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Alternatives to In-Feed Antibiotics: Effects on Broiler Performance and Gut Health

By

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Declaration

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

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

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List of Abbreviations

Acylated starch A	Acetylated high amylose maize starch
Acylated starch B	Butyralated high amylose maize starch
C	Celsius
CFU	Colony-forming units
CPE	*Clostridium perfringens* enterotoxins
CT	Threshold cycle
D	Day (s)
Dig	Digestible
DNA	Deoxyribonucleic acid
EF-Tu	Elongation factor
FCR	Feed conversion ratio
FI	Feed intake
FOS	Fructo-oligosaccharides
GIT	Gastrointestinal tract
GLM	General liner model
GLP-2	Glucagon-like peptide-2
GOS	Galacto-oligosaccharides
H	Hour (s)
HCl	Hydrochloric acid
HI	Heat increment
HP	Heat production
LMH	Leghorn male hepatoma cell line
LV	livability
ME	Metabolisable energy
MEI	Metabolisable energy intake
MOS	Mannanoligosacaritdes
MTBSTFA	N-tert-butyldimethlsilyl-N-methyltrifuoroacetamide
NE	Necrotic enteritis
NSPs	Non-starch polysaccharides
NSW	New south wales
PCR	Polymerase chain reaction

PFO	Pyruvate: ferredoxin oxidoreductase
PLC	Phospholipase C
RQ	Respiratory quotient
SAS	Statistical Analysis System
SB	Sodium butyrate
SCFA	Short chain fatty acid (s)
SEM	Standard error mean
SM	Salinomycin
UNE	University of New England
VH:CD	Villus: crypt ratio
WG	Weight gain
YCW	Yeast cell wall
ZB	Zinc bacitracin

List of Publications

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Summary

This thesis examined the efficacy of several alternative feed additives in enhancing performance and enteric health in broilers in the absence of antibiotics. Chapter 1 provides background information, leading to the objectives of conducting the experimental studies. This is followed by a review of literature in Chapter 2, covering the general characteristics of the gastrointestinal tract, gut microflora and the factors affecting gut health. The review also covers enteric diseases, namely necrotic enteritis, factors predisposing birds to necrotic enteritis and methods of controlling it.

Chapter 3 evaluates the efficacy of yeast cell wall extract derived from *Saccharomyces cerevisiae* as a replacement for zinc bacitracin and the anticoccidial ionophore salinomycin, using an established necrotic enteritis challenge model. The results showed that weight gain, feed intake and livability of challenged birds were lower than for unchallenged birds on d 24 and 35 ($P < 0.05$). All of the additives had a greater positive impact on weight gain, feed intake and livability in challenged compared to unchallenged birds. Birds given zinc bacitracin, yeast cell wall extract or salinomycin significantly improved in weight gain and livability when compared to control birds given no additives. Challenged birds fed yeast cell wall extract exhibited decreased crypt depth, increased villus height and increased villus to crypt ratio.

Chapter 4 investigates whether acetylated high amylose maize starch or butyralated high amylose maize starch play a positive role in digestion in broiler chickens and particularly in ameliorating the severity of necrotic enteritis in broilers under experimental disease challenge. The results showed that on d 24 and 35, all challenged birds had lower ($P < 0.001$) livability, weight gain and feed intake compared to unchallenged birds. Birds fed acetylated high amylose maize starch and butyralated high amylose maize starch diets had higher ($P < 0.001$) weight gain and feed intake compared with those fed the control diet. Birds fed acetylated or butyralated high amylose maize starch diets had poorer ($P < 0.002$) feed conversion ratio at d 35. Butyralated high amylose maize starch increased jejunal villus to crypt ratios, ileal and caecal butyrate levels and decreased caecal pH. Acetylated high

amylose maize starch significantly improved ileal acetate content and decreased caecal pH.

Chapter 5 determines the efficacy of acylated starches on performance, heat production and energy efficiency of broiler chickens during an induced outbreak of necrotic enteritis. The data demonstrate that *Eimeria* sp. and *Clostridium perfringens* challenge reduces growth performance, heat production, respiratory quotient, heat increment, metabolisable energy and metabolisable energy intake of birds fed the control, acetylated or butyralated high amylose maize starch. However, birds fed antibiotics exhibited nearly total resistance to the challenge with necrotic enteritis.

Chapter 6 describes the effect of encapsulated sodium butyrate on growth performance and gut health in broilers fed wheat or corn based diets, at normal or high protein levels and normal or low energy levels. The inclusion of encapsulated sodium butyrate at 1 g/kg had no effect on growth performance, ileal and caecal pH and levels of SCFAs, but increasing the inclusion rate to 2 g/kg improved bird performance.

Chapter 7 discusses the major findings on the effects of some feed additives, namely microencapsulated sodium butyrate, yeast cell wall extract and acylated starches, on broiler chickens production and gut health, and the role of yeast cell wall extract and acylated starches in ameliorating the severity of necrotic enteritis in broilers under experimental disease challenge. In conclusion, necrotic enteritis is characterized by necrosis and inflammation of the gastrointestinal tract with a significant decline in growth performance. However, yeast cell wall extract and acylated starches can be used as tools for reducing the severity of necrotic enteritis outbreaks.

The series of experiments reported in this thesis suggests that the yeast cell wall extract consists mainly of mannano-oligosaccharides, butyralated starch and acylated starch products are able to partially ameliorate the impact of necrotic enteritis in broiler chickens by improving gut health, but are unable to replace antibiotics in terms of effectiveness against severe challenges. Further studies are required to investigate the modes of action of these feed additives in improving gut health and reducing the severity of necrotic enteritis.

Chapter 1 General Introduction

Poultry production has undergone a substantial increase compared to other animal food-producing industries since 1970 (Yegani and Korver, 2008). Improvements in housing, genetic selection for growth rate, and advances in feed formulation achieved by matching nutrient requirements of the birds and nutrient contents of the feedstuffs, have resulted in higher meat yield, improved feed conversion and lower mortality rates (Choct et al., 1999; Cooper and Songer, 2009). As growth rate and feed conversion ratio improve, the bird's nutrition and health care are becoming more demanding (Choct et al., 1999). The nutritional and health status of poultry are interlinked with gut health which includes gut microbial balance, and macro and micro-structural integrity of the gut and immune system. The health of the gastrointestinal tract (GIT) affects digestion, absorption and metabolism of nutrients, disease resistance and immune response (Kelly and Conway, 2001; Yegani and Korver, 2008). The disturbances of these processes can result in enteric disease (Dekich, 1998). This makes it important to pay attention to gastrointestinal health, because any slight change is mostly accompanied by disruption of gut health and thus overall performance.

Enteric diseases are one of the most important illnesses in the poultry industry because of high economic losses due to decreased weight gain, increased mortality rates, feed conversion ratio, medication costs, and risk of contamination of poultry products for human consumption (Timbermont et al., 2011). Several pathogens including viruses, bacteria, parasites and other infectious and non-infectious agents are incriminated as possible causes of enteric diseases either alone or in synergy (Reynolds, 2003). Many conditions have been associated with gastrointestinal problems such as diarrhea, wet droppings, dysbacteriosis, intestinal colibacillosis, malabsorption syndrome, coccidiosis and necrotic enteritis. Enteric disorders are frequently associated with an overgrowth of *C. perfringens*. Infections with this bacterium in poultry can cause necrotic enteritis, necrotic dermatitis, cholangiohepatitis, as well as gizzard erosion (Hafez, 2011). Necrotic enteritis is the most common clostridial enteric disease in poultry, which typically occurs in broiler chickens but has also been diagnosed in various avian species including turkeys, waterfowl, and ostriches (Cooper et al., 2013). Necrotic enteritis is characterized by

necrosis and inflammation of the gastrointestinal tract with a significant decline in growth performance and, in clinical cases, a massive increase in flock mortality. The total cost of necrotic enteritis outbreaks globally is estimated to be over $2 billion annually (Van der Sluis, 2000).

Antibiotics have been used as an effective tool to improve animal performance, by selectively modifying the gut microflora, decreasing bacterial fermentation, reducing thickness of the intestinal wall and suppressing bacterial catabolism. All these are important to improve health, nutrient availability and growth performance (Carlson and Fangman, 2000). Dietary antimicrobials not only improve poultry growth and feed conversion efficiency, but also control enteric disease outbreak (Kim et al., 2011). The use of antibiotics in feed and for treating animals is second only to the medical use (Dahiya et al., 2006). It has been estimated that 11.15 million kg of antibiotics are used in animal feed in the USA alone each year (Union of Concerned Scientists. 2001) and 4.7 million kg or 35% of all antibiotics administered in Europe in 1999 were used in animal feed (B, 1999). Hence, antibiotics have come under increasing scrutiny by government regulators, scientists and consumers because of the emergence of antibiotic-resistant "superbugs". European countries have now prohibited the use of in-feed antibiotics in poultry feed. Without the use of in-feed antibiotics, the Animal Health Institute of America has estimated that the USA will require an additional 12 million pigs, 23 million cattle and 452 million chickens to reach the levels of production attained by the current practices (Dahiya et al., 2006).

With a ban of in-feed antibiotics in European countries, the incidence of necrotic enteritis has increased on the broiler farms of these countries (Casewell et al., 2003; Hofacre et al., 2003). At the same time, the focus on alternative strategies has increased to secure animal health and thus the efficiency of livestock production. These alternative strategies include modulation of gut microflora, augmentation of immune response and pathogen reduction through management, vaccination, nutritional strategies and/or feed additives. The major focus of this thesis is to investigate the efficacy of feed additives, namely microencapsulated sodium butyrate, yeast cell wall extract and acylated starch on gut health and productivity in broilers, and the role of yeast cell wall extract and acylated starch in ameliorating the

severity of necrotic enteritis in broilers under experimental disease challenge. The key areas of the study were:

- Response of broiler chickens to microencapsulated sodium butyrate using growth performance and intestinal acidity and short chain fatty acid levels.
- The roles of yeast cell wall extract to modulate enteric health and growth performance under a necrotic enteritis challenge.
- The efficacy of acylated starch on performance and gut health, and
- Heat production and energy balance and efficiency in broiler chickens under an experimental disease challenge.

Chapter 2 Literature Review

2.1 General characteristics of the gastrointestinal tract

The poultry gastrointestinal tract (GIT) consists of the beak, mouth, esophagus, crop, proventriculus, gizzard, small intestine and large intestine. The GIT acts as a gateway for various nutrients to gain access to the circulatory system. In general, the principle functions of the digestive system are degradation and absorption of the nutrients that are required for maintenance, growth, and reproduction. Briefly, the ingested feed materials are moistened, fragmented, acidified and mixed with endogenous enzymes. Macronutrients are digested into amino acids, dipeptides, monosaccharides, monoglycerides and free fatty acids that can be absorbed (Svihus, 2014).

Chickens cannot moisten or break down food in the mouth. Therefore whole food particles can enter the crop through the esophagus. As the crop is not a secretory organ it does not have any direct nutritional or absorptive role. However, it does have a considerable role in digesta miniaturisation, which may help enzymatic digestion and grinding further down the digestive tract (Svihus, 2014). The pH of the crop is variable. Feed for monogastrics usually has a pH between 5.5-6.5 (Ao et al., 2008). Thus one would expect the crop pH to be similar to that of the feed. However, Svihus (2014) observed that the average pH of crop content collected from meal-fed broilers was 4.8. Thus, it is possible that the crop may contain some enzymes and secretion from saliva and feed and that some bacteria are present in the crop. Fuller and Brooker (1974) proposed that bacterial fermentation takes place in the crop, resulting in lactic acid production, which may have a role in decreasing the crop pH.

A small amount of the digesta from the crop is continuously passed to the true stomach compartments, the proventriculus and gizzard. The retention time in the proventriculus and gizzard varies between 30-60 minutes (Shires et al., 1987; Van der Klis et al., 1990; Danicke et al., 1999) with a pH range between 3-4 (Svihus, 2011). Gastric juices including pepsin and hydrochloric acid are secreted by the proventriculus and are mixed with digestive contents in the gizzard due to muscular movements. In the gizzard, grinding and mixing of fed particles with enzymes produced be proventriculus occur (Svihus, 2014) and the small digesta particles will be passed into the intestinal tract for further digestion and absorption.

The small intestine (duodenum, jejunum and ileum) is the site for most digestion and nutrient absorption. The duodenum is the proximal part of the intestine, extending from the gizzard to the pancreatic and biliary ducts. The acidic contents of the gizzard are mixed with pancreatic and bile juices (Duke, 1986) for less than 5 minutes (Noy and Sklan, 1995). Next to the duodenum is the jejunum. Meckel's diverticulum is used as a landmark to separate the jejunum and ileum. This segment extends from the pancreatic ducts to the Meckel's diverticulum, and major nutrients are digested and absorbed in this segment (Duke, 1986). The retention time in the jejunum is reported to be between 40-60 minutes, which is roughly half the retention time of the ileum (Weurding et al., 2001; Rougière and Carré, 2010). The ileum is the last segment of the small intestine, starting from Meckel's diverticulum and ending at the ileo-caecal junction. The main role of this segment is water and mineral absorption, although digestion and absorption of some starch, fat and protein may take place (Svihus, 2014).

The pair of blind pouches located at the junction of the small and large intestine are called caeca (McLelland, 1989). The role of the avian caeca is reabsorption of water and salts and fermentation of uric acid and carbohydrate (Svihus et al., 2013).

2.2 Intestinal microflora of poultry

The gastrointestinal microflora consists of different kinds of microorganisms, including bacteria, protozoa and fungi, among which the bacteria are the predominant microorganisms. The bacteria in the GIT can become attached to the epithelium or digesta or be free living in the intestinal lumen (Gabriel et al., 2006). The bacterial population is diverse in different parts of the GIT and population concentrations tend to rise from proximal to distal GIT (Richards et al., 2005) and each part of the GIT has its own microbial profile (Lu et al., 2003; Amit-Romach et al., 2004). Many factors can affect the composition of the avian gut flora, including strain, species, diet, age, sex, stress, infection and antibiotic administration (Smith, 1965b; Hume et al., 2003; Lu et al., 2003; Pedroso et al., 2006).

The bacteria in the digestive system range from a relatively aerobic environment in the crop and duodenum to a strictly anaerobic one in the caeca. At this stage, *Lactobacillus* spp. including *L. salivarius*, *L. fermentum* and a type resembling *L.*

salivarius have been isolated and characterised from the crop (Fuller, 1973). Although the environment in both the gizzard and the proventriculus are likely to be unfavorable for the growth of various bacteria, resulting in lower bacterial populations in these parts than that found in other parts of the GIT, a low number of *E. coli,* streptococci, enterococci and yeasts have been observed in the gizzard (Smith, 1965b, a). The low population of bacteria in these parts is probably due to a low pH (Gabriel et al., 2006).

In the small intestine, facultative anaerobic organisms including streptococci, lactobacilli and *E. coli* comprise 60-90% of the small intestine microflora and the majority of predominant bacteria isolated from the duodenum, ileum and caecum are Gram-positive (Salanitro et al., 1978). The ileal environment is more favorable to the growth of bacteria because of lower bile salt and enzyme concentration and lower oxygen pressure (Gabriel et al., 2006). The predominant cultured microbiotas present in the ileum include *Lactobacillus* (33.8-59%), *Streptococcus* (8.9-16.8%), *E. coli* (14.7-33%) and eubacteria (9-24.3%) (Salanitro et al., 1974). Using new molecular methodologies, based on 16S rRNA, Lu et al. (2003) found that the *Lactobacillus* (70%), *Clostridiaceae* (11%), *Streptococcus* (6.5%), and *Enterococcus* (6.5%) were dominant in the ileum of broilers.

In poultry, the caeca have a complex microflora (Wei et al., 2012). Total numbers of caecal organisms per gram of digesta in the adult chicken are much higher than in other segments of the GIT (Jamroz et al., 1998). Salanitro et al. (1978) proposed that nearly the entire microbial population of the caecum are anaerobes including Gram-positive (highest numbers) cocci, eubacteria, and clostridia and Gram-negative gemmiger, fusobacteria, and bacteroides. More recently, molecular techniques have been used to examine the ecology of the caecal microflora. Based on 16S rDNA analysis, (Zhu et al., 2002) found a total of 1656 nucleotide sequences which belong to 50 different phylogenetic groups or subgroups. Gong et al. (2000a,b) observed that *Fusobacterium prausnitzii*, ruminococci, *Clostridia* spp and *E. cecorum* were the predominant groups of bacteria in the caecal mucosa. Using the same technique, Lu et al. (2003) showed that 65% of the sequences were related to *Clostridaceae* with other abundant sequences being related to *Fusobacterium* (14%), *Lactobacillus* (8%) and *Bacteriodes* (5%).

2.3 Gut microflora in relation to fermentation

Carbohydrates are digested and absorbed in the proximal gut, whereas the residual and the indigestible carbohydrates can be fermented in the distal gut (Hooper et al., 2002). Such fermentation can be observed in most parts of the GIT in poultry, but most investigators agree that the caeca are the principal place of fermentation (Jozefiak et al., 2004). The carbohydrates which can be fermented in the distal gut can be polysaccharides, oligosaccharides, disaccharides, non-starch polysaccharides, starch and/or resistant starch (Jozefiak et al., 2004; Pan and Yu, 2013). The fermentation of these carbohydrates by intestinal bacteria produces short chain fatty acids (SCFAs), mainly butyrate, acetate and propionate (Pan and Yu, 2013), which can be utilised by the host as carbon and energy sources (van der Wielen et al., 2000b; Hooper et al., 2002; Koutsos and Arias, 2006; Tellez et al., 2006).

Gut microflora can degrade or ferment protein from different sources in the distal gut. The presence of proteolytic bacteria (bacteriodes, clostridia, propionibacteria and streptococci) in the caecum can catabolise the undigested protein or uric acid to ammonia, which can be absorbed by the host to synthesise some amino acids such as glutamine (Macfarlane et al., 1986; Vispo and Karasov, 1997; Svihus et al., 2013). Thus, the gut microflora themselves can be a source of amino acids (Metges, 2000). However, most of these bacterial proteins cannot be absorbed by the host, because the caecum of birds does not have the ability to absorb protein (Pan and Yu, 2013). These bacterial proteins can be utilised when birds are raised on hard floors, because the birds may ingest faeces and then the bacterial protein can be digested and absorbed in the proximal gut (Vispo and Karasov, 1997; Koutsos and Arias, 2006). However, fermentation of nitrogen will also initiate less favorable outcomes, for example production of toxic substances such as phenols, thiols, amines, ammonia and indoles, and increase the pH of the lower part of the GIT, which encourages the proliferation of pathogenic bacteria (Juśkiewicz et al., 2004; Lan et al., 2005).

2.3.1 Short chain fatty acids

Normal gut microflora play an important role in intestinal growth, gut morphology and health. The SCFAs produced as a result of fermentation in the intestinal tract can be absorbed across the epithelium and enter a variety of metabolic pathways (Hooper et al., 2002). It has been reported that SCFAs have a direct stimulatory effect on

gastrointestinal cell proliferation, through the increase of plasma glucagon-like peptide-2 (GLP-2) and ileal proglucagon, glucose transporter (GLUT2) expression and protein expression, which may explain the stimulating effect of gut microflora on intestinal growth (Tappenden and Mcburney, 1998; Le Blay et al., 2000; Blottiere et al., 2003). This was supported in work done by Muramatsu et al. (1993) where fermented carbohydrates stimulated increased microbial fermentation and SCFA production, which resulted in a higher gut weight. In addition, gut microflora can also affect gut morphology. Supplementation of probiotics, prebiotics and fermented feed increased both villus height and villus height:crypt depth ratio (Xu et al., 2003; Feng et al., 2007; Chiang et al., 2010; Chae et al., 2012; Sun et al., 2013). Such intestinal morphology changes are not a direct effect of these dietary supplementations, but an indirect effect through the manipulation of the gut microflora profile (Xu et al., 2003). In addition to energy producing activity, the production of SCFAs in the distal intestine results in pH reduction and inhibition of acid sensitive microorganisms (Mroz et al., 2006). It also has been reported that SCFAs improved protein digestibility through lowering the pH of the GIT and thus increasing pepsin activity (Afsharmanesh and Pourreza, 2005). Langhout (2000) showed that SCFAs reduce pathogenic bacteria by controlling the colonisation of pathogenic bacteria on the gastrointestinal wall, thus inhibiting damage to epithelial cells. Furthermore, increased SCFAs have been shown to improve digestibility of calcium, phosphorus, magnesium and zinc which serve as substrate in the intermediary metabolism (Garcia et al., 2007).

2.4 Factors affecting gut health

Recently, maintenance or enhancement of gut health has been a major topic for human and animal research. Gut health can be described in a variety of ways including microflora balance, macro- and micro-structural integrity of the gut and immune system ability (Choct, 2009). A wide range of factors associated with diet, infectious agents, and stress can affect the components of the chicken gut and alter growth performance and feed efficiency (Choct, 2009).

2.4.1 Diet composition

Diet has the greatest potential effect on intestinal digestion and health. The wide range of feed ingredient components that escapes digestion and absorption modulates intestinal microflora and the digestion process (Yegani and Korver, 2008; Pan and Yu, 2013). For instance, anti-nutritional compounds, especially NSPs that are present in all poultry diets, are resistant to enzymatic digestion and subsequently create a viscous environment in the intestinal lumen and sticky droppings (Choct and Annison, 1992a, b). High digesta viscosity prolongs transit time and decreases nutrient digestibility (Choct et al., 1996). Decreased digesta passage time facilitates bacterial colonisation and activity in the gut (Waldenstedt et al., 2000). Wheat, barley, or rye based diets, which have high levels of NSPs, create a favorable environment for *C. perfringens* proliferation and predispose young chicks to necrotic enteritis (Kalshusdal and Hofshagen, 1992; Annett et al., 2002; Dahiya et al., 2006; McDevitt et al., 2006). In addition to anti-nutrient factors, poultry diets with high protein content, those rich in animal protein such as meat and bone meal or fish meal, and imbalanced amino acid profiles, have a significant effect on gut microbial composition and encourage the proliferation of pathogenic bacteria (Juśkiewicz et al., 2004; Lan et al., 2005; McDevitt et al., 2006; Wu et al., 2014).

2.4.2 Infectious agents

The GIT of birds is constantly exposed to a variety of harmful factors. These factors can have negative effects on health, GIT condition and performance of birds. Enteric diseases are one of main concern to the poultry industry because of production losses, increased morality, increased risk of contamination of poultry products for human consumption, and concerns over animal welfare (Timbermont et al., 2011). Enteric diseases in poultry can be caused by different infectious agents including viruses, bacteria, parasites and other infectious and non-infectious agents (Reynolds, 2003). As stated earlier, the principle functions of the GIT are degradation and absorption of the nutrients that are required for maintenance, growth, and reproduction. Biological, chemical, or physical disturbances of the GIT functions can result in enteric diseases (Dekich, 1998).

Several viral diseases have been related to enteric disease such as reoviruses, astroviruses, adenoviruses, enteroviruses and coronaviruses (Guy, 1998). The main

impact of viral diseases on poultry are depressed growth performance, reduced flock uniformity and a likely contribution to development of other GIT diseases (Guy, 1998). Although these enteric viral diseases can occur in birds of all ages, they are predominantly found in young birds. Many factors contribute to the outcome of these infections, such as management, nutrition, environmental factors, virulence of virus, age and immune status of birds (Guy, 1998).

Food borne toxins can also cause enteric disease. The most common examples of food borne toxins are mycotoxins and biogenic amines (Dekich, 1998). Mycotoxin such as T-2 toxin are characterized by necrosis, hemorrhage, and inflammation of the GIT, with significant reduction of villus height and the mitotic activity of the crypt (Yegani and Korver, 2008), which results in economic losses due to reduced performance and impaired health status (Sklan et al., 2003). Biogenic amines such as spermine, putrescine, spermidine, histamine and cadaverine are present in animal protein products (Yegani and Korver, 2008). They are involved in the incidence of malabsorption syndrome, which is categorized by hyperplasia in the proventriculus (Stuart et al., 1985).

Parasites are a common problem wherever poultry are raised. Protozoal parasites are common in the commercial poultry industry and may cause severe or moderate diseases (Yegani and Korver, 2008). The protozoa that typically infect the poultry industry belong to the phylum Apicomplexa which includes *Eimeria, Haemoproteus, Sarcocystis, Leucocytozoon*, *Toxoplasma*, and *Cryptosporidium*. Amoeba of the genera *Endolimax* and *Entamoeba* and flagellates such as *Chilomastix, Trypanosoma, Trichomonas, Histomonas,* and *Hexamita* are also found in poultry. Recently, microsporidian, *Encephalitozoon cunicule* has been reported in chickens (Reetz, 1993). Coccidiosis (*Eimeria* spp.) in the fowl has been well documented and is recognized as being of major economic importance in the poultry industry. Several *Eimeria* species have been investigated and each species has been found to cause disease in a separate part of the GIT (Williams, 2005). The protozoan parasites of the genus *Eimeria* cause intestinal malabsorption, villus atrophy, intestinal leakage of plasma proteins, interruption of nutrient digestion, reduced weight gain, reduced feed and water intake, increased intestinal passage time and increased susceptibility to other disease agents (Williams, 2005; Yegani and Korver, 2008).

Enteric bacterial infections in poultry cause intestinal damage and can contribute to production losses and increased mortality of a flock. Several enteric bacterial diseases have been recognized in poultry. Necrotic enteritis, spirochetosis and ulcerative enteritis are primarily enteric diseases that infect the GIT, while other bacterial infections such as mycobacteriosis, colibacillosis, erysipelas, fowl cholera and salmonellosis affect the intestine in addition to a variety of organ systems (Porter, 1998). In this part of this review, some factors affecting intestinal health have been briefly presented. However, necrotic enteritis as a common bacterial disease in the poultry industry will be discussed in detail as the focus of this study.

2.5 Necrotic enteritis in poultry

Necrotic enteritis (NE) is an economically important bacterial disease for the meat chicken industry because of production losses, reduced welfare of birds and increased risk of contamination of poultry products for human consumption (Timbermont et al., 2011). Necrotic enteritis was first recorded in Australia by Bennetts (1930) and later described by Parish (1961b) in the United Kingdom. NE has been recognized in broilers (Cowen et al., 1987), turkeys (Droual et al., 1995) and layers (Dhillon et al., 2004). Free living birds like quail (Berkhoff, 1985) and wild crows (Asaoka et al., 2003) can also develop NE. The causative agent of NE is *C. perfringens* (Dahiya et al., 2005). Many other factors contribute to the pathogenesis of this disease including excess dietary protein (mainly animal protein), environmental stress and *Eimeria* infection that may damage intestinal mucosa (Kaldhusdal and Skjerve, 1996). Necrotic enteritis is characterized by necrosis and inflammation of the gastrointestinal tract with a significant decline in growth performance and, in clinical cases, a massive increase in flock mortality (Van der Sluis, 2000). It has been estimated that the total cost of clinical and subclinical necrotic enteritis can be as high as two billion dollars annually (Van der Sluis, 2000).

2.5.1 Epidemiology

Necrotic enteritis has a high mortality rate with severe economic losses. The disease has been reported in many countries, including the United Kingdom (Parish, 1961b), Australia (Nairn and Bamford, 1967), Canada (Helmboldt and Bryant, 1971) and France (Casewell et al., 2003). The primary causative agent of NE is *C. perfringens*

and the source of *C. perfringens* is ultimately the chickens themselves (Cooper and Songer, 2009). Outbreaks of NE in poultry, past and present, have been associated with *C. perfringens* contamination of the chickens' feed (Nairn and Bamford, 1967; Eleazer and Harrell, 1976; Hofacre et al., 1986). Occurrences of NE are also affected by season, (Kaldhusdal and Skjerve, 1996), dietary restriction (Olkowski et al., 2006), bedding on high fiber litter (Branton et al., 1997) and management-related stress (Craven, 2000).

Necrotic enteritis usually occurs in broiler chicks at 2-6 weeks of age (Cooper and Songer, 2010). Generally, NE is not typically known as a seasonal disease, although the occurrences of NE between different latitudes appear to contradict this. Kaldhusdal and Skjerve (1996) suggested that uni-variable regression analysis in south-eastern Norway indicated that NE occurred more often during the months October–March than during the months April–September, whilst in Canada it mostly appeared in July-October (Long, 1973). In the United Kingdom, the peak incidence of NE is during winter (Hermans and Morgan, 2007).

2.5.2 Aetiology

The primary cause of NE in poultry is *C. perfringens* type A and, to a lesser extent, type C (Engström et al., 2003). A high bacterial population in the GIT is associated with the appearance of necrotic lesions (McDevitt et al., 2006). The intestinal numbers of *C. perfringens* in NE-affected and healthy birds are different. The *C. perfringens* population is found to be 10^7- 10^9 colony-forming units (CFU) per gram of the intestinal contents of NE-affected birds, compared to less than 10^4 CFU/g of the intestinal contents in healthy birds (Kondo, 1988).

