler production when antimicrobial agents are totally banned from use in feed. However, this thesis represents a small step towards understanding how nutritional strategies can be deployed to minimise the economic, welfare and health impacts of NE in broiler chickens. Therefore, much needs to be done to understand this fully and, in particular, elucidate the mechanisms by which the gut microbiome influences the outcome of NE outbreaks in broiler chickens.