2.5.3 *Clostridium perfringens* as a causative agent of necrotic enteritis

Clostridium perfringens is a Gram-positive, spore forming anaerobic bacterium, able to produce several enzymes and toxins responsible for NE symptoms and lesions (Van Immerseel et al., 2004). *C. perfringens* is classified into five strains A, B, C, D and E, based on the production of major lethal toxins (Songer and Meer, 1996). In avian species, types A and C are the most commonly linked to NE disease (Engström et al., 2003). *C. perfringens* can be found in poultry litter, feces, soil, dust and in healthy bird intestinal contents (Dahiya et al., 2005). It is expected that small

numbers of *C. perfringens* are resident, transiently or permanently, in the GIT of most bird species (Cooper and Songer, 2009). When poultry meat is analyzed for *C. perfringens*, in some cases up to 84% of meat samples are positive (Craven et al., 2001b). It is reported that colonization or contamination of poultry by *C. perfringens* occurs during the early life of the animal, and can commence in the hatchery environment (Van Immerseel et al., 2004). *C. perfringens* is found in eggshell, paper pads and chicken dander in the hatchery (Craven et al., 2001a). Craven et al. (2003) indicated that the *C. perfringens* contamination found in broiler carcasses can begin in the breeder hen, and be transmitted through the hatchery and growing area. Free-living birds such as crows have high counts of *C. perfringens* in their intestinal droppings, which indicate that wild birds also suffer from NE (Asaoka et al., 2003). Craven et al. (2001b) found that swabs taken from poultry farms showed an incidence of *C. perfringens* from a variety of sources, including live flies, walls, dirt outside the entrance, fans, floor, nipple-drinker drip-cups, water pipes, litter material, chick delivery-tray liners and boots of farm staff before chicks were placed. Even feed samples taken after 2 weeks following bird placement had an incidence of *C. perfringens*. This indicates that different sources and strains of *C. perfringens* can colonize in the birds and produce NE.

Morphology and growth characteristics

Clostridium perfringens was previously known as *Clostridium welchii* (Helmboldt and Bryant, 1971). It is a large Gram-positive rod-shaped (0.6-2.4 x 1.3-9.0 μm) bacterium which is spore forming, non-motile, encapsulated, fermentative and catalase negative (Cato et al., 1986). Colonies of *C. perfringens* are smooth, round, glistening, and surrounded by an outer and inner zone of hemolysis (Ribeiro et al., 2007). Its generation time can be very fast (8–10 min), and is accompanied by gas production (Stevens and Bryant, 2002). The temperatures required for the minimum, optimal and maximum growth of *C. perfringens* are 10-14ºC, 43°C and 50-52ºC, respectively (Li and McClane, 2006b). Its endospores can tolerate 100ºC for 2 h (Parish, 1961a). The optimum pH for *C. perfringens* growth is 7 (Li and McClane, 2006a) and it requires 13 amino acids for its optimum growth (Cato et al., 1986).

Clostridium perfringens can grow on a wide variety of culture media, including blood agar (Nairn and Bamford, 1967), Cooked Meat Medium (Shane et al., 1985),

Brain Heart Infusion agar (Cooper et al., 2009), Fastidious anaerobe agar (Engström et al., 2003), Tryptose Sulphite Cycloserine (TSC) agar (Hauschild and Hilsheimer, 1974) and Reinforced Clostridial agar (RCA) (Byrne et al., 2008). Byrne et al. (2008) reported that TSC and RCA Agar are the best media for culturing *C. perfringens* and RCA for *C. perfringens* spore recovery. However, TSC media with or without the addition of egg yolk is the best, as black colonies can be observed after 24 hours of anaerobic incubation.

Toxins to produce necrotic enteritis

Clostridium perfringens is able to produce several types of toxins, while individual strains only produce a subset of these toxins (Van Immerseel et al., 2008). *C. perfringens* strains (A, B, C, D and E) are classified according to the production of four major extracellular toxins (alpha (α), beta (β), epsilon (ε) and iota (ι)), while various strains can also produce other toxins, including β 2 toxin, perfringolysin O [θ-toxin], collagenase [κ-toxin], enterotoxin, theta toxin etc. (Petit et al., 1999). Type A produces α-toxin, type B produces α, β and ε toxins, type C produces α and β toxins, type D produces α and ε toxins and type E produces α and ι toxins (Brynestad and Granum, 2002). In poultry, *C. perfringens* types A and C are most commonly linked with NE disease. The subclinical form of CP infection and NE are caused by type A and to a lesser extent by type C (Engström et al., 2003). However, *C. perfringens* strains B, D and E do not play a role in poultry NE (Van Immerseel et al., 2004). The roles of some of these toxins are well understood. For example, all five toxinotypes produce α-toxin, which is the main virulence in gas gangrene (Awad et al., 1995). The ε and β toxins are responsible for enterotoxaemia in domestic livestock including lambs, calves, goat and piglets. In developed countries, most domesticated livestock are immunized against disease with toxoid vaccines (Van Immerseel et al., 2008).

The ι toxin consists of active protein (Ia) and a binding protein (Ib). The ι toxin is lethal, dermonecrotic and causes disruption of cytoskeleton action and cell barrier integrity (Petit et al., 1999). The theta-toxin is a pore-forming cytolysin that can lyse red blood cells (Tweten, 1997). Theta-toxin modulates the host inflammatory response, causing leukocyte accumulation within blood vessels and the extracellular matrix of host tissues (Bryant et al., 1993; Bryant and Stevens, 1996; Ellemor et al.,

1999). The *C. perfringens* enterotoxins (CPE) are responsible for antibiotic-associated diarrhea and *C. perfringens* type A food poisoning diarrhea. They alter the plasma membrane permeability through cell damage and interact with tight junction proteins and affect tight junction function and structure (McClane, 2001). Recently, β-2 toxin and NetB toxins were discovered. The β-2 toxin has been associated with equine, porcine and bovine gastro-enteritis and also been confirmed in poultry *C. perfringens* type A (Bueschel et al., 2003). The NetB toxin is now known to be essential in the pathogenesis of necrotic enteritis in poultry (Keyburn et al., 2008).

Alpha-toxin

Over the years, α-toxin has been reported to be the main virulence factor in poultry NE (Van Immerseel et al., 2008). A zinc metalloenzyme is produced by all *C. perfringens* strains (Brynestad and Granum, 2002). Alpha toxin has phospholipase C (PLC) and sphingomyelinase activity (Flores-Díaz and Alape-Girón, 2003). The substrates of α-toxin are phosphatidylcholine and sphingomyelin, which are components of the epithelial cell membrane of the GIT (McDevitt et al., 2006). Hydrolysis of the cell membrane produces ceramide (from sphingomyelin) and diacylglycerol (from phosphatidylcholine) (Donelli et al., 2003). The formation of diacylglycerol results in activation of protein kinase C, and stimulation of the arachidonic cascade (McDevitt et al., 2006). This induces the synthesis of inflammatory mediators, such as thromboxane, leukotrienes, prostacyclin and platelet-agglutinating factors (Titball and Rood, 2002). These mediators induce blood vessel contraction, platelet aggregation and myocardial dysfunction, which lead to death (McDevitt et al., 2006).

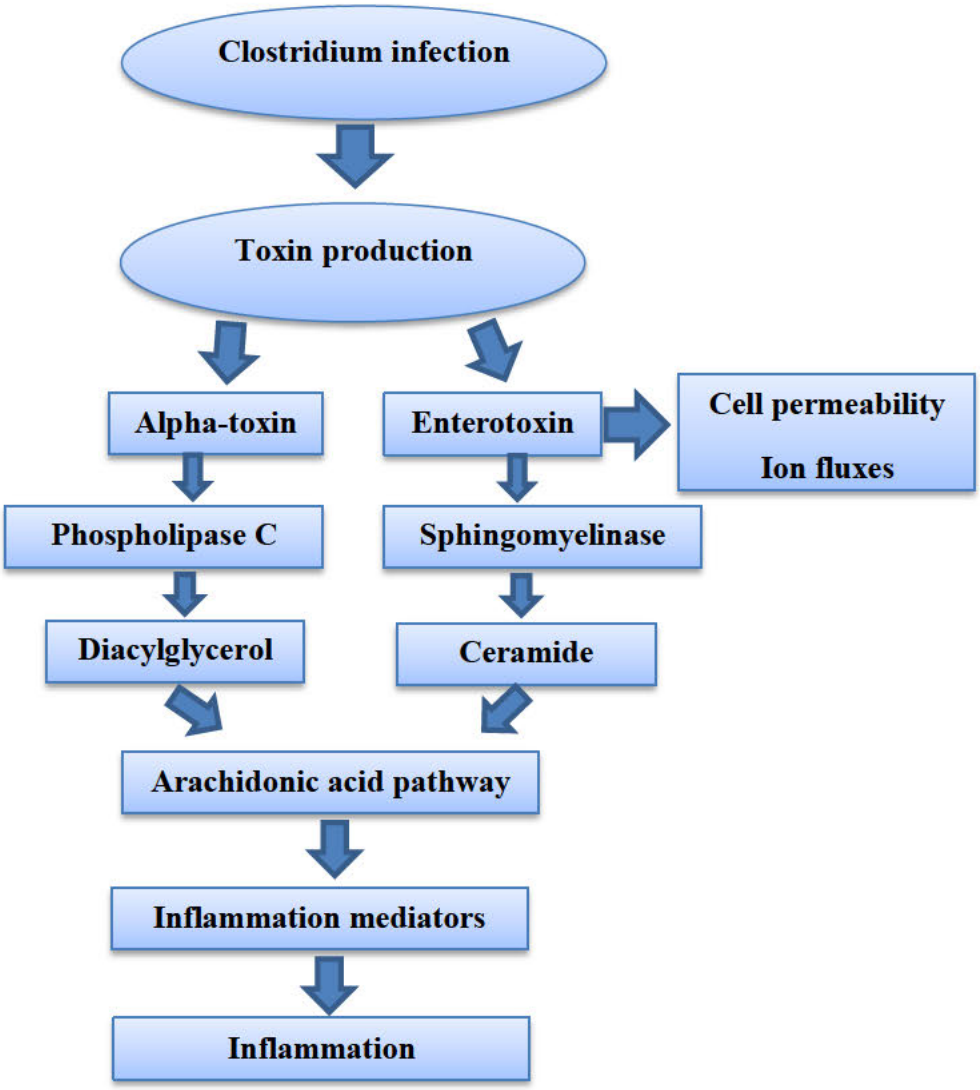

Figure 2.1 Mode of action of α-toxin. Modified from (McDevitt et al., 2006)

Although for more than three decades α-toxin was believed to be the main virulent factor in the pathogenesis of NE, the importance of α-toxin in the pathogenesis of NE is still questionable. Early studies on the factors inducing NE showed that intro-duodenal infusions of both large volumes of *C. perfringens* broth culture (Al-Sheikhly and Truscott, 1977b) and crude toxins (Al-Sheikhly and Truscott, 1977a) into chickens, resulted in typical NE lesions. From these studies the authors concluded that α-toxin was the main toxin produced by *C. perfringens*, and that α-toxin was the most virulent factor in the pathogenesis of NE. Fukata et al. (1988) found that 21 out of 56 germ-free chickens, inoculated with either purified α-toxin or a supernatant of broth cultures of *C. perfringens* died after inoculation, whereas no bird died after receiving a culture supernatant neutralised by anti-alpha-toxin serum. Later on, Hofshagen and Stenwig (1992) found a significantly higher amount of α-

toxin in isolates from birds with NE compared to isolates from birds without NE. In a more recent study, Cooper and Songer (2009) suggested that immunisation with α-toxin gave substantial protection against NE and Rehman et al. (2009) concluded that α-toxin can damage the intestinal mucosal barrier. Contradicting these studies, an *in vitro* study (Gholamiandekhordi et al., 2006) demonstrated no difference in α-toxin production between *C. perfringens* isolated from healthy flocks and those isolated from NE outbreaks flocks. The most convincing evidence that α-toxin of *C. perfringens* was not a major factor in producing NE in chickens came from a study using an α-toxin negative mutant of *C. perfringens* strain from a NE outbreak. This study demonstrated that the constructed α-toxin gene (plc) induced the same type of NE lesion as that of the wild type strain (Keyburn et al., 2006). Other studies also argue against the role of α-toxin in NE. In mice, α-toxin-negative mutants are unable to cause gas gangrene, but they do promote inflammatory responses (Awad et al., 1995). Furthermore, histological analysis of tissue in early stages of NE lesion development is inconsistent with the α-toxin phospholipase C or shingomyelinase activities (Olkowski et al., 2008).

Net B

In the past few years some studies have proposed that α-toxin is not an essential virulence factor in NE. Recently, a new pore-forming toxin named NetB (necrotic enteritis toxin B-like) was suggested by Keyburn et al. (2008) as a virulence factor for the development of NE. NetB toxin showed limited amino acid similarity with pore-forming β-toxin (38% identity) and the α-toxin of *Staphylococcus aureus* (31% identity). This toxin was identified from *C. perfringens* type A strain isolated from infected chickens. Both recombinant and native NetB showed cytotoxic activities against the chicken leghorn male hepatoma cell line (LMH) and the mechanism of action appears to include the formation of a hydrophilic pore in the cell membrane with a functional diameter of 1.6–1.8 nm (Keyburn et al., 2008). A complemented netB mutant was able to cause necrotic lesions in the gut of experimentally infected broilers (Keyburn et al., 2008). Additional evidence for the role of netB in disease comes from the finding that chickens suffering from NE carry the NetB gene and produce highly conserved NetB toxin (Keyburn et al., 2010). This finding has been confirmed by surveys in North America, where all *C. perfringens* isolates from birds

infected with NE carry the NetB gene, while only a small percentage of isolates from healthy birds carry the gene (Chalmers et al., 2008b; Martin and Smyth, 2009). Keyburn et al. (2010) reported that NetB positive *C. perfringens* strain isolates from diseased birds were able to produce NetB *in vitro* and therefore only strains producing NetB were able to induce disease. This was supported by findings of Smyth and Martin (2010) where all NetB positive isolates induced NE in challenged birds, whereas none of the NetB negative isolates produced disease.

Although several studies have demonstrated that NetB is an essential virulence factor in NE disease, some studies have found that *C. perfringens* strains isolated from birds clearly suffering from NE disease do not have NetB (Chalmers et al., 2008a; Keyburn et al., 2008; Martin and Smyth, 2009).The question therefore arises as whether other secreted toxins interact to induce NE. The contradictory evidence given by several studies on the role of NetB toxin and α-toxin in NE in chickens argues for the role of other toxins, as yet unidentified virulence factor(s), which effectively produce the disease.

2.5.4 Outbreaks of necrotic enteritis

Necrotic enteritis is a common bacterial disease in broiler growing areas of the world. It usually occurs in broiler chickens at 2-6 weeks of age (Cooper and Songer, 2010). Under field conditions the disease occurs in two clinically different forms; clinical and subclinical.

Clinical signs

The clinical form of NE is associated with signs such as ruffled feathers, relative immobility, depression, anorexia, diarrhea and decreased appetite (Helmboldt and Bryant, 1971; Al-Sheikhly and Truscott, 1977b; Al-Sheikhly and Al-Saieg, 1980; Gazdzinski and Julian, 1992). Wet litter is also sometimes an early indicator of the disease (Riddell and Kong, 1992). Birds displaying clinical signs generally die within a few hours, with mortality rates up to 1% per day (Helmboldt and Bryant, 1971). In the acute form of NE, characterized by a sudden increase in flock mortality, birds usually die without premonitory signs (Kocher and Choct, 2008).

Diarrhea and associated wet litter were noted in the wood shaving litter of birds suffering from NE (Kalshusdal and Hofshagen, 1992). Elwinger and Teglöf (1991) found a direct correlation between poor litter condition and sticky droppings. In field surveys conducted globally (Van der Sluis, 2000) and in the United Kingdom (Hermans and Morgan, 2003), researchers associated NE with diarrhea or wet litter. Williams (2005) noted that in the United Kingdom, any sudden increase in litter moisture of poultry farms is associated with NE and antibiotic therapy is initiated. Helmboldt and Bryant (1971) suggested that acute NE may be associated with diarrhea or wet litter, but is not always so (Nairn and Bamford, 1967). Apart from leaking drinkers, feed quality, ventilation and house temperature, mycotoxins and infection with viruses or protozoa or bacteria and high stocking density contribute to wet litter (Williams, 2005; Butcher and Miles, 2011).

Subclinical signs

In subclinical forms of NE there is no peak of mortality and no clinical signs are present (Timbermont et al., 2011). Subclinical forms of NE are usually associated with reduced feed intake and weight gain and increased feed conversion ratio (Kalshusdal and Hofshagen, 1992; Lovland and Kaldhusdal, 2001). They are also associated with hepatitis and cholangiohepatitis (Van Immerseel et al., 2004). During subclinical infection, bacteria can reach the portal blood stream and bile duct. Colonisation of high numbers of *C. perfringens* in hepatic tissue result in cholangiohepatitis (Timbermont et al., 2011). Diseased livers are greatly enlarged and have a pale reticular pattern with white or red foci and histophathological lesions characterized by bile duct hyperplasia, cholangitis, fibrinoid necrosis and sometimes focal granulomatous inflammation (Løvland and Kaldhusdal, 1999). Onderka et al. (1990) reported that inoculation of birds with *C. perfringens* produced cholangiohepatitis, enlarged liver, tan-colored liver with red and white foci and oedematous gall bladder. During meat inspection at a slaughter house, infected livers were found, without any clinical signs in the flock (Timbermont et al., 2011). Although clinical forms of NE may cause high levels of mortality, the subclinical form of NE is more important than the clinical form because it may persist in the flock without any clinical manifestation. Thus, untreated birds suffering from

subclinical NE can cause huge economic loses in the poultry production industry (Dahiya et al., 2006).

Intestinal gross lesion

The small intestine is the principle site of tissue damage associated with NE lesions. Although gross lesions are usually restricted to the duodenum, jejunum and ileum (Timbermont et al., 2011) lesions can also occur in the caeca (Van Immerseel et al., 2004). The small intestine of infected birds is friable, dilated, hyperemic, thin walled and filled with gas, and the mucosal surfaces are covered by tan-orange pseudo-membranes and occasional hemorrhages (Broussard et al., 1986; Olkowski et al., 2006). Microscopic examination shows a strong inflammatory reaction to *C. perfringens* in the early stages of NE. The lamina propia is infiltrated and hyperemic with several inflammatory cells, especially at the interface of the basal domain of enterocytes and lamina propria. These areas are edematous, permitting for the extensive disorder of the structural integrity between the enterocytes and the lamina propria (Olkowski et al., 2006). Histopahological examination of later stage NE lesions shows extensive villous necrosis (Broussard et al., 1986), coagulation of necrosis of the mucosa in all segments of the small and large intestine (Olkowski et al., 2006) and a clear line of demarcation between necrotic and viable tissue. An accumulation of heterophilic granulocytes at the junction is seen (Long, 1973; Al-Sheikhly and Al-Saieg, 1980). Hemorrhage or patchy congestion is present over all the lamina mucosa, particularly in the vicinity of the crypts. The crypts are usually misshapen and inflated by pink mucus and necrotic cellular debris (Olkowski et al., 2006).

Predisposing factors for necrotic enteritis

Clostridium perfringens type A and to a lesser extent type C is documented as a primary causative agent of NE in poultry, while contributory factors that alter the gastrointestinal tracts environment and create a favorable environment for *C. perfringens* overgrowth in the gut are essentially required to produce the clinical and subclinical signs and lesions of NE. Factors that predispose the bird to NE are nutrition (Kalshusdal and Hofshagen, 1992; McDevitt et al., 2006; Timbermont et al., 2011; Shojadoost et al., 2012), management (Craven et al., 2001a; Craven et al.,

2001b; McDevitt et al., 2006) and infection agents (Williams, 2005; McDevitt et al., 2006; Timbermont et al., 2011; Shojadoost et al., 2012).

Coccidiosis

Coccidiosis is an enteric disease caused in the fowl by numerous *Eimeria* species. Some species (*E. praecox, E. mitis or E. acervulina*) produce less severe clinical coccidiosis than others (*E. brunetti* , *E. maxima, E. necatrix* , and *E. tenella*) (Williams, 2005). The intestinal damage caused by coccidia is an essential predisposing factor for NE (Al-Sheikhly and Al-Saieg, 1980; Williams, 2005; Rodgers et al., 2014), allowing *C. perfringens* overgrowth and production of toxins (Van Immerseel et al., 2008). Intestinal damage during *Eimeria* infection will result in leakage of plasma proteins into the lumen of the intestinal tract, which is a rich nutrient substrate and favorable for *C. perfringens* proliferation and toxin production (Van Immerseel et al., 2004). Collier et al. (2008) suggested that coccidial infection induces mucogenesis as a result of a host mucogenic response, providing a growth advantage for *C. perfringens*.

For these reasons, several studies have used *Eimeria* spp. in conjunction with *C. perfringens* to induce NE experimentally. *Eimeria maxima, Eimeria acervulina* and *Eimeria necatrix* are known to be the most suitable species to induce NE (Al-Sheikhly and Al-Saieg, 1980; Hofacre et al., 1998; Van Immerseel et al., 2004; Williams, 2005). *Eimeria* vaccine has also been used to enhance *C. perfringens* to induce NE (Gholamiandehkordi et al., 2007; Timbermont et al., 2009). The time and dose of administration of the virulent *Eimeria* or coccidial vaccine are important for inducing experimental NE. The time should not be more than 4-5 days before *C. perfringens* challenge (Williams et al., 2003; Williams, 2005). The doses of *Eimeria* administration needed for experimentally inducing NE are different according to the models which have been used. For example, Wu et al. (2014) used a suspension of 5000 sporulated oocysts each of *Eimeria maxima* and *E. acervulina,* and 2500 oocysts of *E. brunetti;* Williams et al. (2003) used 30,000 sporulated oocysts of *Eimeria maxima* and Gholamiandehkordi et al. (2007) suggested using attenuated coccidal vaccines at 10-times the recommended vaccination doses.

Stress and immunosuppression

Any stressful condition in broiler chickens could predispose them to NE, because it could change the intestinal environment in such a way that the risk of induction of NE is raised (McDevitt et al., 2006). For example, alteration in feeding regime (moving from a starter diet to a grower diet) in young chicken causes such stress in the gastrointestinal tract. Increases in stocking density are also frequently associated with NE (McDevitt et al., 2006). Immunosuppressive agents such as infectious bursal disease, Marek's disease, chicken anemia virus and Gumboro disease have been suggested as causing an increase in the severity of NE (Williams et al., 2003; McReynolds et al., 2007; Lee et al., 2011; Timbermont et al., 2011). Indeed, in several studies, infectious bursal disease vaccine has been used as a predisposing factor to experimentally induce NE (McReynolds et al., 2004; Gholamiandehkordi et al., 2007; McReynolds et al., 2007; Timbermont et al., 2009). This has been done by inoculation with usual doses of nfectious bursal disease vaccine (intermediate class) (Gholamiandehkordi et al., 2007; Timbermont et al., 2009) or at 10-times the recommended vaccination dose rate with infectious bursal disease vaccine (intermediate plus) (Nikpiran et al., 2008).

Nutritional factors

The key risk factor for the development of NE is alteration of the GIT environment which creates a favorable environment for *C. perfringens* growth. Diet is now widely recognized as having a strong impact on the incidence of NE in broiler chickens (Annett et al., 2002; Drew et al., 2004). Evidence arising from several studies has shown that there is a relationship between cereal type used in the diet, dietary protein levels and anti-nutritional factors and the incidence of NE (Kalshusdal and Hofshagen, 1992; Riddell and Kong, 1992; McDevitt et al., 2006).

Type of dietary cereals

It is widely believed that a diet with high levels of indigestible water soluble NSPs strongly influences the incidence of NE in broilers. Numerous studies have revealed that a diet comprising cereals such as barley, rye and wheat, which contain high amounts of NSPs such as arabinoxylans or β glucans, increase the digesta viscosity and enhance the development of NE (Kalshusdal and Hofshagen, 1992; Annett et

al., 2002; Dahiya et al., 2006; McDevitt et al., 2006). The different levels of starch and NSPs of cereals and other feedstuffs which are not digestible by the enzymes that are present in the bird's GIT (Iji and Tivey, 1998; Juśkiewicz et al., 2004) act as substrates for the gut microflora and provide an opportunity to gut microflora bacteria, including pathogenic bacteria, for proliferation (Choct et al., 1996; Iji and Tivey, 1998; Apajalahti et al., 2004; McDevitt et al., 2006). Higher NSPs in diets lead to increased digesta viscosity, prolonged transit time and decreased nutrient digestibility (Choct et al., 1999), which may be responsible for promoting the proliferation of *C. perfringens*, predisposing birds to NE (Annett et al., 2002). The NSPs are also hydrophilic, which encourages birds to drink more water in order to maintain homeostasis. The increased water intake also increases water excretion, affecting litter quality and thus allowing the pathogenic bacteria to proliferate (McDevitt et al., 2006). Furthermore, some NSPs interact with epithelial protein and glycoproteins, increasing the mucin secretion from the tissues (Kleessen et al., 2003). This provides an opportunity to pathogenic microorganisms to adhere to the mucin and proliferate (McDevitt et al., 2006).

It has been proven that the cereal component of poultry diets influences the development of NE. In birds experimentally challenged with *C. perfringens*, Riddell and Kong (1992) found that mortalities of birds receiving barley, rye and wheat ranged from 26-35%, whereas birds that were fed a supernatant of digested maize showed mortality of 0-12%. Mortality due to coccidiosis-challenged birds with a wheat base diet was lower than mortality in a corn based diet (Branton et al., 1987). In an in vitro study, Annett et al. (2002) showed that the proliferation of *C. perfringens* was lower in the extract of digested maize than in the extract of digested barley or wheat. It has also been reported that the numbers of *C. perfringens* in the intestines of broilers fed 50% rye were 1.2-1.5 $\log_{10}$ higher than birds fed a corn based diet (Craven, 2000).

Dietary animal products

The level of crude protein, protein source, and the amino acid content of a diet are all associated with the incidence of NE. Poultry diets with high protein content or those rich in animal protein such as meat and bone meal or fish meal predispose birds to NE (Kaldhusdal and Skjerve, 1996; Williams et al., 2003; Williams, 2005; Wu et al.,

2010; Wu et al., 2014). Diets that have a high protein concentration or imbalanced amino acid profiles reduce the digestibility of these compounds in the upper part of digestive system (McDevitt et al., 2006). Thus the indigestible protein in the lower part of GIT acts as substrate for the gut microflora (Timbermont et al., 2011). The fermentation of protein produces unfavorable outcomes such as phenols, thiols, amines, ammonia, indoles and increases the pH of the lower part of the GIT, which encourages the proliferation of pathogenic bacteria such as *Clostridium* SP. (Juśkiewicz et al., 2004; Lan et al., 2005). Another possible explanation of the association between fish or meat meal and NE could be related to their higher zinc, glycine and methionine concentrations. Fish meal is relatively high in zinc (NRC, 1984), glycine and methionine (Dahiya et al., 2007). In an *in vitro* study, Baba et al. (1992) proposed that dietary zinc increased the production of α-toxin and protected the α-toxin destruction by trypsin. Glycine is an amino acid that stimulates the growth of *C. perfringens* (Dahiya et al., 2007) and is positively correlated to the *C. perfringens* population in the intestine (Wilkie et al., 2005).

Inducing NE experimentally in chickens having a high dietary content of animal protein, or changing the diet to one with high protein before challenging birds, seems to increase the severity of NE and may be important to predispose the birds to NE (Shojadoost et al., 2012). It has been reported that changing the diet to a high protein diet before challenging birds with *C. perfringens* increased the severity of NE (Timbermont et al., 2010). In another study,Wu et al. (2014) postulated that broiler chickens receiving fish meal had significantly changed gut microflora, which may play an important role in predisposing birds to NE. Interestingly, however, when birds were challenged with *C. perfringens* subsequently, fishmeal did not play a critical role in predisposing the chickens to NE compared at least to *Eimeria* and was thus removed from the NE challenge model (Rodgers et al., 2014).Consumption of diets containing a lower energy-to-protein ratio can also be a contributing factor to predisposing birds to NE. A diet which contains lower energy-to-protein ratio, increases feed intake and nitrogen content of digesta and excreta (McDevitt et al., 2006). The increased nitrogen content of digesta and excreta can lead to an enhanced substrate for *C. perfringens* (Lan et al., 2005; Timbermont et al., 2011).

Anti-nutritional factors

Anti-nutritional factors such as lectins, trypsin inhibitors and tannins may also predispose birds to NE. Lectins from wheat and soybeans are proteins and glycoproteins, which interact vigorously with epithelial tissues (Pusztai and Bardocz, 1996) and cause damage, change in microflora population and immune response in animals (Pusztai and Bardocz, 1996; Lan et al., 2005). Lectins alter bacterial attachment to the GIT and change the extent and rates of bacterial growth (Giovannini et al., 1996; McDevitt et al., 2006). Trypsin inhibitors, found in soybean meal, reduce the digestibility of protein and thus increase nitrogen concentration in the lower GIT, which provides suitable conditions for the growth of proteolytic bacteria such as *C. perfringens* (Clarke and Wiseman, 2005; McDevitt et al., 2006). Tannins are present in many dietary ingredients such as rapeseed meals and beans, and can interact strongly with protein, leading to tissue damage that may predispose birds to NE (Robins and Brooker, 2005; McDevitt et al., 2006). There are also other compounds such as mycotoxins, glucosinolates, alkaloids and polyphenols which interact with bacteria, altering bacterial proliferation (McDevitt et al., 2006) and which may play a role in predisposing birds to NE.

Physical form of diet

The physical form of poultry diets may affect the physiological and morphological characteristics of the GIT (Engberg et al., 2004). Published reports in this area of research are inconsistent. Highly ground feed allows *C. perfringens* to grow faster than coarse ground feed, which can lead to occurrence of NE in the field (Engberg et al., 2002). It has been suggested that coarsely ground mash stimulates gastric function, including secretion of hydrochloric acid, and simultaneously increases the retention time of feed in the proventriculus and gizzard. Branton et al. (1987) observed that use of a coarsely ground wheat diet decreased mortality from NE to 18.1%, whereas a finely ground wheat diet resulted in mortality of 28.9%. In contrast, Gabriel et al. (2003) found feeding whole wheat to broilers, experimentally challenged with coccidiosis, reduced weight gain and enhanced development of *Eimeria tenella* in the caecum.

2.6 Methods of controlling necrotic enteritis

2.6.1 Antibiotics

Antibiotics have been used in the animal feed industry for more than 50 years. Antibiotics at sub-therapeutic levels have been used as an effective tool to improve animal performance, by selectively modifying the gut flora, decreasing bacterial fermentation, reducing thickness of the intestinal wall and suppressing bacterial catabolism. All of these are important to improve health, nutrient availability and growth performance (Carlson and Fangman, 2000). Antibiotics have played a major role in the suppression of clinical NE, but there is no specific medicine employed for clostridial diseases (Williams, 2005). Several studies have been conducted on the effects of different antibiotics such as avoparcin, virginiamycin, zinc bacitracin (ZB), tylosin, salinomycin and others in controlling NE. For instance, in some *in vitro* studies, avoparcin and avilamycin had strong antibacterial effects on all poultry isolates of *C. perfringens* (Devriese et al., 1993; Watkins et al., 1997). Avoparcin reduced intestinal *C. perfringens* counts and prevented NE in broilers challenged with *C. perfringens* (Hofshagen and Kaldhushal, 1992). Multiple studies have shown that tylosin (Collier et al., 2003) and virginiamycin (Stutz and Lawton, 1984) reduces mortality and intestinal numbers of *C. perfringens* when NE is present. The inclusion of bacitracin in broiler feed has also been shown to reduce intestinal gut lesion scores and mortality caused by NE (Broussard et al., 1986; Brennan et al., 2003; Hofacre et al., 2003). Furthermore, dietary zinc bacitracin has been shown to increase body weight of birds under NE challenge conditions (Ao et al., 2012). Finally, our results have shown that antibiotics (zinc bacitracin and salinomycin) protected birds from NE by controlling performance decline, improving gut health, and reducing gut lesions and *C. perfringens* population. In an NE challenge model, salinomycin decreased the severity of lesions and *C. perfringens* counts in the intestinal tracts (Engberg et al., 2000; Jackson et al., 2003). However, public concern over the use of in-feed antibiotics and the emergence of antibiotic-resistant "superbugs" has led many countries to ban the use of dietary antimicrobials.

As a result of consumer pressure to reduce in-feed antibiotics in animal feed, Sweden was the first country to ban the use of antimicrobials for growth-promoting purposes (Kocher and Choct, 2008). In 1995, Denmark banned the use of avoparcin and

virginiamycin in animal feed (Casewell et al., 2003). In 1997, the European Union restricted the use of some antibiotics in animal feed and followed this by a general ban on the use of all antibiotics in all animal feed in 2006 (Kocher and Choct, 2008). After the banning of sub-therapeutic antibiotics in animal feed, the incidence of NE in poultry farms increased in many European Union countries (Van Immerseel et al., 2004). For example, the incidence of NE in France was 4% in 1995 and increased to 12.4% in 1999 (Drouin, 1999). Similarly, in the USA, who also stopped using in-feed antibiotics, the incidence of NE and other diseases like gangrenous dermatitis, botulism and cholangiohepatitis has increased (Shane, 2004). Thus, the question has now become 'how to control this disease in the absence of antibiotics'. To investigate a method for controlling NE, the factors that predispose birds to develop NE must be better understood and alternative dietary supplements and management strategies to control NE must be developed.

2.6.2 Alternative strategies to prevent and control necrotic enteritis

Based on the literature, there are three basic strategies used to control NE. These strategies are: amplification of immune response, pathogen reduction, and dietary modification and/or use of feed additives (Dahiya et al., 2006). Augmentation of immune response by vaccination and pathogen reduction strategies, including farm biosecurity and poultry house sanitation, will not be addressed here. However, this paper will review numerous strategies for controlling NE, including dietary modification and the use of feed enzymes, probiotics, prebiotics, organic acids and acylated starch and vaccination strategy will be discussed in the following sections.

Dietary modifications and feed enzymes

Modification of diet and the addition of enzymes cannot provide total protection against NE, but may reduce the risk of NE by improving digestion. For example, dietary cereals such as barley, rye and wheat contain high amounts of arabinoxylans or β glucans, mannans, cellulose, lignin and ingredients, which cannot be digested by poultry. These non-digestible feed consituents increase the digesta viscosity and encourage the development of NE (Dahiya et al., 2006; McDevitt et al., 2006). Also, poultry diets with a high protein content, or those rich in animal protein such as meat and bone meal or fish meal predispose birds to NE (Kaldhusdal and Skjerve, 1996;

Williams et al., 2003; Williams, 2005; Wu et al., 2010; Wu et al., 2014). Thus, reducing indigestible carbohydrates and proteins that predispose birds to NE may decrease the risk of NE.

The use of various feed enzymes has become standard in all poultry feed over the past two decades. It has been demonstrated that inclusion of exogenous enzymes to wheat, barley, oat or rye based diets can significantly decrease digesta viscosity in the small intestine (Bedford and Classen, 1992; Choct et al., 1999). Choct et al. (1999) reported that the addition of xylanase to wheat base diets decreased digesta viscosity and fermentation, increased nutrient digestion and digesta passage, and reduced the amount of nutrients available to the microflora, which, in turn, may reduce the bacterial population in the small intestine. Sinlae and Choct (2000) also demonstrated that dietary supplementation of xylanase reduced numbers of *C. perfringens*. Jackson et al. (2003) proposed that β mannanase would reduce the severity of challenge by *Eimeria spp.* and *C. perfringens* in broiler chickens, and also reduce NE lesion scores in the intestine. However, supplementation of pentosanase in a wheat based diet did not have any beneficial effect on the susceptibility to NE in broiler chickens. Despite the contradictory results from published studies on the effect of feed enzymes on various bacterial populations, including *C. perfringens* in the broiler intestine, the supplementation of exogenous enzymes alone cannot provide complete protection against NE (Elwinger and Teglöf, 1991; Riddell and Kong, 1992). Enzymes have no direct effect on *C. perfringens*, but only change the intestinal environment (Kocher and Choct, 2008).

Probiotics

Probiotics have been defined as a "live microbial feed supplements, which beneficially affect the host animal by improving its intestinal balance" (Fuller, 1990). The characteristic of ideal probiotics are that they must be; from host origin, resist to gastric acids and bile, persist in the intestinal tract, adhere to epithelium or mucus, produce inhibitory compounds, alter immune response and modulate the microflora activity (Patterson and Burkholder, 2003). The modes of action of probiotics include stimulating the immune system, maintaining gut microflora by competitive exclusion, altering metabolism through increased digestive enzyme activity, decreasing bacterial enzyme activity and ammonia production, and neutralizing

enterotoxins (Walker and Duffy, 1998; Collins and Gibson, 1999; Simmering and Blaut, 2001; Patterson and Burkholder, 2003). The mechanisms of competitive exclusion of pathogens include competitive nutrients and mucosal binding sites, or production of SCFAs, low pH and bacteriocins, which are bactericidal or bacteriostatic for pathogenic bacteria (Ohland and MacNaughton, 2010).

A number of studies have reported the potential of undefined (normal gut flora) or defined (characterized bacterial strain) probiotics on the colonization of pathogenic bacteria. Mead (2000) reported that normal gut flora preparations have shown efficacy against food borne pathogens such *as Escherichia coli, Yersinia enterocolitica, C. botulinum, C. perfringens, Salmonella, and Campylobacter jejuni.* Morishita et al. (1997) showed that *Lactobacillus acidphilus* and *Streptococcus faecium* reduced the colonisation of *C. jejuni* in the jejunum by 27%. A number of studies have been conducted on the effects of probiotics on NE in chickens. It has been reported that commercial probiotics reduce gross intestinal lesions from NE and improve feed efficiency (Hofacre et al., 1998). Craven et al. (1999) proposed that feeding normal gut flora to broiler chickens would reduce the *C. perfringens* colonization and decrease the incidence of NE. Hofacre et al. (2003) showed that mortality due to NE was reduced from 60 to 30% when birds were treated with lactic acid bacteria. La Ragione and Woodward (2003) found that colonization and determination of *C. perfringens* was suppressed when 1 day old and 20 day old birds were inoculated with 10^9 spores of *Bacillus subtilis* strain, then challenged 24 h later with 10^5 CFU of *C. perfringens*, colonization and determination of *C. perfringens* was suppressed. Jayaraman et al. (2013) found that dietary supplementation of *B. subtilis* in experimentally challenged broiler chickens with *Emeria* and *C. perfringens* improved FCR, intestinal lesion scores, *C. perfringens* counts, and villus height to crypt depth ratios when they were compared with an infected control.

Prebiotics

Prebiotics are generally defined as indigestible feed ingredients that selectively stimulate the growth or activity of beneficial bacteria that are already resident in the GIT (Gibson and Roberfroid, 1995). The potential effects of prebiotics on animals were recognized in the 1980s (Hajati and Rezaei, 2010). For a feed ingredient to be classified as a prebiotic, it must be neither digested nor absorbed in the upper part of

digestive system; it must be a selective substrate for one or a limited number of useful bacteria in the GIT, stimulate the bacteria to grow and be metabolically activated, be able to alter the intestinal microflora toward a healthier composition and be palatable as a food ingredient (Collins and Gibson, 1999; Hajati and Rezaei, 2010). Substances used as prebiotics are non-digestible carbohydrates, certain lipids, proteins and peptides. Lactose is a disaccharide which has been used as a prebiotic in chickens (Hajati and Rezaei, 2010). The predominant prebiotics are fructo-oligosaccharides (FOS), xylo-oligosaccharides, glycol-oligosacchriades galacto-oligosaccharides (GOS), gluco-oligosaccharides, lactitol, lactulose, malto-oligosaccharides, and trans-galacto-oligosaccharides (Gibson and Roberfroid, 1995; Patterson et al., 1997; Collins and Gibson, 1999). In general, prebiotics can prevent the colonization of bacterial pathogens in the GIT (Bengmark, 2001), lower the gut PH through SCFA production (Gibson and Wang, 1994), and stimulate the immune system (Monsan and Paul, 1995).

Mannooligosaccharides (MOS) also have been considered as prebiotic. They are obtained from the yeast cell wall of *Saccharomyces cerevisiae* yeast (Hofacre et al., 2003). The yeast cell wall consists of protein, glucans, and mannan (Klis et al., 2002). MOS that are harvested from yeast cell walls consist of mannoproteins, chitin, β (1,3) glucan, and β (1,6) (Kollár et al., 1997). The exact mode of action of MOS is unclear, but β-glucans and mannans are primary functional units. The mannans act as a receptor for type-1 fimbriae which are used by some harmful bacteria such as *Escherichia coli* and *Salmonella* to attach to the gastrointestinal wall (Oyofo et al., 1989). β glucans act as microbial recognition receptors of the innate immune system (Gantner et al., 2003) and both mannans and β-glucans structures stimulate the immune system (Spring et al., 2000). The effects of MOS supplementation on the broiler immune system, gut microflora and gut morphology have been well documented. For instance, Shanmugasundaram and Selvaraj (2012) reported that dietary prebiotics (killed whole yeast cells) increased IL-10 mRNA by 9 fold in comparison to the control. Shanmugasundaram et al. (2013) also proposed that 0.2% of killed whole yeast cell prebiotics increased body weight gain, macrophage nitric oxide production and caecal tonsil interleukin-1 mRNA amounts in broilers challenged with coccidia. Ghosh et al. (2011) reported that antibody titer against Newcastle disease was significantly higher in birds fed a yeast cell wall preparation.

In the same study, birds were orally infected with *S. pullorum* at 45 days of age. After the oral infection, *Salmonella* counts were lower in digesta in birds fed the yeast cell wall preparation. This was supported by a number of studies, where dietary MOS were effective in reducing *Salmonella* infection (Fernandez et al., 2000; Spring et al., 2000). It has also been reported that the mannan oligosaccharides improved intestinal morphology, such as increasing villi height and altering mucosal architecture (Iji et al., 2001), and decreasing the crypt depth of the mucosa of the small intestine (Yang et al., 2009).

Some studies have been conducted on the effects of probiotics on *C. perfringens* in reducing the severity of NE. Data from Sims et al. (2004) showed that 6 week old turkeys in a MOS treatment group had significantly fewer *C. perfringens* in their large intestines compared to control birds. This finding is supported by (Kim et al., 2011) who reported that dietary supplementation with MOS decreased *C. perfringens* populations in broilers' small intestines. Dietary MOS also reduced the abundance of *C. perfringens* in the broiler caeca at 21 days of age (Yang et al., 2008a). During NE infection, Mohamed and Hafez (2011) proposed dietary MOS reduced mortality by 12% compared to the positive control group. In the same study, dietary MOS supplementation showed a reduction in the severity of lesion scores which reached 0.57 in contrast to 1.8 in the control group. Also, our unpublished results showed that yeast cell wall extract was effective in reducing lesions and improving livability compared with the challenged control. In contrast, Hofacre et al. (2003) suggested neither addition of MOS nor FOS to the broilers diet had a significant effect on intestinal lesion scores and mortality caused by NE.

Organic acids

Organic acids have been used for decades in feed, to protect feed from microbial and fungal damage. These acids are added to foods as preservative agents and can also be used to control microbial contamination (Kum et al., 2010). In fact, the organic acids that have antimicrobial activity are simple monocarboxylic acids such as formic, acetic, propionic and butyric acids, or are carboxylic acids bearing a hydroxyl group on the alpha carbon such as lactic and tartaric acids. It has been noted that salts of some of these acids have performance benefits. Some short chain carboxylic organic acids, such as sorbic and fumaric acids containing double bonds also have antifungal

activity (Dibner and Buttin, 2002). Several of these organic acids can be produced in small quantities as a result of the fermentation activity of the GIT (Dorsa, 1997) where anaerobic microflora are predominant (Dixon and Hamilton, 1981).

The effects of organic acids on broiler chickens have been well documented. Hassan et al. (2010) proposed that the inclusion of organic acids to broiler diets enhanced growth and feed utilisation and feed conversion ratio. Acidification with different organic acids has been shown to reduce pathogenic bacteria toxicity through control of pathogenic colonization on the gastrointestinal wall, inhibiting damage to epithelial cells (Langhout, 2000). The basic principle mode of action of organic acids is that non-dissociated organic acids can diffuse through lipophilic bacteria and mold membrane, disrupting the enzymatic reaction and the transport system of the bacteria (Cherrington et al., 1991). As described by Lambert and Stratford (1999), after penetration of organic acids into bacterial cytoplasm the non-ionized organic acids decompose to H (H^+) ions and (A^-) ions. By the time of the decline of the pH inside the bacteria, a specific mechanism (H^+- ATP ase pump) will act to return the intracellular levels to normal pH. This process requires energy, which will lead to reduced energy accessibility for the proliferation of cells and eventually stop the bacterial growth or even kill it. Izat et al. (1990) observed that dietary supplementation of buffered propionic acid significantly reduced the total number of *E. coli* in the intestinal tract. Thompson and Hinton (1997) found that organic acids are bactericidal for *Salmonella* serotype Enteritidis PT4. Chaveerach et al. (2004) demonstrated that organic acids in the drinking water of young chicks could have a potential effect on *Campylobacter* infection. Czerwiński et al. (2012) found a negative correlation between *Enterobacteriaceae* numbers and the concentration of un-dissociated propionate, acetate and butyrate in the caeca. Dietary addition of organic acids can also improve the digestibility of protein and amino acids (Afsharmanesh and Pourreza, 2005), calcium, phosphorus, magnesium and zinc (Garcia et al., 2007). Also, it has been reported that organic acid supplementation can improve gastrointestinal villi height and cell proliferation (Adil et al., 2010). Other activities of organic acids associated with acidification include the increase of pancreatic secretions, and microbial phytase activity and the improvements of digestive enzyme activity (Dibner and Buttin, 2002).

Recently, microencapsulated organic acids have been used in poultry diets. The rationale behind using coated organic acids is that non-protected organic acids are digested and absorbed in the upper part of digestive tract, while coating organic acids prevents dissociation in the upper part of the digestive tract and directs their bioactivity towards the lower gastrointestinal tract. The applications of microencapsulated organic acids on animals are limited. Smulikowska et al. (2009) suggested that there is no growth promoting response to coated sodium butyrate or its salt when in birds raised under optimized conditions. This finding has been supported by a number of other researchers (Smulikowska et al., 2009; Zhang et al., 2011; Czerwiński et al., 2012). Jerzsele et al. (2011) suggested that protected sodium butyrate had no beneficial effects in birds challenged with *C. perfringens,* but a combination of sodium butyrate with essential oils protected with vegetable fat increased body weight gain, villus height and decreased gross lesion scores compared with a control.

Resistant starch

Resistant starch is the fraction of ingested starch which is not digested by enzymes in the small intestine and therefore escapes into the large bowel (Asp and Björck, 1992). Resistant starch can be a protective agent against many pathogenic organisms in the gut through the production of SCFAs as well as encouraging the proliferation of beneficial organisms (Topping and Clifton, 2001). Indeed, SCFAs have an important physiological and nutritional function in maintaining the large bowel in humans through a number of chronic and acute actions. Resistant starch has been classified into four classes (Englyst et al., 1992):

1. Physically trapped, i.e. partially milled grains
2. Resistant raw starch, i.e. high amylose maize starch
3. Retrograded starch, i.e. cooled and cooked
4. Chemically modified, i.e. esterified (Acylated starches).

Acylated starches are a relatively new innovation and are used to deliver specific SCFAs to the hind gut for therapeutic, clinical and public health application. Acylated starches are resistant to small intestine digestion and esterified acids released by bacterial enzymes, and are available for utilization and absorption by gut

microbes and colonocytes (Abell et al., 2011). Thus, the acylating carbohydrates, such as starch, with specific SCFAs offer a degree of specificity in SCFA delivery. Feeding trials in rats (Annison et al., 2003; Abell et al., 2011) and humans (Clarke et al., 2007) have confirmed the ability of acylated starches to deliver specific acids that had been esterified. It has been reported that esterified acid is released in the large bowel by bacterial lipases and esterases and the residual starch available for fermentation further increases large bowl SCFA levels (Annison et al., 2003).

Vaccination

Vaccination has been an effective way to prevent humans and animals from many infectious diseases. It can enhance specific immunity of the organisms to viral and bacterial diseases. Vaccines have also been successfully applied to control numerous clostridial diseases in livestock animals (Walker, 1992). Therefore, vaccination against NE disease is proposed to provide an alternative treatment for NE in poultry especially when more convincing evidences was revealed that the toxin NetB is responsible for the disease (Keyburn et al., 2008; Keyburn et al., 2013).

Vaccination strategies have been put forward for the control of NE mainly in broiler chickens. These strategies were extensively reviewed recently by (M'Sadeq et al., 2015). Prior to the discovery of NetB, the earlier vaccination focused on toxins that may not be associated to NE by and large, for example, α-toxin. Thus, the vaccines developed only had limited success in controlling NE (Hoang et al., 2008; Zekarias et al., 2008; Cooper et al., 2009), while the partial protective effect of α-toxin based vaccine may be due to the association of α-toxin protein with cell membrane that can have immune interaction to perform such protection (Keyburn et al., 2013). On the other hand, vaccination against coccidiasis has been also used to protect birds from NE occurrence (Jackson et al., 2003; Williams et al., 2003; Tsiouris et al., 2013; Bangoura et al., 2014). As *Eimeria* infection is widely recognised as one of the most important NE prodisposing factors in broilers, it seems reasonable that the reduced risk of coccidiasis in birds would allievate NE outbreak in the flock.

The most important step forward to develop vaccines to immune the birds against NE occurred following the discovery of NetB toxin which is now considered a major virulence factor for birds to develop NE (Keyburn et al., 2008). A recombinant NetB

C. perfringens (rNetB) was constructed and attenauted as a vaccine by (Keyburn et al., 2013). The birds imunised with rNetB were significantly protected against NE challenged with a mild dose of virulent bacteria, while the effectiveness of the vaccination was not so when a more robust challenge was performed. Alternatively, when the birds were imunised with a combination of rNetB, bacterin and cell free toxoid, significant protection against moderate and severe challenge was observed. It was suggested that in vitro levels of NetB produced by virulent *C. perfringens* isolates were too low to produce strong immune repsonse in the birds and thus the combined vaccination of birds with rNetB and other cellular or cell-free antigens may be necessary. Concurrently, Jang et al. (2012) compared four *C. perfringens* recombinant proteins as vaccine candidates using the Montanide™ ISA 71 VG adjuvant in an experimental model of NE. When the broilers were immunized with purified clostridial recombinant NetB toxin, pyruvate: ferredoxin oxidoreductase (PFO), α-toxin, or elongation factor-Tu (EF-Tu), significantly reduced gut lesions were observed. Furthermore, birds immunized with NetB toxin exhibited significantly increased body weight gains and greater NetB toxin antibody titers. The authors suggested that vaccination with NetB toxin or PFO, in combination with ISA 71 VG enhances protective immunity against NE in broiler chickens. Fernandes da Costa et al. (2013) employed similar stratedgy but used only a formaldehyde NetB toxoid or NetB genetic toxoid (W262A) without attetuated NetB containing *C. perfringens* strains or recombinants. The immunisation of poultry with these toxoids resulted in the induction of antibody responses against NetB and provided partial protection against the disease. Furthermore, Mot et al. (2013) used crude supernatant containing active toxin or formaldehyde-inactivated supernatant (toxoid) of a netB positive *C. perfringens* strain and administered vaccination at earlier days post hatch. It was found that double vaccination at 3 d and 12 d resulted in a significant decrease in the number of chickens with NE lesions although single vaccination with crude supernatant at 3 d also pruduced significant protection. However, the efficacy of vaccination using toxoid was lower compared with crude supernatant, and vaccination of 1-day-old chickens with crude supernatant or toxoid did not induce protection, a way supposed to be practical in the broiler production. To be more pratical for the industry, Keyburn et al. (2013) performed maternal immunization in hens injected subcutaneously with genetically toxoided rNetB(S254L) alone, *C. perfringens* type A toxoid and toxoid combined with rNetB (S254L). They observed

strong serum immunoglobulin Y response to NetB immunized with rNetB (S254L) formulations with anti-NetB antibodies transferred to the eggs and then onto the hatched progeny. It was confirmed that birds derived from hens immunized with rNetB (S254L) combined with toxoid and challenged with a homologous strain (EHE-NE18) had significantly lower levels of disease in a subclinical form compared to birds from adjuvant only vaccinated hens. They demonstrated that maternal immunization with a NetB-enhanced toxoid vaccine is promising for the control of NE in broiler chickens.

Although the protection of birds against NE by vaccination using NetB or other antigen related vaccines or in combiantion, more pratical protocols and effective vaccines have to be extensively examined to achieve full protection of bords against the disease. Apparently, the vaccines targeting agaist NetB antigen and possibly other undiscovered toxins responsible for NE should be explored through a pratical vaccination regime for industry application. Undoubtedly, other beneficial combination of different vaccines, such as non-inactivated supernatants, formalin-inactivated crude toxoids, immunogenic proteins and modified toxins including those indirectly protect against NE is also important aspects to be investigated so as to achieve desired immunization levels suitable in a commercial application.

Other potential measures in controling NE

Recent progress on the characterization of NetB toxin has suggested other possibilities to control NE. NetB has been characterized as a pore-forming toxin with a heptameric structure (Savva et al., 2013; Yan et al., 2013) where the polymerization of the proteins lead to the toxicity of the toxin to the epithelial cells of the chiken intestine. It has been recognized that the cholestrerol plays a key role in the ability of NetB to oligomerize and form functional pores (Savva et al., 2013). This may provide some hinds on the development of measures to reduce toxicity of NetB and thus to control NE in birds. Furthermore, the detailed information on the residues important for binding and toxicity will also facilitate vaccine development against NE.

Apart from the toxin NetB which directly produce toxicity to the intestinal tissues, a bacteriocin from NetB positive *C. perfringens* characterized by Timbermont et al.

(2014), perfrin, was considered important in the pathogenesis of NE in broilers. This bacteriocin inhibits growth of other *C. perfringens* strains and leads to extensive and selective growth of *C. perfringens* secreting toxins that cause gut lesions. Consequently, it is reasonable to speculate that mechanisms preventing the production of such bacteriocin in the gut would be able protect birds from infection of NE at least to some extent.

Selenium regulates major physiologic pathways of humans and animals as a essential micronutrient. It can enhance the immune and antioxidant systems. Recently, selenium was used for the in ovo injection to brioler eggs at 18 d of embryo age and the birdst were later challenged with NE, and the protection effect was observed to increase the levels of antibody against NetB and α-toxin (Lee et al., 2014b). Again, the same group injected selenium in ovo that was incorporated into hydrolyzed soybean protein (B-Taxim [BT]) and observed similar protection of challenged birds against NE (Lee et al., 2014a). Accordingly, it seems promising that selenium administered in single or incorporated in hydrolyzed soybean protein shows certain degree of protection thus worth to pursue further for industrial implementation.

2.7 Conclusion

The GIT is an important part of complete health, homeostasis and production in an avian system. Many factors associated with infectious agents and diet can negatively affect the chicken gut health which may affect the health status and production performance of birds. Phasing out of in-feed antibiotics from poultry feed in Europe and recent removal or reduction of these compounds in the other parts of the world, either by government regulation or voluntarily, in countries including Australia and USA, is a challenge to the poultry industry. Studies from Denmark and Sweden have confirmed that the key problem of in-feed antibiotic withdrawal from poultry diets is the control of NE. Currently, much extensive multidisciplinary research has been done to alleviate the problems associated with antibiotic withdrawal from poultry diets, but to date no single preventive therapy has been established to substitute in-feed antibiotics. Although several nutriceutical supplements including probiotics, prebiotics, essential oils, organic acids, enzymes and hen egg antibodies have been used to reduce the incidence of NE, no product has been as effective as antibiotics in terms of controlling NE. However, recent investigations have shown that the use of

alternatives to in-feed antibiotic products in poultry diet such as preobiotics, organic acids and others have improved gastrointestinal health, integrity and reduced the intestinal colonization of pathogenic bacteria. Therefore, using alternatives to in-feed antibiotics with a better understanding of the relationship between nutrition and NE, and limiting exposure to infectious agents through biosecurity and vaccination, might be a tool to reduce the incidence of NE and improve gut health in the absence of in-feed antibiotics.

Chapter 3 Use of Yeast Cell Wall Extract as a Tool to Reduce the Impact of Necrotic Enteritis in Broilers

Abstract

The use of a yeast cell wall (YCW) derived from Saccharomyces cerevisiae (Actigen®) has been proposed as an alternative to in-feed antibiotics. This experiment was conducted to investigate the efficacy of YCW as an alternative to zinc bacitracin (ZB) or salinomycin (SM) using a necrotic enteritis (NE) challenge model. A feeding study was conducted using 480 d-old male Ross 308 chicks assigned to 48 floor pens. A 2 x 4 factorial arrangement of treatments was employed. The factors were: challenge (- or +) and feed additive (control, ZB at 100/50 mg/kg, YCW at 800/400/200 mg/kg, or SM at 60 mg/kg in starter, grower, and finisher, respectively). Diets based on wheat, sorghum, soybean meal, meat and bone meal and canola meal were formulated according to the Ross 308 nutrient specifications. Birds were challenged using a previously established protocol (attenuated Eimeria spp oocysts) on d 9 and 10^8-10^9 *C. perfringens* (type A strain EHE-NE18) on d 14 and 15). Challenged and unchallenged birds were partitioned to avoid cross contamination. Challenged birds had lower weight gain (WG), feed intake (FI) and livability (LV) compared to unchallenged birds on d 24 and d 35 ($P < 0.05$). Birds given ZB, YCW or SM had improved WG and LV when compared to control birds given no additives. Challenge × additive interactions were observed for FI and WG on d 24 and d 35 ($P < 0.01$). The additives all had a greater positive impact on FI, WG and LV in challenged than unchallenged birds. All challenged birds showed higher necrotic enteritis (NE) lesion scores in the small intestine sections when compared to unchallenged birds ($P < 0.01$). Birds fed YCW exhibited decreased crypt depth, increased villus height and increased villus:crypt (VH:CD) ratio in challenged birds. YCW was as effective as ZB and SM in preventing performance decline from NE in the current study. This study indicates that YCW has promise as a tool for controlling NE outbreaks.

3.1 Introduction

Necrotic enteritis (NE) is a common bacterial disease in broiler growing areas of the world. The disease was first recorded in Australia by Bennetts (1930) and later

described by Parish (1961b) in the United Kingdom. The causative agent of NE is *C. perfringens* (CP), a gram positive, spore forming anaerobic bacterium that can be found in poultry litter, feces, soil, dust and in healthy bird intestinal contents (Dahiya et al., 2005). Excess dietary protein (mainly animal protein) and *Eimeria* infection affect NE pathogenesis (Kaldhusdal and Skjerve, 1996). Toxins produced by CP under certain situations are considered important for the pathogenesis of the disease (Keyburn et al., 2006). The CP toxin netB affects intestinal integrity causing a subclinical form and a clinical form resulting in economic loss (Kocher and Choct, 2008). The subclinical form of NE is more financially damaging than the clinical form, and the total cost of clinical and subclinical necrotic enteritis can be as high as $0.05per bird (Van der Sluis, 2000).

NE has been traditionally controlled in broilers through the use of dietary antibiotics, such as virginiamycin, lincomycin, zinc bacitracin (ZB) and others (Williams, 2005). In 2006, the European Union banned the routine use of antibiotics in animal feed. Immediately after the ban it was shown that the incidence of NE and other instances of intestinal dysbacteriosis increased in many European countries (Pattison, 2002). Although the US, Australia and many Asian countries still allow the routine use of antibiotics in feed to promote growth and control intestinal disease, it is widely believed that these countries will follow the initiatives of the EU and ultimately ban their use. Virginiamycin for example, an antibiotic suspected to be associated with the presence of vancomycin resistant plasmids (Thal and Zervos, 1999), has been banned as a routine in-feed antibiotic in Australia and the USDA announced in April 2014 the voluntary withdrawal of 19 antimicrobials from use in food-producing animals. Therefore, new methods for controlling NE must be investigated.

Prebiotic products that are generally recognized as safe have been examined as replacements for in-feed antibiotics. Prebiotics are poorly digestible components that selectively stimulate the growth and/or activity of one or a limited number of bacteria in the gut (Gibson and Roberfroid, 1995). The microflora stimulated by prebiotics may result in competitive exclusion of pathogens by producing antimicrobial metabolites, competing for limiting nutrients or attaching to receptor sites that would otherwise be occupied by pathogens (Vandeplas et al., 2010). Yeast cell wall (YCW) derived from *Saccharomyces cerevisiae* are rich in mannanoligosacarides (MOS), β-glucans, α-methyl-D-mannoside, D-mannose and several other compounds

(Shanmugasundaram and Selvaraj, 2012). These compounds have positive effects on broiler performance and health and may vary in concentration in commercially available products derived from yeast. Morales-López et al. (2010) reported that the addition of MOS, derived from YCW improved broiler growth performance. The objective of the current study was to investigate the efficacy of a YCW (Actigen®) as a replacement for ZB and the anticoccidial ionophore antibiotic, salinomycin (SM) using an established NE challenge model (Wu et al., 2014).

3.2 Material and Methods

The experiment was approved by the Animal Ethics Committee of the University of New England (Approval No: AEC11/127).

3.2.1 Animal husbandry

A total of 480 d-old male Ross chicks were obtained at hatching from the Baiada hatchery in Tamworth, NSW, Australia. Birds were vaccinated against Newcastle, Mareks and infectious bronchitis at the hatchery according to the commercial vaccination schedule of Baiada. Chicks were randomly assigned to 48 hardwood litter floor pens (75 x 60cm) 10 per pen. Temperature and lighting were adjusted according to the husbandry guidelines for the Ross 308 strain (Aviagen, 2012). Each pen was equipped with a tube feeder and 2 water cup drinkers. Water and feed were provided *ad libitum*. Mortality was recorded daily while cumulative pen weight and feed intake (FI) were recorded on d10, d 24 and d 35.

3.2.2 Diets

Levels and trade-names of dietary additives were: control, no additive; ZB, 100 mg/kg in starter and 50 mg/kg in grower and finisher (Albac-150®, Pfizer Company,Australia); YCW, 800 mg/kg in starter, 400 mg/kg in grower and 200 mg/kg in finisher (Actigen®, Alltech Australia, Dandenong, Vic.); SM, 60 mg/kg in starter, grower and finisher (Saccox-120®, Huvepharma, purchased from BEC Feed Solutions P/L, Brisbane, Qld.) Diets were based on wheat and soybean meal with 20% sorghum and 3% meat meal in starter and 4% in grower and finisher and 5%, 7% and 10% solvent canola meal in starter grower and finisher respectively (Table

3.1). All diets were formulated and mixed using pre-analyzed lots of all major ingredients wheat, sorghum, soybean meal, meat meal and canola meal to meet Ross 308 nutrient specifications (Aviagen, 2007). Additives were then incorporated into sub-lots of each basal diet, thoroughly mixed and pelleted at 65° C.

3.2.3 Challenge

Challenged birds were inoculated with 5000 attenuated vaccine strain sporulated oocysts each of *E. maxima* and *E. acervulina* and 2500 sporulated oocysts of *E. brunetti* in 1 mL of 1% (w/v) sterile saline on d 9 (Bioproperties, Glenorie NSW 2157, Australia) and with 10^8–10^9 of CP (type A strain EHE-NE18, CSIRO Livestock Industries, Geelong, Australia) on d 14 and 15 (Wu et al., 2014).

3.2.4 Sample Collection

On d 13 (pre-Clostridial and post-Eimeria challenge) and again on d 16 (post challenge), 2 birds were euthanized by cervical dislocation and intestinal tissue collected for morphometric analyses. Approximately 1 cm of the jejunum was collected. The intestinal samples were opened and gently flushed clean with phosphate buffered saline (PBS, pH 7.4) and then fixed in 10% buffered formalin for 24 h. Formalin was subsequently replaced by 70% ethanol for storage. On d 16, intestinal tissues (duodenum, jejunum, and ileum) were scored for coccidiosis and NE lesions. On d 35, foot pads were scored for presence of dermatitis lesions.

Table 3.1 Ingredient and nutrient composition of the basal diets (g/kg)

Ingredients	Starter	Grower	Finisher
Wheat	373.4	415.7	464.2
SBM, Arg	267.7	200.2	124.2
Sorghum	200.0	200.0	200.0
Canola meal solvent	50.00	70.00	100.0
Canola oil	39.34	49.22	52.17
Meat meal	30.00	40.00	40.00
Limestone	11.78	7.95	6.74
Dical Phos	11.07	4.86	2.67
D,L-methionine	3.65	2.71	1.87
L-lysine HCl	3.52	2.36	1.99
L-threonine	1.97	1.19	0.85
Salt	2.49	2.19	1.63
Na bicarb	2.38	1.50	1.50
Choline Cl 70%	1.12	0.58	0.57
Vitamin premix [1]	0.75	0.75	0.75
Trace mineral premix [2]	0.50	0.50	0.50
Allzyme SSF 0.2g/kg	0.20	0.20	0.20
Nutrient composition			
ME (kcal/kg)	3025	3150	3200
Crude protein	225	207	188
Digestible arginine	13.10	11.40	9.90
Digestible lysine	12.70	11.00	9.40
Digestible MC	9.40	8.40	7.30
Digestible tryptophan	2.47	2.08	1.82
Digestible isoleucine	8.78	7.67	7.72
Digestible threonine	8.30	7.30	6.30
Digestible valine	10.01	9.00	8.20
Calcium	10.5	8.5	7.60
Non-phytate P	5.0	4.2	3.80
Sodium	2.2	1.9	1.90
Chloride	3.0	2.5	2.40
Linoleic	17.3	19.4	2.00

[1] Vitamin concentrate supplied per kilogram of diet: retinol, 12000 IU; cholecalciferol, 5000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 μg; biotin, 200 μg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg.

[2] Trace mineral premix supplied per kilogram of diet: Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

3.2.5 Measurement

Performance

Weight gain (WG) and FI and were recorded, and feed conversion ratio (FCR) were calculated from pen data collected on d 10, 24 and 35. Feed conversion ratio was calculated as the total feed consumed divided by the live pen weight plus the weight of mortalities, culls and sampled birds during each period. FI was calculated as WG × FCR. Livability (LV) was calculated as live birds plus sampled birds divided by initial bird count and expressed as percent.

Lesion scoring

Lesions from 2 birds per pen were scored and averaged. The NE lesions were scored 0 to 4 with a higher number indicating more severe lesions following the standards of Prescott et al. (1978) and Broussard et al. (1986). Similarly, coccidiosis lesion scores of 0 to 4 were assigned following the Bayer HealthCare Baycox® internet resource (2013). Foot pads were scored for lesions on d 35 on a scale of 0 to 9 using the method of Allain et al. (2009).

Gut morphology

Fixed samples were dehydrated, cleared and embedded in paraffin wax for subsequent histological analysis. Consecutive longitudinal sections (7 μm) were cut with microtome (Microm International GmbH, Walldorf, Hessen, Germany) and placed individually onto Superfrost® slides and stained with Haematoxylin and Eosin. Sections were examined by light microscopy (Olympus CX41 microscope) using a 10× objective and colour images captured with the software Analysis 5.0 (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

3.2.6 Statistical analysis

The SAS statistical package (PROC GLM) was used to determine significance and interactions of main effects (SAS, 2013). Birds were completely randomized into treatment pens. A 2 × 4 factorial arrangement of treatments was employed. Factors were: challenge – unchallenged or challenged; additive – control (no additive), ZB,

YCW or SM. When interactions were observed ($P < 0.05$), Duncan's multiple range test was used to separate individual treatment means. The SAS statistical package (PROC NPAR1WAY WILCOXON) was used to determine statistical significance of livability and lesion scores.

3.3 Results

3.3.1 Broiler performance

Performance results are presented in Tables 3.2- 3.4. The supplementation of antibiotics and YCW, and *Eimeria* inoculation on d 9 did not affect bird performance ($P > 0.05$), and no interactions were observed ($P > 0.05$) during the d 0 to d 10 period (Table 3.2). The average WG was 309 g, which is 22% higher than Ross 308 male performance expectations (Aviagen, 2012).

Table 3.2 Bird performance fed different diets from d 0 to 10

Main effects	Feed intake g/bird	Weight gain g/bird	FCR	Livability %
Challenge				
No	341	309	1.103	98.3
Yes	343	309	1.110	97.9
Additive[1]				
None	343	309	1.110	98.3
ZB	336	305	1.102	99.2
YCW	339	310	1.091	96.7
SM	351	313	1.121	98.3
P-value				
Challenge	0.674	0.985	0.523	0.714
Additive	0.204	0.312	0.303	0.467
Challenge × Additive	0.657	0.188	0.362	-

[1] None= control diet (No additive); ZB= control diet + zinc bacitracin; YCW= control diet + yeast cell wall extract; ZB= control diet + salinomycin

Table 3.3 shows that between d 0 and 24, the challenged birds had lower FI, WG, LV and poorer FCR compared to the unchallenged birds ($P < 0.01$). Weight gain of the challenged birds was 11% lower than the unchallenged birds (1296 vs. 1458) and FCR was 4.1 points poorer. Similarly, LV of the challenged birds was 16% lower than the unchallenged birds. In the birds given ZB, YCW or SM; WG, FCR and LV were all improved relative to controls ($P < 0.05$). Challenge × additive interactions

were present for FI and WG ($P < 0.01$) and a strong tendency of their interaction observed for FCR ($P = 0.059$). The interactions indicated that supplementation of ZB, YCW or SM exerted a greater positive impact on the performance of birds challenged with NE than on that of the unchallenged birds in comparison with the treatments without additives. Weight gain, FI and LV of the challenged birds supplemented with SM were not different from those in the unchallenged birds in the treatment without additives ($P > 0.05$). Weight gain of the challenged birds supplemented with YCW and ZB was less than that of the unchallenged birds with no additive but higher than the challenged controls without additive ($P < 0.05$). Livability of the challenged birds supplemented with ZB was not different than the unchallenged birds with no additive ($P > 0.05$).

Table 3.3 Bird performance fed different diets from d 0 to 24

Treatment means	Feed Intake g/bird	Weight gain g/bird	FCR	Livability %
No challenge none	1907[a]	1446[a]	1.318	96.7[a]
No challenge ZB	1851[ab]	1459[a]	1.269	96.7[a]
No challenge YCW	1910[a]	1477[a]	1.293	95.0[a]
No challenge SM	1860[ab]	1449[a]	1.283	95.0[a]
Challenge None	1539[c]	1092[c]	1.413	51.7[c]
Challenge ZnB	1749[b]	1341[b]	1.303	90.0[ab]
Challenge YCW	1728[b]	1297[b]	1.333	81.7[b]
Challenge SM	1861[ab]	1453[a]	1.281	96.7[a]
Main Effects				
Challenge				
No	1882[a]	1458[a]	1.291[b]	95.8[a]
Yes	1719[b]	1296[b]	1.332[a]	80.0[b]
Additive[1]				
None	1723[b]	1269[b]	1.366[a]	74.2[b]
ZB	1800[ab]	1400[a]	1.286[b]	93.3[a]
YCW	1819[ab]	1387[a]	1.313[b]	88.3[a]
SM	1861[a]	1451[a]	1.282[b]	95.8[a]
P-value				
Challenge	0.001	0.001	0.005	0.003
Additive	0.040	0.001	0.001	0.048
Challenge × Additive	0.002	0.001	0.059	-

[abc] Means sharing the same superscripts are not significantly different from each other at ($P < 0.05$).

[1] None= control diet (No additive); ZB= control diet + zinc bacitracin; YCW= control diet + yeast cell wall extract; ZB= control diet + salinomycin

Performance results from d 0 to d 35, given in Table 3.4, show WG, FI and LV to be markedly poorer in the challenged birds compared to the unchallenged birds ($P <$

0.05). The additives, ZB, YCW and SM improved WG and LV across challenged groups relative to birds fed diet with no additive ($P < 0.01$). Birds fed YCW consumed more feed ($P < 0.01$) than those fed ZB and no additive.

Challenge × additive interactions were observed from d 0 to d 35 for WG and FI ($P < 0.01$) but not for FCR ($P > 0.05$). Birds fed ZB, YCW or SM had higher WG, FI and LV relative to birds fed no additive when challenged with NE whereas no difference was observed between unchallenged birds.

Table 3.4 Bird performance fed different diets from d 0 to 35

Treatment means	Feed intake g/bird	Weight gain g/bird	FCR	Livability %
No challenge none	3798[a]	2541[a]	1.494	91.7[ab]
No challenge ZB	3668[a]	2597[a]	1.413	96.7[a]
No challenge YCW	3997[a]	2699[a]	1.481	91.7[ab]
No challenge SM	3782[a]	2575[a]	1.469	93.3[ab]
Challenge None	3165[b]	2105[b]	1.508	51.7[c]
Challenge ZnB	3672[a]	2474[a]	1.484	90.0[ab]
Challenge YCW	3826[a]	2561[a]	1.494	81.7[b]
Challenge SM	3947[a]	2704[a]	1.460	93.3[ab]
Main Effects				
Challenge				
No	3811[a]	2603[a]	1.465	93.3[a]
Yes	3653[b]	2461[b]	1.486	79.2[b]
Additive[1]				
None	3482[c]	2323[b]	1.501[a]	71.7[b]
ZB	3670[bc]	2536[a]	1.449[b]	93.3[a]
YCW	3911[a]	2630[a]	1.487[ab]	86.7[a]
SM	3865[ab]	2639[a]	1.465[ab]	93.3[a]
P-value				
Challenge	0.045	0.008	0.138	0.005
Additive	0.001	0.001	0.065	0.035
Challenge × Additive	0.005	0.004	0.268	-

[abc] Means sharing the same superscripts are not significantly different from each other at ($P < 0.05$).

[1] None= control diet (No additive); ZB= control diet + zinc bacitracin; YCW= control diet + yeast cell wall extract; ZB= control diet + salinomycin

3.3.2 Lesion scores

Necrotic enteritis lesion scores were affected by both challenge and additives (Table 3.5). The NE challenge was effective in inducing gross NE lesions in tissues of the duodenum, jejunum and ileum ($P < 0.01$), whereas birds in the unchallenged groups

had no detectable lesions as expected. Birds fed ZB and SM had fewer lesions in the duodenum than birds given no additive and YCW across challenged groups ($P < 0.05$). Compared with no additive treatment, ZB and SM reduced lesion scores whereas YCW did not show difference in the challenged birds. Although NE lesions were less apparent in ileal as compared to duodenal and jejunal tissues, the challenged birds fed the control diet showed a higher lesion score in this section than the birds in the unchallenged groups.

Coccidiosis lesion scores and foot pad lesion scores are shown in Table 3.6. An increase in coccidiosis lesion scores was detected in the duodenum and ileum on d 16 as the results of the NE challenge ($P < 0.05$). There were no significant differences between treatments in challenged groups with *Eimeria* sp. No significant differences of foot pad lesion scores were observed between treatments at d 35.

Table 3.5 Duodenum, jejunum and ileum necrotic enteritis lesion scores of birds fed different diets at d 16

Treatment means	Duodenum	Jejunum	Ileum
No challenge none	0^{b}	0^{c}	0^{b}
No challenge ZB	0^{b}	0^{c}	0^{b}
No challenge YCW	0^{b}	0^{c}	0^{b}
No challenge SM	0^{b}	0^{c}	0^{b}
Challenge None	1.58^{a}	1.87^{a}	0.92^{a}
Challenge ZB	0.67^{b}	0.5^{bc}	0.08^{b}
Challenge YCW	2.04^{a}	2.04^{a}	0.5^{ab}
Challenge SM	0.63^{b}	0.92^{b}	0.45^{ab}
Main Effects			
Challenge			
No	0^{b}	0^{b}	0^{b}
Yes	1.23^{a}	1.33^{a}	0.49^{a}
Additive[1]			
None	0.79^{ab}	0.93^{ab}	0.45
ZB	0.33^{b}	0.25^{c}	0.04
YCW	1.02^{a}	1.02^{a}	0.25
SM	0.31^{b}	0.46^{bc}	0.22
P-value			
Treatment	0.001	0.001	0.001
Challenge	0.001	0.001	0.002
Additive	0.176	0.053	0.146

[abc] Means sharing the same superscripts are not significantly different from each other at ($P < 0.05$).

[1] None= control diet (No additive); ZB= control diet + zinc bacitracin; YCW= control diet + yeast cell wall extract; ZB= control diet + salinomycin

Table 3.6 Duodenum, jejunum and ileum coccidiosis and foot pad lesion scores of birds fed different diets

Main Effects	Coccidiosis lesion scores on d 16			Foot pad lesion scores on d 35
	Duodenum	Jejunum	Ileum	
Challenge				
No	0.19[b]	0.17	0.04[b]	2.79
Yes	0.52[a]	0.31	0.42[a]	2.88
Additive[1]				
None	0.25	0.13	0.21	2.25
ZB	0.71	0.42	0.21	3.08
YCW	0.29	0.38	0.29	2.75
SM	0.17	0.04	0.21	3.25
P-value				
Challenge	0.032	0.359	0.003	0.31
Additive	0.203	0.150	0.962	0.289

[ab] Means sharing the same superscripts are not significantly different from each other at ($P < 0.05$).

[1] None= control diet (No additive); ZB= control diet + zinc bacitracin; YCW= control diet + yeast cell wall extract; ZB= control diet + salinomycin

3.3.3 Gut Morphology

The morphology of the jejunum was measured before and after inoculation with CP. At d 13, i.e., before CP challenge, the birds given no additives and the YCW diets had longer jejunal villi ($p < 0.01$) compared to those in the ZB and SM group (Table 3.7). Crypt depth was reduced by YCW and SM treatment ($P < 0.01$) when compared with that in the non-additive and ZB groups. Birds fed YCW had higher villus: crypt (VH:CD) relative to the no additive and ZB groups ($P < 0.01$). No significant differences were observed between non additive and SM on VH:CD ratio, but birds fed ZB had lower VH:CD relative to the YCW and SM treatments. In the challenged birds, jejunal villi were longer ($P < 0.05$) in birds given YCW compared to those given ZB and SM. YCW increased the VH:CD relative to ZB and control groups.

At d 16, the challenged birds had lower villi, VH:CD and higher crypt depth compared to the unchallenged birds ($P < 0.01$) (Table 3.8).The birds given no additives and the YCW diet had longer jejunal villi ($P < 0.01$) compared to those in the ZB and SM group. Crypt depth was reduced by YCW ($P < 0.01$) when compared with that in the non-additive and ZB groups. The jenunal villus height of non-challenged birds was consistently longer in birds fed the ZB diet than those fed the YCW, SM or control diet. No significant differences were observed between birds fed YCW and control diets, but SM had shorter jejunal villi when compared with

other treatments. The jejunal crypt depth was decreased in ZB compared with YCW group, while no differences were noted when compared with control and SM groups. The ZB diet significantly increased the VH:CD ratio when compared with the YCW, SM or control diet. However, the VH:CD of birds fed YCW was lower than other treatments. Under the challenge condition, dietary supplementation with YCW showed positive effects on jejunal morphology. Chickens receiving YCW and SM had increased villus height ($P < 0.05$), VH: CD ratio ($P < 0.05$) and decreased crypt depth ($P < 0.05$) with respect to the ZB and control diets. Moreover, no significant differences were observed between YCW and SM on crypt depth and VH: CD ratio, but the villus height in birds fed the YCW diet was higher than that of birds fed the SM diet.

Table 3.7 Jejunum villous height, crypt depth and villus: crypt ratio of birds fed different diets at d 13

Treatment means	Villi height *um* VH	Crypt depth *um* CD	VH:CD
No challenge none	778^{ab}	149^{a}	5.6^{bc}
No challenge ZB	724^{d}	144^{ab}	5.3^{c}
No challenge YCW	758^{bc}	138^{bc}	5.8^{ab}
No challenge SM	706^{d}	133^{c}	5.6^{abc}
Challenge None	773^{ab}	143^{ab}	5.6^{bc}
Challenge ZB	727^{cd}	147^{ab}	5.3^{c}
Challenge YCW	796^{a}	138^{bc}	6.1^{a}
Challenge SM	736^{cd}	138^{bc}	5.7^{abc}
Main effect			
Challenge			
No	743^{b}	141	5.6
Yes	758^{a}	141	5.7
Additive[1]			
None	776^{a}	146^{a}	5.6^{bc}
ZB	725^{b}	145^{a}	5.3^{c}
YCW	777^{a}	138^{b}	6.0^{a}
SM	721^{b}	136^{b}	5.7^{ab}
P-value			
Challenge	0.028	0.842	460
Additive	0.001	0.001	0.001
Challenge × Additive	0.118	0.285	0.744

^{abcd} Means sharing the same superscripts are not significantly different from each other at ($P < 0.05$).

[1] None= control diet (no additive); ZB= control diet + zinc bacitracin; YCW= control diet + yeast cell wall extract; ZB= control diet + salinomycin.

Table 3.8 Jejunum villous height, crypt depth and villus: crypt ratio of birds fed different diets at d 16

Treatment means	Villi height *um* VH	Crypt depth *um* CD	VH:CD
No challenge none	780[b]	156[de]	5.2[b]
No challenge ZB	843[a]	151[e]	5.9[a]
No challenge YCW	778[b]	171[d]	4.7[c]
No challenge SM	726[c]	156[de]	5.2[b]
Challenge None	600[e]	277[a]	2.7[e]
Challenge ZB	522[f]	260[b]	2.1[f]
Challenge YCW	733[c]	233[c]	3.6[d]
Challenge SM	681[d]	220[c]	3.3[d]
Main effect			
Challenge			
No	779.[a]	158[b]	5.2[a]
Yes	635[b]	245[a]	2.9[b]
Additive[1]			
None	715[a]	183[c]	4.3
ZB	668[c]	210[a]	3.8
YCW	756[a]	193[b]	4.2
SM	704[b]	188[bc]	4.2
P-value			
Challenge	0.001	0.001	0.001
Additive	0.001	0.001	0.383
Challenge × Additive	0.001	0.001	0.001

[abcdf] Means sharing the same superscripts are not significantly different from each other at ($P < 0.05$).

[1] None= control diet (no additive); ZB= control diet + zinc bacitracin; YCW= control diet + yeast cell wall extract; ZB= control diet + salinomycin.

3.4 Discussion

Necrotic enteritis infection was successfully induced in the present study as shown by necrotic lesions in the small intestine and depression of BW, FI, FCR and LV. These typical of NE outbreaks reported by others (Mikkelsen et al., 2009). In the current study, there were no differences among treatments for FI, WG, FCR and LV percentage during the first 10 d post hatch. This is in agreement with findings of other researchers (Santin et al., 2001; Benites et al., 2008) who reported that the addition of MOS from YCW in broiler diets had no impact on WG, FI and mortality during the first week of age. Kim et al. (2011) and Reisinger et al. (2012) also reported that MOS was unable to improve WG, FI, FCR and LV from d 1 to 14.

In the last decade, there has been increased interest in using prebiotics, such as MOS derived from the cell wall of the yeast *Saccharomyces cerevisiae*, in broiler diets.

However, there are limited reports on the effect of MOS to control NE in broilers. The results of the current study showed no benefit of YCW on WG, FI, FCR and LV in unchallenged birds from d 0 to 24 and 0 to 35. Similar results for broilers have been observed when MOS supplementation failed to improve live body weight, FI and FCR at d 21 and 35 (Baurhoo et al., 2007; Yang et al., 2007; Yang et al., 2008b; Shanmugasundaram and Selvaraj, 2012). In the current study, however, YCW was effective in curbing performance decline in birds challenged with CP and *Eimeria*. This was not the case in previous studies where MOS had no positive effect in birds challenged with *C. perfringens* (Hofacre et al., 2003; Nollet et al., 2007; Ao et al., 2012; Shanmugasundaram et al., 2013).

In the present study, NE lesion scores at d 16 differed between the challenged and unchallenged treatments and between the challenged sub-treatments. Hofacre et al. (2003) reported that supplementing MOS in broiler diets challenged with CP had no effect on NE lesion score. This was in agreement with Ao et al. (2012) who emphasized that MOS administered to broiler chicks had no effect in minimizing NE lesion score development in the small intestine after 3 and 6 d of challenge with CP. Nollet et al. (2007) showed that dietary MOS had no effect on coccidiosis lesion score for different *Eimeria* species. In the current study, the chicks fed YCW had significantly higher NE scores when compared to chicks fed ZB and SM in the duodenum and jejunum one d after the last CP *inoculation*. This may be due to differences in the mode of action of YCW as compared to ZB and SM. Antibiotics have a direct effect on the organisms themselves. They are directly bactericidal causing death of bacteria or bacteriostatic preventing bacterial growth. YCW may act by enhancing immunity and shifting the gut microflora to reduce the damaging effect of clostridia.

The current study also examined foot-pad lesions, which are a good visual indicator of bird welfare. There are several factors contributing to the etiology of foot pad dermatitis that include diet, litter type or quality, and management. The current results suggest that ZB, SM or YCW had no effect on foot pad lesions.

There was no effect of YCW on villus height compared to the unchallenged control (non-additive) group. This was in agreement with findings that dietary addition of MOS had no significant effect on jejunal villus height at d 14 under a normal

environment (Sun et al., 2005; Baurhoo et al., 2007; Yang et al., 2007). In the current study, NE challenge negatively affected jejunal morphology. At d 13 after *Eimeria* inoculation villi height were increased. At d 16 after CP infection villi height were greatly reduced and VH:CD ratio were decreased. In the challenged birds on d 16, YCW was protective to the gut as it resulted in increased villus height, decreased crypt depth and increased VH:CD ratio compared to the control (non-additive) group. The VH:CD ratio is an important indicator of intestinal recovery and health. A high ratio indicates a long, mature and functionally active villus, in company with a thin crypt with constant renewal of cells. Ao et al. (2012) found that dietary MOS had no significant effect VH:CD ratio at d 14, but significantly increased villus height to crypt depth ratio at d 21 after CP infection. This is expected because both NE outbreaks have profound effects on the gut in poultry.

3.5 Conclusion

The current study results showed that NE challenge increased crypt depth and thus VH:CD ratio. Also, YCW, ZB and SM were all effective in mitigating performance decline, mortality and lesions associated with NE, possibly through improving gut integrity by increasing villous high and VH:CD ratio as demonstrated. While YCW was effective in reducing lesions and improving LV compared with the challenged control, it was slightly less effective than ZB and SM in preventing mortality caused by NE. Perhaps it is important to remember that the aim of using alternatives such as YCW is to maintain performance and health, while minimizing mortality and morbidity when there is an NE challenge in an antibiotic-free production situation.

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STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

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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Principal Supervisor — 15/12/2014 Date

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Chapter 4 Improved Performance and Gut Health by Dietary Supplementation of Acylated Starch in Necrotic Enteritis Challenged Broilers

Abstract

Resistant starch has been reported as a protective agent against pathogenic organisms in the gut and to encourage the proliferation of beneficial organisms. This study examined the efficacy of acetylated high amylose maize starch (Acylated starch A) and butyralated high-amylose maize starch (Acylated starch B) in reducing the severity of necrotic enteritis (NE) in broilers under an experimental challenge. A total of 720 d-old male Ross 308 chicks were assigned to 48 floor pens in 2 rooms with a 2 × 4 factorial arrangement of treatments. Factors were a) challenge: positive or negative (by room); and b) feed additive: control, antibiotics, acylated starch A or acylated starch B. Birds were challenged according to a previously reported protocol. The results showed that on d 24 and 35, all challenged birds had lower ($P < 0.001$) livability (LV), weight gain (WG) and feed intake (FI) compared to unchallenged birds. Challenged birds fed acylated starch A and acylated starch B diets had higher ($P < 0.001$) WG and FI compared with those fed the control diet. However, antibiotics completely protected the birds from NE. The unchallenged birds fed acylated starch A and acylated starch B had higher ($P < 0.01$) FI at d 24 and 35. Birds fed acylated starch B had increased ($P < 0.001$) jejunal villus height/crypt depth (VH:CD) ratios at d 15, increased ileal ($P < 0.001$) and caecal ($P < 0.001$) butyrate levels at d 15 and 24 and decreased ($P < 0.01$) caecal pH at d 15. Acylated starch A increased ($P < 0.001$) ileal acetate content at d 24 and decreased ($P < 0.01$) caecal pH at d 15. In conclusion, these results demonstrated that challenged birds fed acylated starch improved WG compared to control group. Although acylated starch negatively affected FCR without challenge, it offered a degree of specificity in short chain fatty acid (SCFA) delivery.

4.1 Introduction

Necrotic enteritis (NE) is an economically important bacterial disease for the meat chicken industry in the world (Kocher and Choct, 2008). The causative agent of NE is *C. perfringens* which is a Gram-positive, anaerobic bacterium that can be found in

poultry litter, excreta, soil, dust and in the intestinal contents of healthy birds (Williams, 2005). Necrotic enteritis is characterized by necrosis and inflammation of the gastrointestinal tract with a significant decline in growth performance and, in clinical cases, a massive increase in flock mortality (Van der Sluis, 2000). Traditionally, NE has been controlled by antibacterial feed additives such as virginiamcin, lincomycin, bacitracin, tylosin, penicillin, and avoparcin (Williams, 2005). Dietary antimicrobials can not only control NE outbreaks, but can also improve poultry growth and feed conversion efficiency (Kim et al., 2011). However, public concern over the use of in-feed antibiotics and the emergence of antibiotic-resistant "superbugs" has led many countries to ban the use of dietary antimicrobials. Thus new methods for controlling NE must be investigated.

Resistant starch is the fraction of ingested starch which is not digested by digestive enzymes in the small intestine and therefore escapes into the large bowel (Asp and Björck, 1992). Resistant starch can be a protective agent against many pathogenic organisms in the gut through the production of short chain fatty acids (SCFA) as well to encourage the proliferation of beneficial organisms (Topping and Clifton, 2001). Indeed, SCFAs have an important physiological function in maintaining the large bowel in humans through a number of chronic and acute actions. Although the concentration of SCFAs may be manipulated in the gut by provision of appropriate substrates in the diet, it is not easy to selectively increase certain types of SCFAs in a substantial manner. Thus, the acetylating carbohydrates, such as starch, with specific SCFAs offer a degree of specificity in SCFA delivery. Acetylated starches are resistant to small intestine digestion and esterified acids released by bacterial enzymes are available for utilization and absorption by gut microbes and colonocytes (Abell et al., 2011). The vast majority of the studies were done in test animals for human health and nutrition purposes. Thus, the present study was designed to investigate if acylated starch plays a positive role in production animals, such as the chickens, and particularly the efficacy of acetylated high amylose maize starch (Acylated starch A) and butyralated high-amylose maize starch (Acylated starch B) in ameliorating the severity of necrotic enteritis in broilers under experimental disease challenge.

4.2 Materials and Methods

The experiment was approved by the Animal Ethics Committee of the University of New England (Approval No: AEC13-064). The acylated starch products were purchased from the CSIRO Division of Animal, Food and Health Sciences, Adelaide, South Australia.

4.3 Animal husbandry

A total of 720 d-old male Ross 308 chicks were placed in 48 floor pens in the University of New England Animal House Complex, Armidale, NSW, Australia. All the birds were vaccinated against Marek's disease and infectious bronchitis. These birds were randomly assigned to 8 treatments with six replicate pens per treatment and 15 birds each pen. A 2×4 factorial arrangement of treatments was employed with the factors of, challenge: with (+) or without (-); and feed additive in the diets: control, antimicrobials, acylated starch A and acylated starch B. Pens (wire mesh partitioned at 120 × 75 cm) were assigned into two rooms according to challenge (24 pens per room) in the same environmentally controlled facility. The room temperature was set at 33-34°C initially and gradually decreased by 3°C per week until 22-24°C was reached by the third week. Chicks were subjected to artificial fluorescent illumination of 23 hours between d 0-7, then 18 hours from d 7 to 30, and 23 hours from d 30 to 35. Each pen was equipped with a separate tube feeder and nipple drinkers with water and feed provided *ad libitum*. During the trial period, starter diets were fed during d 0-10, grower diets between d 10-24, and finisher diets between d 24-35. The primary determinants of performance, i.e., body weight gain (WG), feed intake (FI), livability (LV) and feed conversion ratio (FCR) were measured at d 10, 24 and 35.

4.4 Dietary treatment

Four diets were formulated with wheat, soybean meal, meat and bone meal, and canola meal according to Ross 308 nutrient specifications (Table 4.1). The diets were thoroughly mixed and pelleted at 65^0C. Treatments were arranged in a 2×4 factorial design, the first being challenge, negative or positive. Dietary treatments were as follows: 1) control diet without additive; 2) control diet supplemented with 0.5 g/kg salinomycin and 0.33 g/kg zinc bacitracin in starter, grower and finisher diets; 3)

control diet supplemented with 50 g/kg acylated starch A in starter, grower and finisher diets; 4) control diet supplemented with 50 g/kg acylated starch B in starter, grower and finisher diets. To minimize potential errors, basal diet was mixed first and then the test ingredients were mixed with the appropriate amount of basal diet. The Concept 5 feed formulation program (Creative Formulation Concepts, LLC, Annapolis, MD, USA) was used to formulate diets. Feed was changed on d 10 and 24. Residual feed was weighed on d 10, 24 and 35. The major ingredients were supplied by the manufacturers. Custom-formulated broiler premixes as well as salinomycin (Sacox 120) were purchased from BEC Feed Solutions P/L, (Brisbane, QLD, Australia) and zinc bacitracin (Albac 150) was purchased from Ridley AgriProducts, (Tamworth, NSW, and Australia).

4.4.1 Necrotic enteritis challenge

The NE challenge was performed based on previous report with modifications (Wu et al., 2014). The *Eimeria acervulina* (batch E1-3/11-064), *E. brunetti* ("Roybru", batch E2-3/11-072) and *E. maxima* (batch E9-6/11-072) were all vaccine strains obtained from Bioproperties Pty. Ltd. (Glenorie, NSW, Australia). *C. perfringens* type A strain EHE-NE18 from CSIRO Livestock Industries (Geelong, Australia) was incubated overnight at 39˚C in 100 mL of sterile thioglycollate broth (USP alternative; Oxoid) followed by subsequent overnight incubations of 1 mL of the previous culture in 100 mL of cooked meat medium (Oxoid), and then in 700 mL of thioglycollate broth (USP alternative; Oxoid) containing starch (10 g/L) and pancreatic digest of casein (5 g/L) to obtain the challenge inoculum. On d 9, challenged birds were inoculated with 5000 sporulated oocysts each of *E. maxima* and *E. acervulina* and 2500 sporulated oocysts of *E. brunetti* in 1 mL of 1% (w/v) sterile saline. Unchallenged birds received 1 mL of 1% (w/v) sterile saline.

On d 14, birds were inoculated twice with 2 mL of *C. perfringens* (EHE-NE18, CSIRO) suspension (3.8×10^8 CFU/mL).

Table 4.1 Ingredient and nutrient composition of the basal diets

Ingredient	Starter	Grower	Finisher
Wheat Aus 3100_10.5	564	615	665
SBM Arg 2400-45.2	302	215	134
Meat meal	30	50	70
Canola solvent	30	50	70
Canola Oil	37	45.4	45.8
Limestone	11.90	7.56	4.56
Salt	1.68	1.35	0.98
Sodium bicarbonate	2	2	2
Dicalcuim phosphate	10.85	4.27	-
L-lysine HCL	2.28	2.73	2.20
D,L-methionine	3.72	3.05	2.21
L-threonine	2.02	1.69	1.27
Choline chloride 70%	0.69	0.50	0.68
Vitamin premix[1]	0.50	0.50	0.50
Trace mineral premix[2]	0.75	0.75	0.75
Nutrient composition			
ME, kcal/kg	2975	3100	3150
Protein	236	221.1	208
Digestible lysine	12	11	9.4
Digestible methionine	6.68	5.83	4.86
Digestible MC	9.4	8.4	7.3
Digestible tryptophan	2.72	2.34	2.01
Digestible threonine	8.30	7.30	6.30
Digestible arginine	13.10	11.40	9.90
Digestible isoleucine	8.63	7.56	6.60
Digestible valine	9.67	8.64	7.74
Calcium,	10.50	9.00	8.50
Non-phytate P	5.00	4.50	4.45
Sodium	1.80	1.80	1.80
Choline, mg/kg	1600	1500	1400
Linoleic acid	15.70	17.37	17.35
Analyzed total starch % in the diets			
Control	34.3	35.6	36.7
Antibiotics	34.6	36.2	36.5
Acylated starch A	36.7	38.2	39.8
Acylated starch B	36.8	38.5	39.7

[1] Vitamin concentrate supplied per kilogram of diet: retinol, 12,000 IU; cholecalciferol, 5,000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 μg; biotin, 200 μg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg.

[2] Trace mineral concentrate supplied per kilogram of diet: Cu (sulfate), 16 mg; Fe (sulfate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulfate and oxide), 120 mg; Zn (sulfate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

4.4.2 Sample collection

On d 15 and 24, three birds and two birds, respectively, were randomly selected from each pen, weighed, and euthanised by cervical dislocation. The body cavities of birds were exposed and digesta samples from the ileum and caeca were collected and stored in 50 mL plastic containers and frozen directly at -20°C for SCFA analysis. Around 1 g of content was used to measure the pH. Approximately 1 g of caeca digesta contents was collected in a 2mL Eppendorf tube, snap-frozen in liquid N2, and stored at -20°C for bacteria quantification. Intestinal tissue of one bird was collected for morphometric analysis. Approximately 1 cm of the jejunum was collected. The intestinal samples were opened and flushed clean with phosphate buffered saline (PBS, pH 7.4) and fixed in 10% buffered formalin for 24 hours. Formalin was subsequently replaced by 70% ethanol for long- term storage. Weights of liver, bursa and spleen of one bird per pen were taken, and the relative organ weight was subsequently calculated as g/100g of body weight. On d 15, intestinal tissues (duodenum, jejunum and ileum) were scored for NE lesions according to Prescott et al. (1978).

4.4.3 Measurements and analysis

Ileal and caecal PH

The ileal and caecal pH values were measured at d 15 and 24. Approximately 1 g of contents was diluted in 9 mL of distilled water. The suspension was mixed with a stirrer and the pH was determined by the EcoScan 5/6 pH meter (Eutech Instrument Pty Ltd., Singapore).

Analysis of short chain fatty acids

The analytical method described by Jensen et al. (1995) was used with modifications for the SCFA analysis. Frozen ileal and caecal samples were thawed and homogenized. Approximately 2 g of wet homogenized digesta samples were weighed, and 1 mL of internal standard 0.01 M ethylbutyric acid was added and mixed with a vortex mixer and then centrifuged at 38625 ×g at 5°C for 20 minutes. Following this, approximately 1 mL of the supernatant, 0.5 mL of concentrated HCl, and 2.5 mL of ether were mixed using a vortex mixer. An internal standard solution

and a blank were also prepared in the same method by replacing the supernatant with 1 mL of the standard acid mixture and 1 mL of water respectively. The mixture was centrifuged at 2060 ×g at 5°C for 15 minutes and 400 µL of the supernatant was transferred to a gas chromatograph vial (2 mL) and mixed with 40 µL of *N*-tert-butyldimethlsilyl-*N*-methyltrifuoroacetamide (MTBSTFA). The sample vials were kept in a heating block at 80 °C for 20 minutes and then left at room temperature for 48 hours. Sample vials run 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 observed in a sample, expressed as µmol/g digesta after $\log_{10}$+1 transformation.

Analysis of starch

The total starch contents of the feed samples were determined using the Megazyme total starch assay (AA/AMG). Briefly, finely ground samples (0.5mm) of approximately 100g were weight accurately into the bottom of pre-weighed 25 mL culture tubes and wet with 5 mL 80% ethanol. A further 2 mL of DMSO were added and heated f at 100 °C for 10 minutes. Then 3 mL of MOPS buffer (50mM, pH 7.0)/ enzyme solution were added followed by 0.1 mL of amyloglucosidase (3300 U/mL on soluble starch at pH 4.5, Megazyme) and incubated at 50 °C for 30 minutes. Glucose was determined colorimetrically after incubating an aliquot (0.1 mL) with 2.25 mL of GOPOD reagent (Megazyme) at 50 °C for 20 minutes and reading the absorbance at 510 nm against a reagent blank.

Gut morphology

Fixed samples were dehydrated, cleared and embedded in paraffin wax for subsequent histological analysis. Consecutive longitudinal sections (7 µm) were placed individually onto Superfrost® slides (Thermo Scientific, Rockville, MD, USA) and stained with haematoxylin and eosin, and villus height and crypt depth were measured by the video pro 32 program following the images captured with a colour video camera (Sony® SSC-DC93P).

Quantification of caecal bacteria

Caecal digesta DNA was extracted by using the ISOLATE II Plant DNA Kit (Bioline, Alexandria, NSW, Australia) following the manufacturer's instructions with slight modifications. Briefly, 300 mg glass beads (0.1 mm; Biospec Products, Bartlesville, OK) and around 200 mg frozen digesta were placed into 2 mL Eppendorf tubes and 450 µL of lysis buffer were added. The samples were shaken on a Mixer Mill MM 300 (Retsch GmbH & Co, Haan, Germany) at a frequency of 30/s for 5 minutes, and heated at 95^{o}C for 5 minutes. The cells were lysed after adding 200 µL and then 100 µL of Extraction Buffer (2M NaCl, 20mM EDTA, 100mM Tris/HCl, 2% CTAB, 2% polyvinylpyrrolidone, pH 8.0) with vortex-mixing following each addition. Following centrifugation at 4,600 × g, 600 µL sample was incubated at 65^{o}C following the addition of 10 µL RNase to remove RNA. A total of 450 µL Binding Buffer were used to capture DNA, 400 µL Wash Buffer PAW1 and 700 µL PAW2 to remove impurity, and 50 µL Elution Buffer to dissolve DNA.

The quantitative real-time PCR of total bacteria, *Enterobacteriaceae*, *lactobacilli* and *C. perfringens* were performed following the method of Wise and Siragusa (2006) . The extracted DNA from caecal digesta was diluted twenty times in sterilized water; TaqMan universal PCR master mix (Applied Biosystems) was used to quantify *C. perfringens* and SensiMix™ SYBR® No-ROX Kit was used to quantify total bacteria, *Enterobacteriaceae,* and *lactobacilli*. Species-specific 16 rRNA primers/probe were used.

For the *C. perfringens*:
CGCATAACGTTGAAAGATGG and CCTTGGTAGGCCGTTACCC;
TaqMan probe: 5'-FAM-TCATCATTCAACCAAAGGAGCAATCC-TAMRA-3';

For the *Enterobacteriaceae:*
F:CATTGACGTTACCCGCAGAAGAAGC and
R: CTCTACGAGACTCAAGCTTGC);

For the *lactobacillus spp.:*
F: CACCGCTACACATGGAG and R:AGCAGTAGGGAATCTTCCA);

For total bacteria:
F: CGGYCCAGACTCCTACGGG and R: TTACCGCGGCTGCTGGCAC).

A total volume of 10 µL was used in PCR reaction. PCR was performed with two replicates for each sample in a Rotorgene 6500 real-time PCR machine (Corbett, Sydney, Australia). A threshold cycle (CT) average from the replicate samples was used for data analysis. Serial dilutions of linearized plasmid DNA (pCR®4-TOPO Vector, Life Technologies, Carlsbad, USA) inserted with respective bacterial amplicons were used to construct a standard curve. The concentrations of the plasmid DNA were measured using NanoDrop ND-8000 (Thermo Fisher Scientific, Waltham, USA) prior to the serial dilutions. The number of target DNA copies was calculated from the mass of 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.

4.4.4 Statistical analysis

The SAS statistical package (Proc GLM) was used to determine statistical significance. Means were separated using Duncan's multiple range test when challenge × feed additive interactions were observed ($P < 0.05$). Means were deemed significantly different at $P < 0.05$ or highly significantly different at $P < 0.01$ or $P < 0.001$. The SAS statistical package (PROC NPAR1WAY WILCOXON) was used to determine statistical significance of LV, lesion scores and quantification of *C. perfringens.*

4.5 Results

4.5.1 Broiler performance

Performance of the birds was not affected by the treatments prior to, but was affected following, the necrotic enteritis challenge as shown in Tables 4.2-4.4. From d 0-10, i.e., one d after challenge with *Eimeria*, there were no differences in performance between the challenged and unchallenged birds (Table 4.2) or between any of the additive treatments. There were no challenge × feed additive interactions observed.

Table 4.2 Performance of birds fed antibiotics, acylated starch A and acylated starch B from d 0 to 10

Main Effects	Initial weight g/bird	Feed intake g/bird	Weight gain g/bird	FCR
Challenge				
No	45	287	261	1.101
yes	45	287	264	1.085
Additive[1]				
None	45	282	257	1.097
Antibiotics	44	286	264	1.085
ASA	45	290	267	1.088
ASB	45	289	262	1.101
SEM	0.118	1.269	1.518	0.007
P-value				
Challenge	0.876	0.86	0.237	0.060
Additive	0.549	0.119	0.109	0.438
Challenge × Additive	0.766	0.913	0.503	0.396

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA = acylated starch A, ASB = acylated starch B

From d 0-24, the effect of the challenge was clearly visible (Table 4.3). The challenged birds performed significantly poorer for WG, FI, FCR and LV ($P < 0.001$). The WG, FI and LV of the challenged birds were 86%, 89% and 89% of the unchallenged birds, respectively and FCR was 5.4 points poorer. Inclusion of feed additives increased WG and FI over the controls ($P < 0.001$). However, birds fed acylated starch B had poorer FCR than those fed control and antibiotics diets ($P < 0.001$). Significant challenge × additive interactions were observed in FI ($P < 0.001$) and WG ($P < 0.001$). Under challenge conditions, the birds fed acylated starch B and acylated starch A significantly increased WG and FI compared with those fed the control diet, while being significantly lower than those fed antibiotics. Challenged birds given antibiotics had significantly higher LV than the control, acylated starch A and acylated starch B. On the other hand, unchallenged birds showed no significant differences between any treatment for WG and LV, while feed intake of unchallenged birds fed acylated starch A and acylated starch B was higher relative to antibiotics and control birds ($P < 0.001$). The WG of unchallenged birds fed acylated starch A and acylated starch B was 4% higher than unchallenged controls.

From d 0-35, bird performance was markedly different upon the challenge and feed additive treatments (Table 4.4). Challenge birds reduced FI, WG and LV ($P < 0.001$

for all the variables). On the other hand, all the feed additives significantly increased WG and FI over the control group ($P < 0.001$) while birds fed acylated starch A and acylated starch B had poorer FCR ($P < 0.001$). Birds fed antibiotics had significantly higher LV than that of birds fed the control, acylated starch A and acylated starch B. Significant challenge × additive interactions were observed for FI ($P < 0.002$) and WG ($P < 0.001$). Challenge, however, did not affect the WG, FCR and LV ($P > 0.05$) of the birds fed antibiotics. Acylated starch A and acylated starch B increased WG and FI of challenged birds compared to the control group ($P < 0.001$) whereas unchallenged birds fed acylated starch A and acylated starch B had higher FI ($P < 0.002$) compared to unchallenged, control birds.

Table 4.3 Performance of birds fed antibiotics, acylated starch A and acylated starch B from d 0 to 24

Treatment means[1]	Feed intake g/bird	Weight gain g/bird	FCR	Livability %
No Challenge None	1806^{b}	1424^{a}	1.268	99^{a}
No Challenge Antibiotics	1749^{bc}	1396^{a}	1.253	97^{a}
No Challenge ASA	1919^{a}	1487^{a}	1.292	96^{a}
No Challenge ASB	1959^{a}	1484^{a}	1.322	99^{a}
Challenge none	1475^{d}	1096^{c}	1.346	79^{b}
Challenge antibiotics	1811^{b}	1428^{a}	1.269	99^{a}
Challenge ASA	1664^{c}	1216^{b}	1.369	81^{b}
Challenge ASB	1681^{c}	1227^{b}	1.370	83^{b}
Main Effects				
Challenge				
No	1858^{a}	1448^{a}	1.284^{b}	98^{a}
Yes	1658^{b}	1242^{b}	1.338^{a}	86^{b}
Additive				
None	1640^{b}	1260^{b}	1.307^{b}	89^{b}
Antibiotics	1780^{a}	1412^{a}	1.261^{c}	98^{a}
ASA	1792^{a}	1352^{a}	1.330^{ab}	88^{b}
ASB	1820^{a}	1355^{a}	1.346^{a}	91^{b}
SEM	24.18	21.99	0.008	1.48
P-value				
Challenge	0.001	0.001	0.001	0.001
Additive	0.001	0.001	0.001	0.128
Challenge × Additive	0.001	0.001	0.057	0.001

[abcd] Means sharing the same superscripts are not significantly different from each other at P < 0.05.

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA = acylated starch A, ASB = acylated starch B

Table 4.4 Performance of birds antibiotics, acylated starch A and acylated starch B from d 0 to 35

Treatment means[1]	Feed intake g/bird	Weight gain g/bird	FCR	Livability %
No Challenge None	3751[b]	2663[a]	1.409	99[a]
No Challenge Antibiotics	3796[b]	2694[a]	1.409	96[a]
No Challenge ASA	4010[a]	2749[a]	1.459	94[a]
No Challenge ASB	4063[a]	2782[a]	1.461	97[a]
Challenge none	3165[d]	2201[c]	1.438	78[b]
Challenge antibiotics	3713[bc]	2647[a]	1.403	96[a]
Challenge ASA	3532[c]	2406[b]	1.468	81[b]
Challenge ASB	3523[c]	2427[b]	1.453	82[b]
Main Effects				
Challenge				
No	3905[a]	2722[a]	1.435	96[a]
Yes	3483[b]	2420[b]	1.441	84[b]
Additive				
None	3458[b]	2432[b]	1.423[b]	88[b]
Antibiotics	3754[a]	2670[a]	1.406[b]	96[a]
ASA	3771[a]	2578[a]	1.463[a]	88[b]
ASB	3793[a]	2605[a]	1.457[a]	89[b]
SEM	44.91	31.72	0.007	1.44
P-value				
Challenge	0.001	0.001	0.529	0.001
Additive	0.001	0.001	0.001	0.238
Challenge × Additive	0.002	0.001	0.481	0.001

[abc] Means sharing the same superscripts are not significantly different from each other at P < 0.05.

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA= acylated starch A, ASB = acylated starch B

4.5.2 Necrotic enteritis lesion scores

Necrotic enteritis lesion scores are shown in Table 4.5. On d 15 (one d after *C. perfringens* inoculation), all challenged birds except those given antibiotics showed a higher intestinal NE lesion score compared to unchallenged birds ($P < 0.001$). Intestinal NE lesion scores of challenged birds fed antibiotics were significantly lower than those of the control, acylated starch A and acylated starch B groups.

4.5.3 Ileal and caecal pH

There were significant differences between challenged and unchallenged birds for ileum and caecum pH at d 15 and 24 (Tables 4.5 and 4.6). The pH values of ileum and caecum digesta in challenged birds were significantly lower than those in

unchallenged birds at d 15 ($P < 0.01$), whereas the pH values in challenged birds were higher than those in unchallenged at d 24 ($P < 0.01$). Acylated starch A and acylated starch B decreased ileal digesta pH compared to antibiotics ($P < 0.05$) at d 15 and 24 and caecal digesta pH compared to control ($P < 0.01$) at d 15. Significant challenge × additive interactions were observed in ileal and caecal digesta pH at d 24. The highest ileal digesta pH was recorded for challenged birds fed antibiotics. Unchallenged birds fed acylated starch A had significantly lower caecal pH than those fed antibiotics.

Table 4.5 Intestinal necrotic enteritis lesion score and the pH values of ileal and caecal content of birds fed antibiotics, acylated starch A and acylated starch B at d 15

Treatment means[1]	NE lesion score	Ileum pH	Caeca pH
No Challenge None	0.00^{b}	6.99^{a}	7.09^{a}
No Challenge Antibiotics	0.00^{b}	7.05^{a}	6.78^{bc}
No Challenge ASA	0.00^{b}	6.81^{ab}	6.75^{bc}
No Challenge ASB	0.00^{b}	6.68^{b}	6.86^{ab}
Challenge none	2.04^{a}	6.58^{b}	6.85^{ab}
Challenge antibiotics	0.00^{b}	6.81^{ab}	6.79^{bc}
Challenge ASA	2.96^{a}	6.57^{b}	6.52^{c}
Challenge ASB	2.21^{a}	6.58^{b}	6.61^{bc}
Main Effects			
Challenge			
No	0.00^{b}	6.89^{a}	6.87^{a}
Yes	1.80^{a}	6.64^{b}	6.70^{b}
Additive			
None	1.02^{a}	6.803^{ab}	6.99^{a}
Antibiotics	0.00^{b}	6.929^{a}	6.78^{b}
ASA	1.48^{a}	6.703^{b}	6.66^{b}
ASB	1.10^{a}	6.676^{b}	6.76^{b}
SEM	0.201	0.040	0.037
P-value			
Challenge	0.001	0.001	0.01
Additive	0.047	0.01	0.01
Challenge × Additive	0.001	0.454	0.362

^{abc} Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA= acylated starch A, ASB = acylated starch B

Table 4.6 The pH values of ileal and caecal content of birds fed antibiotics, acylated starch A and acylated starch B at d 24

Treatment means[1]	Ileum pH	Caeca pH
No Challenge None	6.06[b]	6.47[abc]
No Challenge Antibiotics	6.04[b]	6.69[ab]
No Challenge ASA	6.13[b]	6.10[c]
No Challenge ASB	5.83[b]	6.34[bc]
Challenge none	6.39[b]	6.88[a]
Challenge antibiotics	7.46[a]	6.52[abc]
Challenge ASA	6.01[b]	6.83[ab]
Challenge ASB	6.55[b]	6.97[a]
Main Effects		
Challenge		
No	6.01[b]	6.40[b]
Yes	6.60[a]	6.81[a]
Additive		
None	6.23[b]	6.67
Antibiotics	6.75[a]	6.61
ASA	6.06[b]	6.43
ASB	6.19[b]	6.63
SEM	0.104	0.067
P-value		
Challenge	0.001	0.001
Additive	0.03	0.48
Challenge × Additive	0.02	0.05

[abc] Means sharing the same superscripts are not significantly different from each other at P < 0.05.

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA = acylated starch A, ASB = acylated starch B

4.5.4 Visceral organ weight

Relative weights of liver, spleen and bursa of Fabricius to live body weight showed changes upon the treatments as demonstrated in Tables 4.7 and 4.8. Challenged birds had higher ($P < 0.01$) liver and spleen relative weight at d 15, and higher ($P < 0.01$) liver relative weight at d 24 compared with unchallenged birds. On the other hand, no significant differences in relative weights of liver and spleen to live body weights at d 15 and 24 were detected among the birds fed control diet or diets with different additives. There were significant challenge × additive interactions for bursa of Fabricius relative weight. Unchallenged birds fed acylated starch B had significantly lower bursa of Fabricius relative weight at d 15 than those birds fed antibiotics and control diets. The bursa of Fabricius relative weight of challenged birds was significantly increased by dietary antibiotics ($P < 0.01$) at d 24.

Table 4.7 Relative weights (%) of liver, spleen and bursa of Fabricius to body weight of birds fed antibiotics, acylated starch A and acylated starch B at d 15

Treatment means[1]	Liver	Spleen	Bursa
No Challenge None	3.16	0.078	0.192[a]
No Challenge Antibiotics	3.19	0.77	0.195[a]
No Challenge ASA	3.08	0.083	0.174[ab]
No Challenge ASB	3.45	0.071	0.137[b]
Challenge none	3.58	0.116	0.195[a]
Challenge antibiotics	3.55	0.106	0.165[ab]
Challenge ASA	3.72	0.102	0.167[ab]
Challenge ASB	3.41	0.091	0.179[a]
Main Effects			
Challenge			
No	3.216[b]	0.077[b]	0.174
Yes	3.566[a]	0.104[a]	0.176
Additive			
None	3.370	0.097	0.193[a]
Antibiotics	3.370	0.092	0.180[ab]
ASA	3.397	0.093	0.170[ab]
ASB	3.427	0.081	0.158[b]
SEM	0.062	0.004	0.005
P-value			
Challenge	0.005	0.002	0.849
Additive	0.983	0.511	0.05
Challenge × Additive	0.246	0.810	0.05

[ab] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA = acylated starch A, ASB = acylated starch B

Table 4.8 Relative weights (%) of liver, spleen and bursa of Fabricius to body weight of birds fed antibiotics, acylated starch A and acylated starch B at d 24

Treatment means[1]	Liver	Spleen	Bursa
No Challenge None	2.597	0.103	0.207 [b]
No Challenge Antibiotics	2.842	0.093	0.173 [b]
No Challenge ASA	2.896	0.088	0.175 [b]
No Challenge ASB	2.773	0.098	0.172 [b]
Challenge none	2.770	0.093	0.170[b]
Challenge antibiotics	3.273	0.103	0.260[a]
Challenge ASA	3.210	0.113	0.175 [b]
Challenge ASB	3.244	0.102	0.164 [b]
Main Effects			
Challenge			
No	2.777[b]	0.095	0.182
Yes	3.147[a]	0.103	0.192
Additive			
None	2.683	0.098	0.188[ab]
Antibiotics	3.058	0.098	0.217[a]
ASA	3.098	0.100	0.175[b]
ASB	2.683	0.100	0.168[b]
SEM	0.087	0.003	0.007
P-value			
Challenge	0.04	0.231	0.417
Additive	0.316	0.994	0.05
Challenge × Additive	0.927	0.244	0.01

[ab] Means sharing the same superscripts are not significantly different from each other at P < 0.05.

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA = acylated starch A, ASB = acylated starch B

4.5.5 Ileal and caecal SCFAs

Ileel SCFAs were measured at d 15 and 24 in birds fed treatment diets (Table 4.9). On d 15, ileal formate ($P < 0.03$), propionate ($P < 0.001$) and butyrate ($P < 0.001$) concentrations were all increased by the challenge. Dietary additives had a significant effect on ileal SCFA contents. Birds fed acylated starch B had increased ileal butyrate when compared to acylated starch A, antibiotics and controls ($P < 0.001$), and ileal lactate compared to antibiotics ($P < 0.01$). Dietary acylated starch A and control significantly increased ileal acetate compared to acylated starch B and antibiotics. There was also significant challenge × additive interaction for butyrate ($P < 0.001$) concentration. Unchallenged birds fed acylated starch B had the highest ileal butyrate content out of all other treatments ($P < 0.001$). Challenged birds fed antibiotics showed decreased ileum butyrate ($P < 0.001$) compared to challenged birds fed control, acylated starch A and acylated starch B diets. No significant

differences were detected in ileal butyrate between challenged and unchallenged birds fed acylated starch B.

By d 24, challenged birds had higher ileal levels of formate ($P < 0.019$) and butyrate ($P < 0.01$) than unchallenged birds. Birds fed acylated starch A and acylated starch B had increased ileal acetate and butyrate level ($P < 0.001$), respectively. There were also significant challenge × additive interactions for butyrate ($P < 0.01$) and lactate ($P < 0.01$) concentrations. Challenged and unchallenged birds fed acylated starch B had the highest ileal butyrate content out of all other treatments ($P < 0.001$). Lactate concentration of birds fed the control and acylated starch A was higher than those fed antibiotics under challenge conditions ($P < 0.05$).

On d 15, caecal SCFA contents were markedly different in the challenged versus unchallenged birds (Table 4.10). Caecal lactate of challenged birds was higher than those in unchallenged birds ($P < 0.039$), whereas the caecal acetate and butyrate in challenged birds were lower than those in unchallenged ($P < 0.001$). Caecal butyrate of birds fed acylated starch B ($P < 0.001$) was higher overall dietary treatments. There were no significant differences among additives for acetate, propionate, and lactate. There were significant challenge × additive interactions for acetate ($P < 0.02$) and butyrate ($P < 0.003$) concentrations. No significant differences were detected in caecal acetate of challenged birds, while unchallenged birds fed antibiotics had significantly lower acetate than birds fed acylated starch A. Unchallenged birds fed acylated starch B had the highest caecal butyrate content out of all other treatments ($P < 0.05$).

On d 24, unchallenged birds had higher ($P < 0.01$) caecal acetate and butyrate than challenged birds (Table 4.10). Birds fed acylated starch B had higher caecal butyrate content than those fed control, antibiotics and acylated starch A ($P < 0.001$). birds fed control diet had increased caecal propionate in compare to birds fed acylated starch A and acylated starch B.

Table 4.9 concentration of various short chain fatty acids (µmol/g) in ileal content of birds fed antibiotics, acylated starch A and acylated starch B

Treatment means[1]	D 15					D 24				
	Formic	Acetic	Propionic	Butyric	Lactic	Formic	Acetic	Propionic	Butyric	Lactic
No Challenge None	0.08	6.02	0.00	0.04^{c}	5.90	0.10	1.75^{b}	0.03	0.02^{c}	65.43^{ab}
No Challenge Antibiotics	0.12	4.69	0.00	0.00^{c}	2.6	0.26	2.17^{b}	0.02	0.03^{c}	68.26^{ab}
No Challenge ASA	0.03	7.06	0.01	0.01^{c}	5.07	0.03	4.62^{a}	0.02	0.01^{c}	32.15^{bc}
No Challenge ASB	0.51	1.71	0.00	3.55^{a}	7.90	0.51	2.07^{b}	0.02	2.49^{b}	43.12^{abc}
Challenge none	0.68	5.90	0.23	2.19^{b}	6.857	0.73	2.24^{b}	0.02	0.63^{c}	57.28^{ab}
Challenge antibiotics	0.14	0.71	0.02	0.01^{c}	3.56	0.61	1.65^{b}	0.05	0.05^{c}	23.00^{c}
Challenge ASA	0.63	6.56	0.29	1.93^{b}	6.67	0.84	8.20^{a}	0.02	0.02^{c}	72.69^{a}
Challenge ASB	0.55	3.69	0.32	2.87^{ab}	9.96	1.80	2.54^{b}	0.03	4.47^{a}	47.94^{abc}
Main Effects										
Challenge										
No	0.20^{b}	4.97	0.01^{b}	0.90^{b}	5.38	0.23^{b}	3.46	0.02	0.64^{b}	53.70
Yes	0.50^{a}	4.24	0.21^{a}	1.70^{a}	6.76	0.99^{a}	3.72	0.03	1.29^{a}	50.23
Additive										
None	0.38	5.95^{a}	0.12	1.12^{b}	6.38^{ab}	0.42	1.97^{b}	0.02	0.33^{b}	61.36
Antibiotics	0.13	2.52^{b}	0.01	0.01^{c}	3.10^{b}	0.44	1.91^{b}	0.03	0.04^{b}	43.58
ASA	0.36	6.81^{a}	0.15	0.97^{b}	5.87^{ab}	0.43	7.91^{a}	0.02	0.01^{b}	56.81
ASB	0.53	2.70^{b}	0.16	3.24^{a}	8.93^{a}	1.15	2.32^{b}	0.02	3.48^{a}	45.53
SEM	0.071	0.514	0.029	0.230	0.629	0.164	0.422	0.003	0.247	4.088
P-value										
Challenge	0.03	0.412	0.001	0.001	0.245	0.019	0.521	0.410	0.01	0.648
Additive	0.198	0.001	0.09	0.001	0.01	0.28	0.001	0.545	0.001	0.248
Challenge × Additive	0.250	0.127	0.106	0.001	0.982	0.760	0.696	0.167	0.01	0.01

[abc] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA = acylated starch A, ASB = acylated starch B

Table 4.10 Concentration of various short chain fatty acids (µmol/g) in caecal content of birds fed antibiotics, acylated starch A and acylated starch B

Treatment means[1]	D 15				D 24			
	Acetic	Propionic	Butyric	Lactic	Acetic	Propionic	Butyric	Lactic
No Challenge None	84.68^{abc}	5.29	12.39^{bc}	0.19	86.08	4.70	24.31^{cb}	0.31
No Challenge Antibiotics	74.95^{bc}	2.91	13.35^{bc}	0.16	88.77	4.93	23.40^{cb}	0.10
No Challenge ASA	102.34^{a}	2.91	16.04^{b}	0.21	97.53	4.15	23.59^{cb}	0.47
No Challenge ASB	92.39^{ab}	3.26	32.17^{a}	0.22	107.41	5.02	36.27^{a}	0.39
Challenge none	47.32^{d}	4.25	7.44^{c}	0.12	73.33	5.96	18.16^{cd}	0.13
Challenge antibiotics	65.83^{cd}	3.97	9.45^{bc}	0.52	73.21	4.53	17.00^{cd}	0.09
Challenge ASA	51.62^{d}	4.15	7.38^{c}	2.17	82.37	4.34	11.75^{d}	0.67
Challenge ASB	48.10^{d}	3.98	12.49^{bc}	1.33	76.54	3.65	31.82^{ab}	0.13
Main Effects								
Challenge								
No	88.76^{a}	3.52	18.75^{a}	0.20^{b}	94.11^{a}	4.95	26.89^{a}	0.32
Yes	52.74^{b}	4.10	9.17^{b}	1.10^{a}	75.87^{b}	4.67	19.92^{b}	0.24
Additive								
None	66.00	4.72	9.69^{b}	0.15	80.98	5.83^{a}	21.23^{b}	0.21
Antibiotics	71.30	3.39	11.79^{b}	0.32	81.70	4.75^{ab}	20.84^{b}	0.09
ASA	76.98	3.53	11.71^{b}	1.19	91.03	4.24^{b}	17.67^{b}	0.57
ASB	74.67	3.59	22.33^{a}	0.78	90.57	4.34^{b}	34.04^{a}	0.26
SEM	3.785	0.215	1.351	0.220	3.362	0.221	1.485	0.069
P-value								
Challenge	0.001	0.211	0.001	0.039	0.005	0.504	0.002	0.587
Additive	0.384	0.094	0.001	0.308	0.531	0.036	0.001	0.116
Challenge × Additive	0.02	0.190	0.003	0.347	0.707	0.519	0.629	0.649

[abcd] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA = acylated starch A, ASB = acylated starch

4.5.6 Gut morphology

The morphology of jejunal samples was studied after *C. perfringens* challenge and the data are presented in Tables 4.11 and 4.12. At d 15, the effect of the challenge was clearly visible. The challenged birds had higher muscle thickness ($P < 0.05$), crypt depth ($P < 0.001$) and lower villus height to crypt depth ratios (VH:CD) ($P < 0.001$) than unchallenged birds. Additives had a significant effect on jejunum crypt depth and VH:CD ratios. Antibiotics decreased crypt depth ($P < 0.01$) in comparison to acylated starch A and control. Birds fed antibiotics and acylated starch B had significantly increased VH:CD ratios relative to the control group. There were no challenge × additive interactions observed.

On d 24, there were no differences in muscle thickness, villus height and crypt depth between challenged and unchallenged birds, however birds fed antibiotics had higher ($P < 0.02$) VH:CD ratio when compared to birds fed control diet. There were no challenge × additive interactions observed.

Table 4.11 Jejunal villus height and crypt depth of birds fed antibiotics, acylated starch A and acylated starch B at d 15

Main effects	Muscle thickness *um*	Villi height *um*	Crypt depth *um*	VH:CD
Challenge				
No	169.07[b]	1601.99	192.138[b]	8.520[a]
Yes	192.23[a]	1513.94	231.614[a]	6.651[b]
Additive[1]				
None	200.50	1469.06	223.05[a]	6.686[c]
Antibiotics	161.55	1640.60	187.59[b]	8.780[a]
ASA	188.75	1575.21	227.12[a]	7.137[bc]
ASB	171.79	1546.99	209.74[ab]	7.740[b]
SEM	5.786	30.158	5.415	0.242
P-value				
Challenge	0.034	0.152	0.001	0.001
Additive	0.06	0.266	0.005	0.001
Challenge × Additive	0.321	0.871	0.06	0.109

[abc] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA = acylated starch A, ASB = acylated starch B

Table 4.12 Jejunal villus height and crypt depth of birds fed antibiotics, acylated starch A and acylated starch B at d 24

Main Effects	Muscle thickness *um*	Villi height *um*	Crypt depth *um*	VH:CD
Challenge				
No	198.85	1744.11	217.82	8.270
Yes	212.28	1705.30	240.43	7.345
Additive[1]				
None	210.61	1589.61	244.11	6.793[b]
Antibiotics	207.89	1801.87	205.62	8.958[a]
ASA	197.16	1693.72	233.71	7.586[ab]
ASB	206.59	1813.64	233.07	7.892[ab]
SEM	6.809	34.499	6.383	0.268
P-value				
Challenge	0.346	0.564	0.07	0.06
Additive	0.914	0.075	0.156	0.02
Challenge × Additive	0.473	0.476	0.421	0.224

[ab] Means sharing the same superscripts are not significantly different from each other at P < 0.05.

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA = acylated starch A, ASB = acylated starch B

4.5.7 Quantification of bacteria

After *C. perfringens* challenge on d 14 of the experiment, bacterial numbers in caecal content were analyzed. The challenge led to significant changes in the composition of the caecal microflora. The main difference observed at d 15 was an increase in the numbers of *C. perfringens* ($P < 0.001$), lactobacillus ($P < 0.001$) and enterobacterial ($P < 0.019$) in the caeca of challenged birds, while no significant difference was observed for all the bacteria analyzed in response to additives (Table 4.13). Challenge × additive interaction was observed for C. perfringens quantification (P < 0.001). No significant differences were detected in caecal C. perfringens count among unchallenged dietary groups, while C. perfringens count in challenged birds fed antibiotics were lower (P <0.001) than challenged birds fed control, acylated starch A and acylated starch B. The numbers of C. perfringens in challenged birds supplemented with antibiotics were not different the unchallenged birds fed control, antibiotics, acylated starch A and acelated starch B.

By d 24, numbers of total anaerobic ($P < 0.001$), lactobacillus ($P < 0.024$) and *C. perfringens* ($P < 0.02$) were higher in challenged birds (Table 4.14). The numbers of enterobacteria were significantly higher in birds fed acylated starch B than in birds

fed control and acylated starch A diets and higher in birds fed antibiotics in comparison with birds fed control diets ($P < 0.002$).

Table 4.13 Bacterial quantification (log_{10} CFU) in caecal content of birds fed antibiotics, acylated starch A and acylated starch B at d 15

Treatment means[1]	Total anaerobic	Enterobacteria	Lactobacillus	*C. perfringens*
Challenge none	9.28	7.41	9.17	8.06[a]
Challenge antibiotics	9.60	7.92	9.31	0.00[b]
Challenge ASA	9.57	7.89	9.23	8.58[a]
Challenge ASB	9.52	8.28	9.20	8.46[a]
No Challenge None	9.40	6.70	8.62	0.00[b]
No Challenge Antibiotics	9.58	7.78	8.89	0.00[b]
No Challenge ASA	9.31	7.35	8.52	0.00[b]
No Challenge ASB	9.55	7.35	9.00	0.00[b]
Main Effects				
Challenge				
No	9.46	7.29[b]	8.76[b]	0.00[b]
Yes	9.49	7.87[a]	9.23[a]	6.28[a]
Additive				
None	9.34	7.05	8.89	4.03
Antibiotics	9.59	7.85	9.10	0.00
ASA	9.44	7.62	8.88	4.29
ASB	9.53	7.81	9.10	4.23
SEM	0.038	0.128	0.072	0.681
P-value				
Challenge	0.639	0.019	0.001	0.001
Additive	0.082	0.084	0.453	0.07
Challenge × Additive	0.280	0.686	0.567	0.001

[abc] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA = acylated starch A, ASB = acylated starch B

Table 4.14 Bacterial quantification (log_{10} CFU) in caecal content of birds fed antibiotics, acylated starch A and acylated starch B at d 24

Main Effects	Total anaerobic	Enterobacteria	Lactobacillus	*C. perfringens*
Challenge				
No	9.25[b]	6.94	8.61[b]	0.00[b]
Yes	9.55[a]	7.18	8.94[a]	1.94[a]
Additive[1]				
None	9.43	6.42[c]	8.63	1.62
Antibiotic	9.436	7.42[ab]	8.71	0.00
ASA	9.24	6.87[bc]	8.72	0.79
ASB	9.50	7.54[a]	9.06	1.46
SEM	0.048	0.123	0.076	448
P-value				
Challenge	0.001	0.256	0.024	0.020
Additive	0.175	0.002	0.150	0.504
Challenge × Additive	0.821	0.124	0.450	0.062

[abc] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

[1] None = no additive, Antibiotics = salinomycin + zinc bacitracin, ASA = acylated starch A, ASB = acylated starch B

4.6 Discussion

Necrotic enteritis is an important concern to the poultry industry because of production losses, reduced welfare of birds and increases the risk of contamination of poultry products for human consumption. Thus, the present was conducted to evaluate the efficacy of acylated starch in reducing the severity of NE in broilers under an experimental challenge. In the present study, NE was successfully induced as shown by as shown by necrotic lesions in the small intestine and depression of BW, FI, FCR and LV as expected based on the reports by other researchers (Mikkelsen et al., 2009; Jayaraman et al., 2013).

To the best of our knowledge, there are no published data on the effects of acylated starch on broiler performance and gut health. The current study demonstrated that although acylated starch products did not have the same effect as antibiotics in terms of reducing lesions or preventing mortality, they had positive effects on gut health and bird performance. For instance, Challenged birds fed acylated starch A and acylated starch B diets had significantly higher WG compared with those fed the control diet. This may indicate the potential of acylated starch A and acylated starch B to improve the performance of birds when they are raised under challenge

conditions. The improved body weight gain is probably due to the beneficial effect of acetate and butyrate on gut health. One of the indicators of gut health is the type and composition of the gut microbiota (Choct, 2009). The current results showed that acylated starch A and acylated starch B increased acetate and butyrate concentrations in the ileum and caecum. Both butyrate and acetate are known to have a positive effect on energy metabolism and gut health (Topping and Clifton, 2001). Furthermore, supplementation of acylated starch B at d 15 significantly increased VH:CD ratios, shows a long, matured and functionally active villus, in company with a thin crypt with constant renewal of cells. It has been reported that SCFAs have a direct stimulatory effect on gastrointestinal cell proliferation, through the increase of plasma glucagon-like peptide-2 (GLP-2) and ileal proglucagon, glucose transporter (GLUT2) expression and protein expression (Tappenden and Mcburney, 1998). Thus, increased intestinal surface area and thus nutrient absorption may be the underlying mechanisms for the improved WG.

Although, the challenged birds fed acylated starch A and acylated starch B had higher WG compared to the birds receiving control diets at d 24 and 35, the challenged and unchallenged birds fed acylated starch A and acylated starch B had higher feed intake at d 24 and 35. The increase in FI by birds fed diets containing 5% each of acylated starch A and acylated starch B perhaps was simply an indication that these additives were not digestible, and hence the bird s increased intake to compensate for lower nutrients compared to other treatments. This is plausible because resistant starch behaves as fiber in monogastric animals (Annison and Topping, 1994). The effects of high fiber levels on feed passage and FI in poultry is well documented. For instance, González-Alvarado et al. (2010) reported that adding oat hulls, which are high in lignin and cellulose, increased the passage rate of digesta through the distal part of the gastrointestinal tract in chickens and led to increased feed intake.

Animal and human studies have shown that various types of resistant starch promote indices of large bowel health by increasing bowel SCFAs and lowering pH (Cummings et al., 1996). As expected from this study, the greatest increase was in the butyrate and acetate that had been esterified to the starch. On d 15, birds fed acylated starch B had higher ileal and caecal butyrate concentrations than those fed other treatment diets. Ileal acetate and butyrate concentrations of challenge birds fed

acylated starch A and acylated starch B, respectively, were also significantly higher compared with those fed the antibiotic diets. On d 24, the highest ileal acetate level was recorded for birds fed acylated starch A and highest ileal butyrate level for birds fed acylated starch B in challenged and unchallenged groups. In addition, challenged and unchallenged birds fed acylated starch B had the highest caecal butyrate concentration. The increase in ileal and caecal digesta acetate and butyrate concentrations in the acylated starch A and acylated starch B groups are likely to be the result of the release of esterified acetate and butyrate by bacterial enzymes, rather than fermentation. This may indicate the ability of acylated starch to deliver specific acids that had been esterified in the ileum and caecum in significantly greater amounts. This is consistent with data that have been reported for rats (Annison et al., 2003; Bajka et al., 2006), where butyralated starch increased caecal butyrate concentration. Interestingly, the ileal and caecal SCFAs of unchallenged birds were not affected by dietary treatments on d 15 and 24 except acetate and butyrate. This may suggest that the high-amylose maize starch is not digestible or fermentable by the bacteria present in the ileum and caeca of birds. This was in contrast to data that have been reported previously in rats (Morita et al., 2005) and pigs (Bird et al., 2007) where high amylose starch increased the overall large bowel SCFAs. The current study also demonstrates that birds challenged with *Eimeria* and *C. perfringens* showed changed ileal and caecal SCFA level. This indicates that the inoculation of *Eimeria* and *C. perfringens* strongly affected the gut microflora composition as has been reported recently (Wu et al., 2014). In the challenged birds, NE increased the pathogenic and non-pathogenic caecal bacteria, which may lead to an increase in caecal fermentation.

This study also demonstrated that NE challenge had dramatically changes in gut morphology, micoroflora and relative weights of the liver and spleen. After *C. perfringens* inoculation, Our results showed that challenged birds had significantly lower villus:crypt architecture and higher crypt depth and muscle thickness compared with unchallenged birds showing that the *Eimeria* and *C. perfringens* challenge model was successful. This was in agreement with the findings of Collier et al. (2008) who proved that a severely impaired intestinal morphology results from an *Eimeria* and *C. perfringens* co-challenge. Also, challenged birds showed higher levels of enterobacteria, lactobacilli and *C. perfringens* at d15 and higher levels of

total anaerobic bacteria, lactobacilli and *C. perfringens* at d 24 compared with unchallenged birds. This may be due to the fact that the challenged birds increased mucus production as a result of inflammation of the gastrointestinal tract. It has been proposed that coccidial infection, caused by *Eimeria* inoculation, induces mucogenesis as a result of a host inflammatory response (Collier et al., 2008) and both commensal and pathogenic bacteria can derive significant benefits from mucus synthesized or secreted from host goblet cells (Deplancke and Gaskins, 2001), which may provide a growth advantage for caecal bacteria. Furthermore, Challenged birds showed significantly higher liver and spleen relative weight at d 15, and higher liver relative weight at d 24 compared with unchallenged birds. Enlarged livers can be a subclinical sign of *C. perfringens*. Løvland and Kaldhusdal (1999) postulated that in cases of subclinical necrotic enteritis, due to the high number of *C. perfringens* residing in the small intestine and the intestinal damage, some organisms can get in the biliary ducts and portal blood stream to reach the liver. This was supported by Ibitoye et al. (2012) who reported that heavier livers could be as a result of toxins causing inflammation. The higher spleen weight may reflect an immune challenge faced by the birds.

4.7 Conclusion

To the best of our knowledge, this is the first study demonstrating the efficacy of acetylated starch products on broiler performance and gut health under necrotic enteritis disease challenge. The data confirm that antibiotics completely protected birds from NE, whereas acetylated starches are effective in controlling body weight gain decline under a challenge environment. The data also demonstrate that acetylated starch consumption offers a degree of specificity in SCFA delivery. Dietary acylated starch B was effective in improving VH:CD ratio.

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Chapter 5 The Role of Acylated Starches and Necrotic Enteritis on Performance and Heat Production of Broiler Chickens

Abstract

Necrotic enteritis (NE) is a common bacterial disease of broilers in many parts of the world. It has been estimated that the total cost associated with clinical and subclinical necrotic enteritis could be as high as $2 billion annually (Van der Sluis, 2000). This study was conducted to determine the performance, energy balance and efficiency of broilers fed acetylated high-amylose maize starch (Acylated starch A) and butyralated high-amylose maize starch (Acylated starch B) under an experimental NE challenge. A total of 48 1-d-old Ross 308 male broilers were assigned to floor pens until d 10. On d 11, birds were randomly placed into16 calorimetric chambers with 4 replicates of 3 birds per treatment. Treatments were: control, antibiotics, acylated starch A or acylated starch B. Birds were challenged twice at 14 according to a previously reported protocol. The results showed that heat production (HP), respiratory quotient (RQ), heat increment (HI), weight gain (WG), feed intake (FI) and livability (LV) of birds fed control, acylated starch A and acylated starch B diets were lower than birds fed antibiotics after 19 and 42 h post inoculation ($P < 0.05$). On 3 d, or 65 h post challenge, birds started to recover from NE by increasing FI and WG. During the entire period, from d 14-17, birds fed control, acylated starch A and acylated starch B had lower WG, FI, HP, RQ, metabolisable energy intake (MEI) and metabolisable energy (ME) ($P < 0.01$) than those fed antibiotics. This study indicated that antibiotics were almost completely effective in controlling NE. The data also demonstrate that *Eimeria* sp. and *C. perfringens* challenge reduces growth performance, HP, RQ, ME and MEI of birds fed control, acylated starch A and acylated starch B diets in compare to birds fed antibiotics.

5.1 Introduction

Controlling enteric diseases is one of the most important measures in the poultry industry from an economic and welfare point of view. Viruses, bacteria, parasites and other infectious and non-infectious agents can also cause enteric diseases (Reynolds, 2003). Enteric disorders are frequently associated with an overgrowth of *C. perfringens*. Infections with this bacterium in poultry can cause necrotic enteritis

(NE), necrotic dermatitis, cholangiohepatitis, as well as gizzard erosion (Hafez, 2011). Necrotic enteritis is characterized by necrosis and inflammation of the gastrointestinal tract with a significant decline in growth performance and, in clinical cases, a massive increase in flock mortality. The subclinical form of NE is financially damaging. It has been estimated that the total cost of clinical and subclinical necrotic enteritis is approximately 5 cents per broiler (Van der Sluis, 2000), which, if extrapolated to the global broiler industry today, could amount to $2.5 billion per annum. Traditionally, NE has been controlled by antibacterial feed additives (Williams, 2005). However, public concern over the use of in-feed antibiotics and the emergence of antibiotic-resistant bacteria has led many countries to ban the use of dietary antimicrobials. With a ban of in-feed antibiotics, the incidence of NE increased in the broiler farms (Casewell et al., 2003; Hofacre et al., 2003) and the focus on alternative strategies has been increased to improve gut health or reduce the severity of NE.

Acetylated starches are resistant to small intestine digestion. Under microbial digestion, however, these starches can be degraded to release esterified acids, which are available for utilization and absorption by gut microbes and colonocytes (Abell et al., 2011). Acetylating carbohydrates, such as starch, with specific short chain fatty acids (SCFAs) offer a degree of specificity in SCFA delivery. SCFA can be a protective agent against many pathogenic organisms in the gut and they have an important physiological function in maintaining the health of the large bowel in humans through a number of chronic and acute actions (Topping and Clifton, 2001). Thus, the present study was designed to assess the efficacy of acylated starches on performance, heat production and energy efficiency of broiler chickens under NE challenge.

5.2 Materials and Methods

The experiment was approved by the Animal Ethics Committee of the University of New England (Approval No: AEC13-064). The acylated starch products were purchased from the CSIRO Division of Animal, Food and Health Sciences, Adelaide, Australia.

5.2.1 Animal husbandry

A total of 48 d-old Ross 308 broilers were assigned to floor pens until d 10. On d 11, (3 d before *C. perfringens* inoculation) birds were transferred to 16 closed-circuit calorimetric chambers with 4 replicates of 3 birds each per dietary treatment for heat production determination as previously described by (Sick et al., 2013). The birds were acclimatized to calorimeter chambers (with lids open) for 3 d in a climate controlled room. Three measurements were taken per chamber with each measurement lasting 22 to 23 h. On d 14, preceding initiation of the first run, birds were inoculated twice (morning and afternoon) with 10^8 CFU of *C. perfringens* in 2 mL of broth/buffer and the chambers were closed with cover.

5.2.2 Dietary treatments

Four diets were formulated with wheat, soybean meal, meat and bone meal, and canola meal according to Ross 308 nutrient specifications (Table 5.1). The diets were thoroughly mixed and cold-pelleted (65^0C). The dietary treatments were as follows: 1) control diet without additive; 2) control diet supplemented with 60 mg/kg salinomycin (Saccox 120®) and 50 mg/kg Zn-bacitracin (Albac 150®) in starter and grower diets; 3) control diet supplemented with 50g/kg acylated starch A in starter, grower and finisher diets; 4) control diet supplemented with 50g/kg acylated starch B in starter, grower and finisher diets. To minimize potential errors, basal diet was mixed first and then the test ingredients were mixed with the appropriate amount of basal diet. The Concept 5 feed formulation program (Creative Formulation Concepts, LLC, Annapolis, MD, USA) was used to formulate diets. Feed was changed to grower diet on d 10. All commercial products were supplied by the manufacturers. Custom-formulated broiler premixes and salinomycin were purchased from BEC Feed Solutions P/L (Brisbane, Queensland, Australia) and zinc bacitracin was purchased from Ridley AgriProducts (Tamworth, NSW, and Australia).

Table 5.1 Ingredient and nutrient composition of the basal diets (g/kg)

Ingredient	Starter	Grower
Wheat Aus 3100_10.5	564	615
SBM Arg 2400-45.2	302	215
Meat meal	30	50
Canola solvent	30	50
Canola Oil	37	45.4
Limestone	11.90	7.56
Salt	1.68	1.35
Sodium bicarbonate	2	2
Dicalcuim phosphate	10.85	4.27
L-lysine HCL	2.28	2.73
D,L-methionine	3.72	3.05
L-threonine	2.02	1.69
Choline chloride 70%	0.69	0.50
Vitamin premix[1]	0.50	0.50
Trace mineral premix[2]	0.75	0.75
Nutrient composition		
ME, kcal/kg	2975	3100
Protein	236	221.1
Digestible lysine	12	11
Digestible methionine	6.68	5.83
Digestible MC	9.4	8.4
Digestible tryptophan	2.72	2.34
Digestible threonine	8.30	7.30
Digestible arginine	13.10	11.40
Digestible isoleucine	8.63	7.56
Digestible valine	9.67	8.64
Calcium,	10.50	9.00
Non-phytate P	5.00	4.50
Sodium,	1.80	1.80
Choline, mg/kg	1600	1500
Linoleic acid	15.70	17.37
Analyzed total starch % in the diets		
Control	34.3	35.6
Antibiotics	34.6	36.2
Acylated starch A	36.7	38.2
Acylated starch B	36.8	38.5

[1] Vitamin concentrate supplied per kilogram of diet: retinol, 12,000 IU; cholecalciferol, 5,000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 μg; biotin, 200 μg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg.

[2] Trace mineral concentrate supplied per kilogram of diet: Cu (sulfate), 16 mg; Fe (sulfate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulfate and oxide), 120 mg; Zn (sulfate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

5.2.3 Necrotic enteritis challenge

The NE challenge was performed based on previous experiments conducted at UNE with modifications (Wu et al., 2014). The *Eimeria acervulina* (batch E1-3/11-064), *E. brunetti* ("Roybru", batch E2-3/11-072) and *E. maxima* (batch E9-6/11-072) were all vaccine strains obtained from Bioproperties Pty. Ltd. (Glenorie, NSW, Australia). *C. perfringens* type A strain EHE-NE18 from CSIRO Livestock Industries (Geelong, Australia) was incubated overnight at 39˚C in 100 mL of sterile thioglycollate broth (USP alternative; Oxoid) followed by subsequent overnight incubations of 1 mL of the previous culture in 100 mL of cooked meat medium (Oxoid), and then in 500 mL of thioglycollate broth (USP alternative; Oxoid) containing starch (10 g/L) and pancreatic digest of casein (5 g/L) to obtain the challenge inoculum. On d 9, birds in the challenge room were inoculated with 5000 sporulated oocysts of *E. maxima* and 2500 sporulated oocysts each of *E. acervulina* and *E. brunetti* in 1 mL of 1% (w/v) sterile saline.

On d 14, birds were inoculated twice with 2 mL of *C. perfringens* (EHE-NE18, CSIRO) suspension (3.8×10^8 CFU/mL).

5.2.4 Feed intake and gross energy

Total feed intake (FI) and excreta output were measured during 3 d. Excreta samples were homogenized and subsamples were taken for analysis. Feed and excreta samples were subjected to dry matter and gross energy analysis using an adiabatic bomb calorimeter (IKA® Werke, C7000, GMBH and CO., Staufen, Germany).

5.2.5 Respiratory chambers and heat production measurement

The net energy chambers were constructed of stainless steel, each with dimensions of 100 cm long × 70 cm wide × 76 cm high and fitted out with a wire-mesh cage (89 cm long × 60.5 cm wide × 60 cm high). The chambers were sealed using water. Humidity and temperature sensors with electronic displays were used for each chamber to monitor the humidity and temperature constantly. Pressure was controlled by a barometric sensor connected to an electronic switch to activate a solenoid valve. An air pump (28L/min) was used to circulate chamber air through a plastic bottle (2L) containing 320 g/kg potassium hydroxide with a bubbler assembly

to absorb CO_2 expired by the birds. The air was passed through a trap containing 3 kg of dried silica gel to absorb the humidity. Chamber humidity was maintained at less than 70% and CO_2 levels at less than 5 mL/L. Oxygen was provided to each chamber by using a 490 L cylinder fitted with a controller and a reducing valve to replace the O_2 consumed in the chamber by the birds.

5.2.6 Measurement of O_2 consumption and CO_2 expired

The O_2 consumption was calculated as oxygen cylinder weight at the end of each run minus oxygen cylinder weight at the beginning. The density of O_2 (1.331 g/L at normal temperature and pressure, defined as 20°C and 101.325 kN/m^2) was used for the conversion of weight (g) to volume (L). The potassium hydroxide (KOH) bottle from each chamber was diluted to 2L and subsamples were taken for CO_2 recovery for each run. The CO_2 recovery was achieved according to the method described by Annison and White (1961) and Swain (1980). 1mL precisely of KOH solution was transferred to pre-dried and pre-weighted 15 mL centrifuge tubes in duplicate. In the fume hood, 1.5 mL of NH_4Cl was added to each tube and thoroughly mixed. Later, 5 mL of $BaCl_2$ was added, mixed and centrifuged for 15 minutes at 3500 rmp. The supernatant of each tube was decanted and the pellet resuspended in 5mL of distilled water and centrifuged at 3500 rmp for 30 minutes. The supernatant was decanted and the tubes were dried at 105°C in an oven for 36 h. Finally, the tubes were cooled in a desiccator and weighed. The $BaCO_3$ recovered from 1 mL of KOH solution was recorded. The CO_2 respired was calculated by multiplying the weight of $BaCO_3$ (in 2 L KOH) by 0.2229 (the fraction of molecular weight of CO_2 to the molecular weight of $BaCO_3$).

5.2.7 Total heat production, ME, and net energy

Apparent metabolisable energy was measured according to the method described by (Bourdillon et al., 1990) and modified for total collection. Total heat production was calculated for the closed chambers over the 3-d run. For each run, calculation was postponed approximately for 3 h in order to collect excreta and refill water, feed, silica and KOH. Total heat production values were measured by using a modified equation. The equation is: Kcal total heat = 3.866 x L of O_2 consumed + 1.200 × L of CO_2 expired (Brouwer, 1965; McLean, 1972)

The respiration quotient (RQ) was measured each d for approximately 22 h for 3 d as the ratio of CO_2 expired to O_2 consumed. Heat increment (HI) was calculated as total heat production minus fasting heat production. Heat increment was corrected for zero activity, by using a value 450 kJ/kg $BW^{0.70}$ / bird/ d, which corresponds to the asymptotic HP (at zero activity) over a 24 h fasting, as suggested by (Noblet et al., 2010). NE was measured by using the equation: NE= ME-HI/feed consumed.

5.2.8 Statistical analysis

The SAS statistical package (Proc GLM) was used to determine statistical significance. The data were analysed using one-way ANOVA with different diets as factor and the significance differences between means was determined by Duncan's multiple range test.

5.3 Results

Energy balance and efficiency and performance results from d 14-17 are summarized in Table 5.2. Birds fed control, acylated starch A and acylated starch B had lower HP, RQ, MEI and ME ($P < 0.01$) than those fed antibiotics. Although birds fed control and acylated starch B diets had significantly lower net energy than birds fed antibiotics, no significant differences were detected among all treatments for the net energy to ME ratio. Birds fed antibiotics had higher body weight, WG and FI ($P <0.01$) compared to those fed control, acylated starch A and acylated starch B. No significant differences were detected between birds fed control, acylated starch A and acylated starch B diets.

Table 5.2 MEI, HP, RQ, ME, net energy, net energy to ME ratio and performance of broilers fed various diets measured from 14 to 17d in indirect closed circuit calorimetric chambers.

Parameter[1]	No additive	Anti-biotics	Acylated starch A	Acylated starch B	SEM	P-value
MEI (kJ/kg $BW^{0.7}$)	622.6[b]	1687.7[a]	730.7[b]	557.4[b]	129.00	0.001
HP (kJ/kg $BW^{0.7}$/d)	684.1[b]	830.6[a]	618.6[b]	659.1[b]	25.534	0.004
RQ	0.893[b]	1.058[a]	0.936[b]	0.862[b]	0.024	0.004
ME, kJ/g Feed	9.71[b]	13.01[a]	10.52[b]	9.43[b]	0.443	0.003
Net energy, kJ/g Feed	5.55[b]	10.07[a]	7.86[ab]	6.04[b]	0.659	0.040
Net energy:ME	0.521	0.775	0.742	0.639	0.048	0.253
Body weight g/b	523.5[b]	656.9[a]	507.8[b]	489.2[b]	19.077	0.001
Weight gain g/b	-16.4[a]	234.3[a]	11.2[b]	-25.6[b]	9.767	0.001
Feed intake g/b	117.0[b]	290.3[a]	129.4[b]	109.1[b]	21.251	0.001

[ab] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

[1] MEI = metabolisable energy intake, HP = heat production, RQ = respiratory quotient, ME = metabolisable energy

Performance, RQ, HI and HP results of the first, second and third runs are summarized in Tables 5.3-5.5 and Figure 5.1-5.3. The effect of the challenge was clearly visible in all treatments except for those containing antibiotics as shown by growth, FI and feed conversion ratio (FCR) depression. After 19 and 42 h of *C. perfringens* inoculation, birds fed control, acylated starch A and acylated starch B had decreased weight gain (WG), FI, heat production (HP), RQ and heat increment (HI) compared to those fed diets with antibiotics ($P < 0.05$). The birds fed control acylated starch A and acylated starch B lost 12.4, 17.0 and 11.3g and 30.2, 10.4 and 33.8 g of weight at the first and second d, respectively. However, by the third d, birds started to recover from NE and showed increasing FI and WG, RQ, HP and HI. Weight gain and FI of birds fed control, acylated starch A and acylated starch B were 27.48, 37.78 and 21.61g and 45.38, 58.91 and 44.18g, respectively.

Table 5.3. Performance, RQ, HI and HP of broilers fed various diets measured at first run in indirect closed circuit calorimetric chambers.

Parameter[1]	No additive	Antibiotics	Acylated starch A	Acylated starch B	SEM	P-value
WG g/b	-12.4[b]	61.1[a]	-17.0[b]	-11.4[b]	9.525	0.001
FI g/b	40.7[b]	73.7[a]	39.1[b]	41.2[b]	4.303	0.001
RQ/bird	0.935[b]	1.071[a]	0.950[ab]	0.903[b]	0.024	0.05
HP kJ /bird	473.7[b]	562.5[a]	438.9[b]	466.6[b]	14.383	0.002
HI kJ /bird	181.8[b]	252.4[a]	160.4[b]	187.0[b]	12.554	0.04

[ab] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

[1] WG = weight gain, FI = feed intake, RQ = respiratory quotient, HP = heat production, HI = heat increment

Table 5.4. Performance, RQ, HI and HP of broilers fed various diets measured at second run in indirect closed circuit calorimetric chambers.

Parameter[1]	No additive	Antibiotics	Acylated starch A	Acylated starch B	SEM	P-value
WG g/b	-30.2[b]	69.8[a]	-10.4[b]	-33.8[b]	11.422	0.001
FI g/b	21.8[b]	89.1[a]	21.5[b]	15.1[b]	8.281	0.001
RQ/bird	0.815[b]	1.073[a]	0.903[b]	0.802[b]	0.033	0.002
HP kJ /bird	371.6[b]	615.02[a]	292.3[b]	338.3[b]	36.247	0.001
HI kJ /bird	86.8[b]	281.1[a]	53.1[b]	67.5[b]	30.392	0.001

[ab] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

[1] WG = weight gain, FI = feed intake, RQ = respiratory quotient, HP = heat production, HI = heat increment

Table 5.5. Performance, RQ, HI and HP of broilers fed various diets measured at third run in indirect closed circuit calorimetric chambers.

Parameter[1]	No additive	Antibiotics	Acylated starch A	Acylated starch B	SEM	P-value
WG g/b	27.5[b]	85.2[a]	37.9[b]	21.6[b]	7.677	0.002
FI g/b	45.4[b]	104.7[a]	58.9[b]	44.1[b]	7.721	0.003
RQ/bird	0.908[cb]	1.037[a]	0.955[b]	0.869[c]	0.019	0.002
HP kJ /bird	460.7[b]	679.4[a]	470.5[b]	393.62[b]	31.785	0.001
HI kJ /bird	179.5[b]	318.3[a]	183.4[b]	126.3[b]	21.264	0.001

[ab] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

[1] WG = weight gain, FI = feed intake, RQ = respiratory quotient, HP = heat production, HI = heat increment

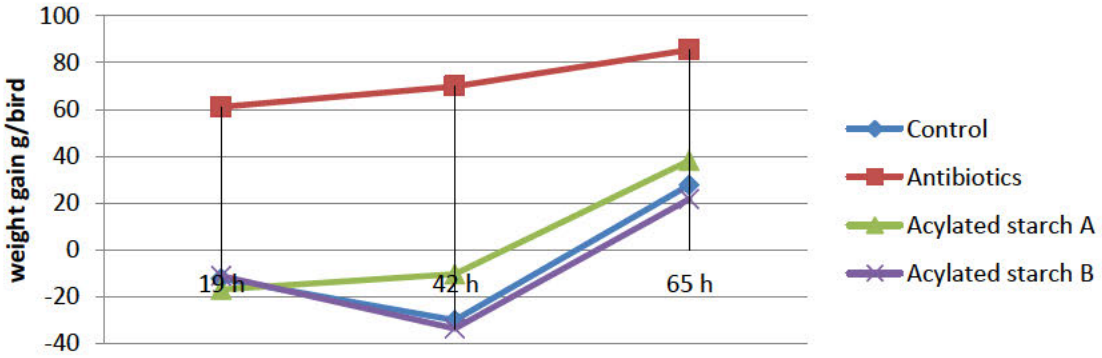

Figure 5.1 Different periods of bird weight gain in response to NE challenge

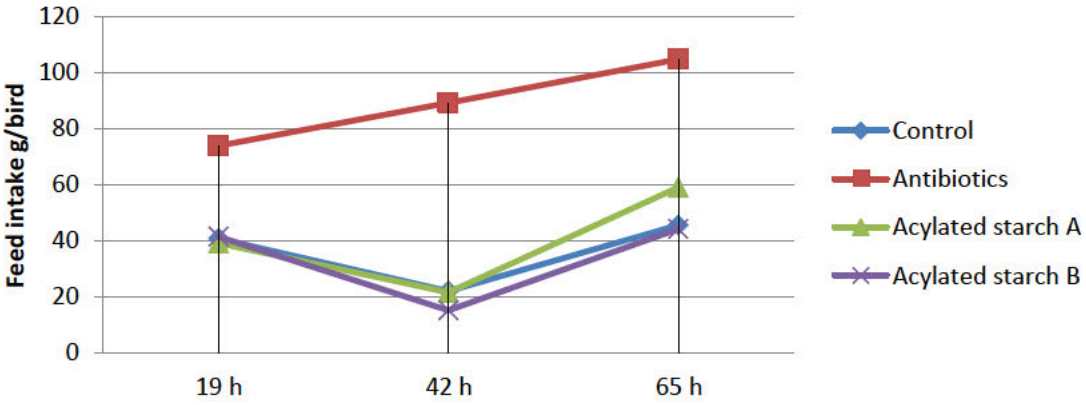

Figure 5.2 Different periods of bird feed intake in response to NE challenge

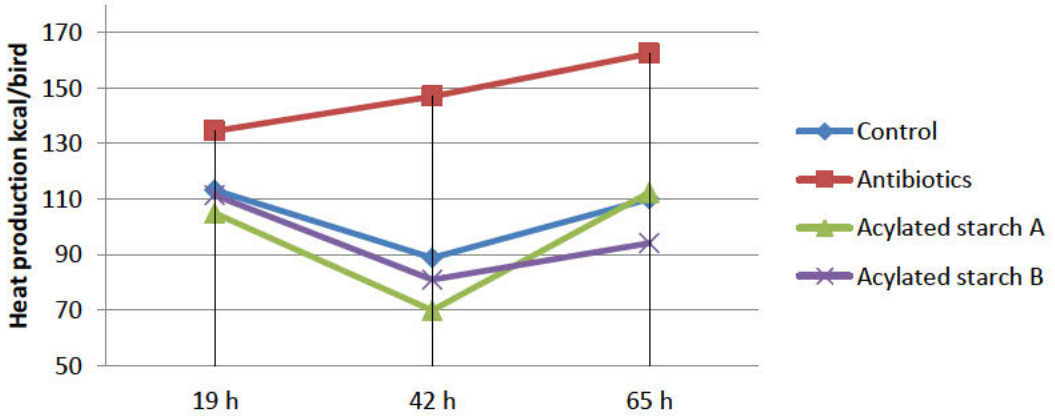

Figure 5.3 Different periods of bird heat production in response to NE challenge

5.4 Discussion

Enteric diseases are a significant concern to the poultry industry due to associated production losses, reduced welfare of birds and increased risk of contamination of poultry products for human consumption (Timbermont et al., 2011). The main purpose of the necrotic enteritis challenge in this experiment was to test the efficacy of two types of acylated starch against infection and to determine the performance, energy balance and efficiency during three d post infection.

Enteric disease has a profound effect on heat production, energy balance and energy efficiency. This study has shown that acute challenged with *Eimeria* and *C. perfringens* reduces HP, RQ, HI, ME of feed, MEI and net energy as compared to birds given protective antibiotics. Necrotic enteritis is characterized by necrosis and inflammation of the gastrointestinal tract, which may have negative effects on the activities of some metabolic functions and body hormone systems. It has been reported that the body temperature of infected turkeys with poult enteritis and mortality syndrome was depressed (Doerfler et al., 1998). Pittman et al. (1976) associated hypothermia in young and adult chickens with bacterial endotoxins. Smith et al. (1978) reported that intravenous injection of 14 d-old birds with 15 mg/kg endotoxin from *Salmonella gallinarum* was accompanied by significant falls in body temperature. This was supported by De Boever et al. (2008) who studied the effect of lipopolysaccharide administration on body temperature. They found that the first injection of lipopolysaccharide produced hypothermia. Pittman et al. (1976) revealed that a fall in body temperature was recorded during 180 minutes after an intravenous

administration of *Salmonella abortus equi* endotoxin. Thus the decreased heat production associated with NE may be due to the metabolic disorder or changes in the body hormone system. Humphrey and Klasing (2004) reported that immunological stress caused by disease has a profound effect on general metabolic processes. The current study showed that all infected birds fed control, acylated starch A and acylated starch B had poorer efficiency of ME and MEI and lower FI when compared to birds fed antibiotics. This may indicate that birds fed control, acylated starch A and acylated starch B diets had metabolic disorders due to the challenge. The lower FI and metabolic disorders may have a direct effect on heat production. Furthermore, the infectious agent resulting in malabsorption or maldigestion can be known as stunting syndrome (Shapiro and Nir, 1995). Rudas et al. (1986) reported that chickens with stunting syndrome experienced hypothyrosis. Doerfler et al. (1998) suggested that depressed body temperature in infected birds with poult enteritis and mortality syndrome was accompanied by highly significant depressions in serum T3 and T4 concentrations. The relationship between body temperature and levels of serum thyroid hormones has been well documented in poultry. Generally, increased heat production in poultry is accompanied by increased serum concentrations of thyroid hormones (Klandorf et al., 1981). However, the current results showed that heat production in birds fed all treatment diets except antibiotics was decreased by the challenge. This may indicate that the heat production driven by T3 decreased as a result of a hypothyroid condition. Of course, a near shut down of the metabolic functions and body systems of the bird would have reduced the energy need for maintenance, such as that required by the sodium pump for instance.

In this study, birds were inoculated with *Eimeria* at d 9 and followed by *C. perfringens* inoculation at d 14. The combination of *Eimeria* and *C. perfringens* infection resulted in growth, FI and FCR depression. Clinical signs of infected birds included ruffled feathers, decreased appetite, and sudden death. All dietary treatments except antibiotics resulted in a loss of weight and decreased FI after *C. perfringens* inoculation. Birds fed antibiotics exhibited nearly total resistance to challenge with NE. Birds fed control, acylated starch A and acylated starch B diets continually decreased body WG, FI and HP after 19 and 42 h post infection. However, in the third d post infection, birds started to recover from NE by increasing

WG, FI and HP. Decreased growth rate and HP in infected birds could be explained by the associated reduced FI. Immunological stress caused by disease challenge results in reduced FI, loss of appetite and lower bird activity. Ferket and Gernat (2006) postulated that the innate immune response resulting in stimulation of the pro-inflammatory cytokine cascade directly changes the bird's behavior. These behavioral modulations subsequently reduce FI (Koutsos and Klasing, 2002), which then reduces WG and HP. During an infection challenge, 70% of reduced performance can be attributed to decreased FI and the remaining 30% is as a result of malabsorption and maldigestion (Klasing et al., 1987). In addition to reduced FI, NE challenge causes intestinal necrosis and epithelium damage, shortening villi and disrupting villus:crypt ratio. These reactions all lead to decreased digestion and nutrient absorption, which result in reduced WG and HP and increased FCR.

5.5 Conclusion

The data confirm that *Eimeria* and *C. perfringens* challenge reduce HP, RQ, ME and MEI of birds fed control, acylated starch A and acylated starch B diets. However, the challenge had no effect on the net energy:ME ratio.

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Chapter 6 The Role of Coated Sodium Butyrate on Performance and Gut Health of Broilers Fed High Protein and Reduced Energy Diets

Abstract

Addition of butyrate or it salts has been reported to have a positive impact on growth performance in broilers due to its bactericidal and bacteriostatic properties. This study investigated the effect of enterically coated dietary sodium butyrate (SB) on growth performance and gut health in broilers. In experiment 1, 2160 d-old male Cobb 500 birds were used in a 2 × 2× 2 factorial plus 1 arrangement of treatments. Factors were: 2 basal diets (corn and wheat), 2 protein levels (normal and high), 2 levels of coated SB (0 and 1g/kg) plus a treatment using 2kg SB in a high protein wheat-based diet. In experiment 2, 408 d-old male Ross 308 chicks were used in a 2 × 2 factorial arrangement of treatments. Factors were: 2 diets normal or DS (down specification (minus 50 kcal/kg relative to normal)), 2 levels of coated SB (0 and 1g/kg). In experiment 1, our results showed no effect of coated SB on performance at 1 g/kg feed across basal diet type or protein level ($P > 0.05$). However, birds fed the high protein wheat diet with 2 g/kg SB were heavier than control birds. Birds fed high protein diets had lower weight gain (WG) than those fed normal protein diets ($P < 0.05$), whereas birds fed corn based diets had higher WG than those fed wheat based diets ($P < 0.05$). High dietary protein across diet type and wheat as compared to corn decreased pH of cecal contents. In experiment 2, results indicated that dietary coated SB had no effect on broiler performance, the level of cecal and ileal SCFA and intestinal pH. Higher inclusion levels may play a beneficial role but this warrants further investigation.

6.1 Introduction

The inclusion of organic acids to broiler diets has been demonstrated to inhibit pathogenic bacteria in the gut and enhance growth (Corduk et al., 2008) and feed conversion ratio (FCR) (Hassan et al., 2010). Acidification reduces pathogen colonization of the gastrointestinal tract and thus inhibits damage to the epithelial cell (Langhout, 2000). Organic acids have been demonstrated to improve the digestibility of protein and amino acids (Afsharmanesh and Pourreza, 2005) and calcium, phosphorus, magnesium and zinc (Garcia et al., 2007). It has been reported

that organic acid supplementation improves gastrointestinal villus height and cell proliferation by increasing the plasma glucagon-like peptide-2 (GLP-2), ileal proglucagon and glucose transporter (GLUT2) (Adil et al., 2010). Dietary organic acids are associated with an increase in pancreatic enzyme secretion, exogenous microbial phytase activity and an overall improvement in digestive enzyme activity (Dibner and Buttin, 2002).

Fermentation of carbohydrates such as dietary fiber and undigested starch in the distal part of the gut are companied by the production of short chain fatty acids (SCFA) such as propionate, acetate and butyrate (Hu and Guo, 2007). In general, the production of SCFA in the hind gut results in a pH reduction and inhibition of acid sensitive microorganisms (Mroz et al., 2006). Czerwiński et al. (2012) found a negative correlation between *Enterobacteriaceae* numbers and the concentration of un-dissociated propionate, acetate and butyrate in the ceca. Also, butyric acid and its sodium salt had a positive effect on intestinal integrity and growth performance (Hu and Guo, 2007; Smulikowska et al., 2009). Furthermore, it can be used as a source of energy for intestinal epithelium and stimulate intestinal epithelial growth (Mroz et al., 2006). The amount of SCFA produced by fermentation in the gut is low in broilers (van der Wielen et al., 2000a) . Dietary supplementation of non-protected organic acids is readily absorbed in the upper part of digestive tract (Molatová et al., 2011). Therefore, supplementation of microencapsulated organic acids into poultry diets may be useful in that the active agent may reach the lower digestive tract and modulate the mucosal morphology and intestinal micro flora. The objective of the study was to investigate the effect of encapsulated sodium butyrate (SB) on broiler performance and gut health.

6.2 Materials and Methods

Both experiments were approved by the Animal Ethics Committee of the University of New England (Approval No: AEC11/064 and AEC13-039)

6.2.1 Animal husbandry

In experiment 1, 2160 d-old Cobb 500 birds obtained from the Baiada hatchery (Tamworth, NSW, Australia) were placed in 54 floor pens assigned to 9 treatments with six replicates each upon arrival at the Kirby Research Station of the University

of New England, Armidale, Australia. Birds were vaccinated against Marek's disease and infectious bronchitis on hatching. Each pen started with 40 chicks from d 0, with 2 weakest chicks culled at d 10. The pens consisted of wire mesh partitions with a dimension of 150 cm long and 150 cm wide. The shed temperature was set at 33-34 °C initially and gradually decreased by 3 °C per week until 22-24 °C was reached by the third week. Chicks were subjected to artificial fluorescent illumination of 23 h between d 0-7, and 18 h from d 8 to 24. Each pen was equipped with a separate feeding trough and 8 nipple drinkers. Water and feed were provided *ad libitum*. The birds were fed starter diets during d 0-10 and grower diets during d 10-24. Mortality was recorded daily while body weight and feed intake (FI) were recorded at d 10 and 24 for the calculation of weight gain (WG) and FCR which was corrected for mortality. On d 8 and 22, three birds from each pen were randomly selected, weighed and killed by cervical dislocation. The abdominal cavity was opened and the content of ileum and caeca were sampled to measure SCFA and pH.

In experiment 2, 408 d-old male Ross 308 birds obtained from the Baiada hatchery (Tamworth, NSW, Australia) were placed in 24 floor pens randomly assigned to 4 treatments with six replicates each in the University of New England's Animal House Complex, Armidale, NSW, Australia. All the birds were vaccinated against Marek's disease and infectious bronchitis. Each pen had 17 chicks from d 0, and 2 were culled at d 10. The pens consisted of wire mesh partitions with a dimension of 120 cm long and 75 cm wide. The management was the same as in experiment 1. The starter diets were fed during d 0-10; grower diets between d 10-24, and finisher diets between 24-35 d. During d 0-35, the birds were fed the treatment diets. The primary determinants of performance were WG, FI, livability (LV) and FCR. Mortality was recorded daily while body weight and FI were recorded at d 10, 24 and 35 for the calculation of WG and FCR. On d 10 and 24, three birds and two birds, respectively from each pen were randomly selected, weighed and killed by cervical dislocation. The content of ileum and caeca were collected and stored in 50 mL plastic containers. Around 1 g of ileal and cecal contents was used to measure the pH and the rest frozen directly at -20 °C for SCFA analysis.

6.2.2 Dietary treatments

Experiment 1

Nine diets were formulated with wheat or corn, soybean meal, canola oil, canola solvent and meat meal as the main ingredients (Tables 6.1 and 6.2) according to the Aviagen Guideline for Ross 308 birds. The diets were assigned as: 1) corn-based diet (C); 2) corn-based diet + 1g/kg SB in starter and 0.5 g/kg in grower (CSB); 3)corn-based diet, high protein (CHP); 4) corn-based diet, high protein + 1g/kg SB in starter and 0.5 g/kg in grower (CHPSB); 5) wheat-based diet (W); 6) wheat-based + 1g/kg SB in starter and 0.5 g/kg in grower (WSB); 7) wheat-based diet, high protein (WHP); 8) wheat-based diet, high protein + 1g/kg SB in starter and 0.5 g/kg in grower (WHPSB); 9) wheat-based diet, high protein + 2g/kg SB in starter and 1 g/kg in grower (WHP2×SB). Raw materials were subjected to Adisseo NIRS nutrient evaluation before final formulation.

Experiment 2

Four diets were formulated with corn, soybean meal, solvent extracted canola meal and meat meal as the main ingredients (Table 6.3) according to the Aviagen Guideline for Ross 308 birds. The dietary treatments were as follows: 1) normal basal diet (ND); 2) normal basal diet + 1g/kg SB (NDSB); 3) down specification diet (minus 50 Kcal/kg relative to normal diet) (DS); 4) down specification diet (minus 50 Kcal/kg relative to normal diet) + 1g/kg SB (DSSB). All raw materials were subjected to proximate and amino acids analysis prior to formulation. Feeds were changed from starter to grower on d 10 and growers to finisher on d 24. Residual feed was weighed on the d of feed changes and at the end of the trial. In both experiments, basal diet was mixed first and then the test ingredients were mixed with the appropriate amount of basal diet

Table 6.1 Composition and nutrient composition of normal and high protein corn base diets (g/kg) (experiment 1)

Ingredients	Starter Normal protein	Starter High protein	Grower Normal protein	Grower High protein
Corn	562.1	429.5	600.0	483.4
Soybean meal	290.2	388.1	235.0	308.4
Canola meal	50.0	70.0	70.0	100.0
Canola oil	26.0	44.8	28.57	45.23
Meat meal	30.0	30.0	40.0	40.0
Limestone	10.48	10.12	6.59	6.21
Dicalcium phosphate	13.58	12.48	7.38	6.30
D,L-methionine	3.70	3.72	2.66	2.66
L-lysine HCl	3.50	1.83	1.99	0.61
L-threonine	1.91	1.45	0.96	0.54
Salt	2.87	3.18	2.37	2.33
Na bicarbonate	2.06	1.59	1.50	1.50
Choline Cl 70%	1.40	1.08	0.86	0.61
Vitamin premix[1]	0.5	0.5	0.5	0.5
Mineral premix[2]	0.75	0.75	0.75	0.75
Salinomycin 12%	0.50	0.50	0.50	0.50
zinc bacitracin 15%	0.33	0.33	0.33	0.33
Nutrient composition				
ME (kcal/kg)	3000	3000	3075	3075
ME (MJ/kg)	12.55	12.55	12.87	12.87
Crude Protein	221	260	207	240
Digestible Arginine	13.22	16.31	11.77	14.16
Digestible Lysin	12.70	13.97	11.00	12.10
Digestible M+C	9.40	10.30	8.40	9.24
Digestible Tryptophan	2.39	3.00	2.04	2.49
Digestible Isoleucine	8.50	10.36	7.50	8.89
Digestible Threonine	0.8.30	0.913	0.730	0.803
Digestible Valine	9.88	11.85	8.99	10.44
Calcium	10.5	10.50	8.50	8.50
Available P	5.00	5.00	4.20	4.20
Sodium	2.20	2.20	1.90	1.90
Chloride	3.20	3.00	2.50	2.20
Linoleic	20.9	23.2	21.9	23.9

[1] Vitamin concentrate supplied per kilogram of diet: retinol, 12000 IU; cholecalciferol, 5000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 µg; biotin, 200 µg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg.

[2] Trace mineral premix supplied per kilogram of diet: Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

Table 6.2 Composition and nutrient content of normal and high protein wheat base diets (g/kg) (experiment 1)

Ingredients	Starter	Starter	Grower	Grower
	Normal protein	High protein	Normal protein	High protein
Wheat	582.6	457.5	635.5	520.9
Soybean meal	257.2	345.0	187.2	253.3
Canola meal	50.0	70.0	70.0	100.0
Canola oil	40.0	53.0	41.63	53.0
Meat meal	30.0	30.0	40.0	40.0
Limestone	11.12	10.65	7.32	6.82
Dical phosphate	11.99	11.39	5.73	5.12
D,L-methionine	3.62	3.84	2.67	2.83
L-lysine HCl	3.56	8.39	2.44	10.2
L-threonine	2.04	1.77	1.28	1.02
Salt	2.56	1.08	2.06	0.58
Na bicarbonate	2.12	4.33	1.50	3.70
Choline	0.98	0.82	0.44	0.33
Vitamin premix[1]	0.5	0.5	0.5	0.5
Mineral premix[2]	0.75	0.75	0.75	0.75
Salinomycin	0.50	0.50	0.50	0.50
Zinc bacitracin	0.33	0.33	0.33	0.33
Xylananse [3]	0.05	0.05	0.05	0.05
Nutrient composition				
ME (kcal/kg)	3000	3000	3075	3075
ME (MJ/kg)	12.55	12.55	12.87	12.87
Crude protein	221	260	204	240
Digestible Arginine	13.10	15.70	11.40	13.4
Digestible Lysin	12.70	18.67	11.00	18.94
Digestible M+C	9.40	10.34	8.40	9.24
Digestible Tryptophan	2.53	3.01	2.15	2.50
Digestible Isoleucine	8.79	10.27	7.69	8.77
Digestible Threonine	8.30	9.13	7.30	8.03
Digestible Valine	9.90	11.54	8.93	10.11
Calcium	10.50	10.50	8.50	8.50
Available P	5.00	5.00	4.20	4.20
Sodium	2.20	2.20	1.90	1.90
Chloride	3.20	3.00	2.40	3.00
Linoleic acid	16.80	19.50	17.20	19.50

[1] Vitamin concentrate supplied per kilogram of diet: retinol, 12000 IU; cholecalciferol, 5000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 μg; biotin, 200 μg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg.

[2] Trace mineral premix supplied per kilogram of diet: Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

[3] Porzyme 93010, Dupont Inc.

Table 6.3 Composition and nutrient content of diets (g/kg) (experiment 2)

Ingredients	Starter		Grower		Finisher	
	Normal[3]	DS[4]	Normal	DS	Normal	DS
Corn	596.5	626.5	653.2	679.7	680.1	725.8
Soybean meal	267.4	258.0	279.3	254.0	262.8	222.6
Meat meal	44.8	32.38	39.09	39.09	-	26.34
Canola meal	60.9	58.81	-	8.66	-	-
Canola Oil	9.8	-	9.64	-	23.81	4.90
Limestone	5.36	7	6.35	6.39	13.85	7.14
Salt	3.6	3.76	2.26	2.25	2.51	2.17
Na bicarbonate	2	2	2	2	2	2.2
Dical phosphate	-	2.23	-	-	7.66	2.09
L-lysine HCL	2.89	2.86	2.60	2.50	2.20	2.14
D,L-methionine	2.21	1.97	2.22	1.96	1.79	1.61
L-threonine	1.27	1.18	0.74	0.70	0.86	0.71
Choline Cl 60%	1.13	1.21	0.51	0.60	0.36	0.43
Vitamin premix[1]	0.5	0.5	0.5	0.5	0.5	0.5
Mineral premix[2]	0.75	0.75	0.75	0.75	0.75	0.75
Phytase	0.1	0.1	0.1	0.1	0.1	0.1
Nutrient composition						
ME, kcal/kg	3000	2950	3075	3025	3150	3100
Protein	235	225	219	212	19.00	19.00
Digestible lysine	12.50	12.00	11.50	11.00	10.00	9.60
Digestible methionine	4.33	4.25	4.52	4.45	4.55	4.35
Digestible M+C	7.00	7.00	7.30	7.30	7.60	7.30
Digestible tryptophan	1.97	1.99	2.01	2.01	2.14	1.99
Digestible threonine	6.40	6.40	6.60	6.60	7.00	6.72
Digestible arginine	10.52	10.50	10.80	10.80	11.00	10.56
Digestible isoleucine	6.50	6.56	6.76	6.78	7.12	6.75
Digestible valine	7.70	7.75	7.84	7.96	8.12	7.94
Calcium,	9.00	9.00	8.50	8.50	9.63	8.00
Available P	4.60	4.50	4.20	4.20	4.00	4.00
Sodium	2.40	2.40	1.80	1.80	1.70	1.70
Choline	1.85	1.85	1.45	1.45	1.25	1.25

[1] Vitamin concentrate supplied per kilogram of diet: retinol, 12000 IU; cholecalciferol, 5000 IU; tocopheryl acetate, 75 mg, menadione, 3 mg; thiamine, 3 mg; riboflavin, 8 mg; niacin, 55 mg; pantothenate, 13 mg; pyridoxine, 5 mg; folate, 2 mg; cyanocobalamin, 16 µg; biotin, 200 µg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg.

[2] Trace mineral premix supplied per kilogram of diet: Cu (sulphate), 16 mg; Fe (sulphate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulphate and oxide), 120 mg; Zn (sulphate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

[3] Normal = starter 3000 kcal/kg, grower 3075 kcal/kg and finisher 3150 kcal/kg

[4] DS = minus 50 kcal/kg relative to normal

6.2.3 Measurement and analyses

Ileal and caecal PH

Approximately 1g of content was diluted in 9 mL of distilled water. The suspension was shaken with a stirrer and the pH was determined using EcoScan 5/6 pH meter (Eutech Instrument Pte Ltd., Singapore) on the sampling d.

Excreta moisture

Three birds from each pen in experiment 2 were transferred to the AME cages at d 21 and 35 to measure the moisture of manure. Ten fresh droppings were collected from each cage, and dried for 24 h at 105 °C. The moisture was calculated using the formula (wet weight – dry mater)/wet weight, and expressed as % wet weight.

Analysis of short chain fatty acids

the analysis described by Jensen et al. (1995) was used to measure SCFA with modifications. Frozen ileal and caecum samples were thawed and homogenized. Approximately 1 g of wet homogenized digesta was weighed and 1 mL of internal standard (0.01 M ethylbutyric acid) was added and mixed with a vortex mixer and then centrifuged at 38625 ×g at 5 °C for 20 minutes. Then 1 mL of the supernatant, 0.5mL of concentrated HCl (36 %) and 2.5 mL of ether were mixed with a vortex mixer. An internal standard solution and a blank were also prepared in the same way but replacing the supernatant with 1 mL of the standard acid mixture and 1 mL of water respectively. The mixture was centrifuged at 2060 × g at 5 °C for 15 minutes and 400 µL of the supernatant was transferred to a gas chromatograph vial (2mL) and mixed with 40 µL of N-tert-butyldimethlsilyl-N-methyltrifuoroacetamide (MTBSTFA). The sample vials were kept in a heating block at 80 °C for 20 minutes and left at room temperature for 48 h and then SCFA measured 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 SCFA determined in the sample, expressed as mg/g digesta. Individual SCFA concentrations were converted to their proportions over the total and expressed in percentage. The SCFA concentration or proportions were transformed with a formula $\log_{10}x+1$ for statistical analysis, where x is the concentration or proportion of SCFA.

6.2.4 Statistical analysis

The SAS statistical package (Proc GLM) was used to determine statistical significance. The significant difference between means was measured by Duncan's multiple range tests when challenge × feed additive interactions were observed ($P < 0.05$). Means were deemed significantly different at $P < 0.05$ or highly significantly different at $P < 0.01$ or $P < 0.001$.

6.3 Results

Experiment 1

6.3.1 Growth performance

From d 0-10, no significant differences were observed in WG, FI, LV and FCR between birds fed different experimental diets (Table 6.4).

From d 0-24, birds receiving wheat base diet had poorer FCR ($P < 0.01$) and lower body WG ($P < 0.01$) compared with those fed corn diet (Table 6.5). High dietary protein negatively affected bird performance with decreased body WG ($P < 0.01$) and FI ($P < 0.01$). No impact on performance was detected in birds supplemented with 1g/kg SB. Grain × protein interaction was detected for WG ($P < 0.002$) and FI ($P < 0.03$). The birds fed WHP or WHPSB diet had decreased WG and FI ($P < 0.05$) compared with birds fed other dietary treatments.

The effects of two levels of SB in high protein wheat-based diets are shown in Table 6.6. In starter and grower phases, birds fed the WHP2×SB diet were heavier compared with those fed WHPSB or WHP diets. But birds fed WHP2×SB did not affect FI, FCR or LV.

Table 6.4 Performance of birds fed different diets from d 0-10

Main effect	weight gain (g/bird)	FCR	Feed intake (g/bird)	Livability %
Grain				
Wheat	235	1.070	247	97
Corn	231	1.059	249	98
SB[1]				
0 g/kg	233	1.071	249.	97
1 g/kg	233	1.059	247	98
Protein				
Normal	231	1.072	248	98
High	235	1.058	248	97
SEM	1.861	0.006	2.471	0.415
P-value				
Grain	0.381	0.446	0.817	0.499
SB	0.891	0.362	0.668	0.368
Protein	0.321	0.286	0.891	0.180
Grain × SB	0.371	0.697	0.363	0.651
Grain × Protein	0.466	0.823	0.665	0.368
SB × Protein	0.769	0.725	0.663	0.262
Grain × SB × Protein	0.899	0.904	0.874	0.499

[1] SB= sodium butyrate

Table 6.5 Performance of birds fed different diets from d 0-24

Treatments[1]	weight gain (g/bird)	FCR	Feed intake (g/bird)	Livability %
C	1255[a]	1.349	1693[a]	91
CSB	1238[a]	1.357	1679[a]	95
CHP	1238[a]	1.324	1638[a]	89
CHPSB	1251[a]	1.329	1664[a]	90
W	1230[a]	1.362	1674[a]	88
WSB	1251[a]	1.366	1709[a]	90
WHP	1142[b]	1.372	1567[b]	88
WHPSB	1156[b]	1.363	1575[b]	89
Main effects				
Grain				
Wheat	1195[b]	1.366[a]	1632	89
Corn	1245[a]	1.340[b]	1669	91
SB[2]				
0 g/kg	1216	1.352	1643	89
1 g/kg	1224	1.354	1657	91
Protein				
Normal	1244[a]	1.359	1689[a]	91
High	1197[b]	1.347	1611[b]	89
SEM	8.013	0.004	10.762	0.826
P-value				
Grain	0.001	0.002	0.059	0.181
SB	0.574	0.788	0.489	0.321
Protein	0.001	0.136	0.001	0.221
Grain × SB	0.453	0.542	0.676	0.860
Grain × Protein	0.002	0.056	0.031	0.380
SB × Protein	0.662	0.587	0.861	0.769
Grain × SB × Protein	0.491	0.693	0.388	0.681

[ab] Means sharing the same superscripts are not significantly different from each other at P <0.05.

[1] C = corn based diet, CSB = corn based diet + 1g/kg sodium butyrate in starter and 0.5 g/kg in grower, CHP = corn based diet, high protein, CHPSB = corn based diet, high protein + 1g/kg sodium butyrate in starter and 0.5 g/kg in grower, W = wheat based diet, WSB = Wheat based diet + 1g/kg Sodium butyrate in starter and 0.5 g/kg in grower, WHP = wheat based diet, high protein, WHPSB = wheat based diet, high protein + 1g/kg sodium butyrate in starter and 0.5 g/kg in grower

[2] SB= sodium butyrate

Table 6.6 Effect of sodium butyrate levels in high protein wheat based diets

Treatments[1]	Weight gain (g/bird)	FCR	Feed intake (g/bird)	Livability %
From d 0-10				
WHP	230[b]	1.070	246	98
WHPSB	233[b]	1.059	247	96
WHP2×SB	250[a]	1.054	264	98
SEM	3.458	0.006	3.587	0.786
P-value	0.016	0.509	0.063	0.787
From d 0-24				
WHP	1142.04[b]	1.372	1567.33	87.50
WHPSB	1156.38[b]	1.363	1575.42	89.17
WHP2×SB	1215.37[a]	1.355	1646.90	88.30
SEM	12.299	0.007	16.865	1.484
P-value	0.019	0.635	0.102	0.879

[ab] Means sharing the same superscripts are not significantly different from each other at P <0.05.

[1] WHP = wheat-based diet, high protein, WHPSB = wheat-based diet, high protein +1g/kg sodium butyrate in starter and 0.5 g/kg in grower, WHP2×SB = wheat-based diet, high protein +2g/kg sodium butyrate in starter and 1 g/kg in grower

6.3.2 Ileal and cecal pH

As shown in Table 6.7, the pH of cecal digesta was lower in birds fed wheat based diets than those fed corn based diets at both d 8 and d 22 ($P < 0.01$). The birds fed high dietary protein decreased cecal digest pH at d 22 compared to those fed normal protein diets ($P < 0.001$). Grain × protein ($P < 0.034$) and SB × protein interactions ($P < 0.045$) were detected for cecal pH at d 8. The birds fed the CHPSB diet had lower cecal pH ($P < 0.01$) compared with those fed a C or CSB diet and the CHP at d 8. However, no significant differences in ileal pH between d 8 and 22 were detected. Ileal and cecal digesta pH did not differ between various levels of SB in high protein wheat based diets.

Table 6.7 The pH values of ileal and caecal content of birds fed different diets

Treatments[1]	Ileum pH		Caeca pH	
	d 8	d 22	d 8	d 22
C	7.998	7.358	7.172[a]	7.118[a]
CSB	8.138	7.385	7.083[a]	7.012[ab]
CHP	7.997	6.837	7.190[a]	6.738[abc]
CHPSB	7.993	6.860	6.607[b]	6.683[abc]
W	7.720	7.408	6.168[b]	6.125[cd]
WSB	7.857	7.358	6.320[b]	6.415[bcd]
WHP	7.933	7.213	6.617[b]	5.907[d]
WHPSB	8.058	7.603	6.367[b]	5.907[d]
Mani effect				
Grain				
Wheat	7.892	7.396	6.368[b]	6.088[b]
Corn	8.032	7.110	7.013[a]	6.888[a]
SB[2]				
0 g/kg	7.912	7.204	6.787	6.472
1 g/kg	8.012	7.302	6.594	6.504
Protein				
Normal	7.928	7.378	6.686	6.668[a]
High	7.995	7.128	6.695	6.309[b]
SEM	0.034	0.103	0.074	0.085
P-value				
Grain	0.063	0.192	0.001	0.001
Additive	0.181	0.653	0.104	0.810
Protein	0.365	0.254	0.937	0.001
Grain × SB[2]	0.667	0.741	0.194	0.414
Grain × Protein	0.059	0.216	0.034	0.973
SB × Protein	0.594	0.619	0.045	0.665
Grain × SB × Protein	0.651	0.614	0.831	0.536

[abc] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

[1] C = corn based diet, CSB = corn based diet + 1g/kg sodium butyrate in starter and 0.5 g/kg in grower, CHP = corn based diet, high protein, CHPSB = corn based diet, high protein + 1g/kg sodium butyrate in starter and 0.5 g/kg in grower, W = wheat based diet, WSB = Wheat based diet + 1g/kg Sodium butyrate in starter and 0.5 g/kg in grower, WHP = wheat based diet, high protein, WHPSB = wheat based diet, high protein + 1g/kg sodium butyrate in starter and 0.5 g/kg in grower

[2] SB= sodium butyrate

Experiment 2

6.3.3 Growth performance

During d 0-10 and d 0-35, no significant differences in WG, FI, LV and FCR were observed between birds fed different experimental dietsn(Table 6.8 and 6.10). However, Between d 0 and 24, the FCR of birds fed ND diet was better than those fed the DS diet (P <0.005) (Table 6.9).

Table 6.8 Performance of birds fed sodium butyrate and different ME from d 0-10

Main effect	Initial weight (g/bird)	weight gain (g/bird)	FCR	Feed intake (g/bird)
Diet[1]				
Normal	43.77	269	1.100	296
DS	43.37	260	1.120	292
Sodium butyrate				
0 g/kg	43.57	262	1.110	291
1 g/kg	43.57	267	1.110	297
SEM	0.133	3.193	0.006	3.057
P-value				
Diet	0.144	0.170	0.051	0.520
Additive	0.985	0.392	0.964	0.318
Diet × Additive	0.260	0.866	0.727	0.754

[1] Normal = starter 3000 kcal/kg, grower 3075 kcal/kg and finisher 3150 kcal/kg, DS = minus 50 kcal/kg relative to normal

Table 6.9 Performance of birds fed sodium butyrate and different ME from d 0-24

Main effect	weight gain (g/bird)	FCR	Feed intake (g/bird)	Livability %
Diet[1]				
Normal	1428	1.290[a]	1842	99
DS	1397	1.311[b]	1831	99
Sodium butyrate				
0 g/kg	1403	1.310	1830	99
1 g/kg	1422	1.296	1843	99
SEM	9.087	0.004	11.288	0.457
P-value				
Diet	0.093	0.005	0.652	1
Additive	0.271	0.216	0.569	1
Diet × sodium butyrate	0.734	0.341	0.485	1

[ab] Means sharing the same superscripts are not significantly different from each other at P <0.05.

[1] Normal = starter 3000 kcal/kg, grower 3075 kcal/kg and finisher 3150 kcal/kg, DS = minus 50 kcal/kg relative to normal

Table 6.10 Performance of birds fed sodium butyrate and different ME from d 0-35

Main effect	weight gain (g/bird)	FCR	Feed intake (g/bird)	Livability %
Diet[1]				
Normal	2670	1.428	3811	97
DS	2643	1.441	3809	99
Sodium butyrate				
0 g/kg	2642	1.440	3803	98
1 g/kg	2670	1.430	3816	98
SEM	17.164	0.004	20.846	0.095
P-value				
Diet	0.455	0.114	0.964	0.242
Additive	0.440	0.237	0.762	0.692
Diet × Additive	0.349	0.807	0.341	0.692

[1] Normal = starter 3000 kcal/kg, grower 3075 kcal/kg and finisher 3150 kcal/kg, DS = minus 50 kcal/kg relative to normal

6.3.4 Ileal and cecal pH and excreta moisture

Ileal and cecal digesta pH data showed no significant differences in birds fed different diets between d 10 and 24. Also, on d 21 and 35 the excreta moisture levels did not differ ($P > 0.05$) (Table 6.11).

Table 6.11 The pH values of ileal and caecal content and excreta moisture percentage of birds fed coated sodium butyrate and different ME

Main effect	Ileum pH		Caeca pH		Excreta moisture %	
	D 10	D 24	D 10	D 24	D 21	D 35
Diet[1]						
Normal	6.962	7.338	6.592	6.868	84.398	85.449
DS	7.021	7.298	6.740	6.816	83.839	84.691
Sodium butyrate						
0 g/kg	6.994	7.302	6.657	6.890	84.082	84.777
1 g/kg	6.988	7.334	6.674	6.793	84.154	85.364
SEM	0.025	0.032	0.050	0.033	0.178	0.472
P-value						
Diet	0.253	0.558	0.140	0.447	0.118	0.454
Additive	0.909	0.641	0.858	0.163	0.835	0.560
Diet × Additive	0.372	0.895	0.137	0.605	0.169	0.965

[1] Normal = starter 3000 kcal/kg, grower 3075 kcal/kg and finisher 3150 kcal/kg, DS = minus 50 kcal/kg relative to normal

6.3.5 Short chain fatty acids

The effects of SB on ileal and cecal SCFA contents are presented in (Tables 6.12 and 6.13). The ileal and cecal concentrations of individual SCFA were not affected by diets.

Table 6.12 Concentration of various short chain fatty acids (µmol/g) in ileal content of birds fed coated sodium butyrate and different ME

Main effect	D 10			D 24		
	Formic	Acetic	Lactic	Formic	Acetic	Lactic
Diet[1]						
Normal	6.234	9.262	0.660	8.450	15.218	0.562
DS	6.351	9.198	0.974	6.839	12.502	0.785
Sodium butyrate						
0 g/kg	6.262	9.344	0.636	8.422	14.599	0.612
1 g/kg	6.323	9.115	0.997	6.867	13.121	0.734
SEM	0.216	0.382	0.152	0.646	0.893	0.185
P-value						
Diet	0.803	0.936	0.293	0.242	0.138	0.550
Additive	0.896	0.774	0.230	0.259	0.410	0.742
Diet × Additive	0.738	0.213	0.138	0.413	0.417	0.099

[1] Normal = starter 3000 kcal/kg, grower 3075 kcal/kg and finisher 3150 kcal/kg, DS = minus 50 kcal/kg relative to normal

Table 6.13 Concentration of various short chain fatty acids (µmol/g) in cecal content at d 24 of birds fed coated sodium butyrate and different ME

Main effect	Formic	Acetic	Propionic	Butyric	Lactic
Diet[1]					
Normal	5.059	73.139	4.567	13.619	0.083
DS	5.291	78.033	4.802	13.620	0.321
Sodium butyrate					
0 g/kg	5.108	73.617	5.174	12.356	0.176
1 g/kg	5.242	77.555	4.196	14.883	0.228
SEM	0.315	2.664	0.297	0.823	0.084
P-value					
Diet	0.734	0.376	0.692	0.999	0.178
Additive	0.844	0.475	0.109	0.146	0.761
Diet × Additive	0.984	0.332	0.372	0.931	0.515

[1] Normal = starter 3000 kcal/kg, grower 3075 kcal/kg and finisher 3150 kcal/kg, DS = minus 50 kcal/kg relative to normal

6.4 Discussion

A major objective of the study was to determine the effect of coated SB supplementation on the growth performance and intestinal metabolites of broiler chickens. Several studies have suggested that dietary SB or its salts had a positive effect on bird performance (Antongiovanni et al., 2007; Taherpour et al., 2009). However, other studies showed no beneficial such effects (Smulikowska et al., 2009; Zhang et al., 2011; Czerwiński et al., 2012).

Growth performance and intestinal metabolites of birds were not affected by dietary SB when it was included at a level of 1 g/kg feed in our study. These results are in agreement with those of Smulikowska et al. (2009), Zhang et al. (2011) and Czerwiński et al. (2012), who reported that the dietary addition of SB did not have any significant effect on WG, FI and FCR. It is suggested that the good hygienic conditions where the birds are grown and the high health status of birds themselves (Smulikowska et al., 2009). Furthermore, in the current study, SB did not affect ileal and cecal pH values and SCFA contents. Similar findings were reported by other researchers where SB did not affect intestinal SCFA contents and pH values (Mahdavi and Torki, 2009; Smulikowska et al., 2009; Czerwiński et al., 2012). On the other hand, the current study showed that SB at a higher dose (2g/kg) significantly increased growth rate compared. This indicates that at high levels of inclusion, SB is effective in improving intestinal environment, and thus protein digestion. Improved protein and amino acid digestibility by other organic acids was reported by Afsharmanesh and Pourreza (2005).

Our data showed that grain type had a clear effect on bird performance. The WG and FCR of birds fed corn-based diets were significantly higher compared to those fed wheat-based diets. The poorer performance of birds fed wheat-based diets is well-known. For instance, Abdollahi et al. (2013) showed that the digestibility of starch, nitrogen, fat, Ca and P, as well as apparent metabolisable energy value of wheat-based diets were lower than corn-based diets. In addition, wheat contains a considerable amount of non-starch polysaccharides (NSPs) which are anti-nutritve in poultry diedts as they impede the digestion and absorption of nutrients (Choct et al., 1999). In the present work, wheat-based diets decreased cecal pH as more SCFAs were produced. This is not surprising as the NSPs move down the digestive tract

undigested, more substrates become available in the lower part of the gut where the microorganisms start to ferment them (Mirzaie et al. (2012). A lower energy content coupled with a higher NSP level would make wheat an inferior cereal for broiler diets compared with corn.

High dietary protein levels negatively affected bird performance. A wheat-based, high protein diet significantly decreased body weight and FI of birds in the current study. However, such an effect was not observed in corn-based diets. Our results also showed that high dietary protein decreased cecal pH over and above the effects observed in similar diets with normal level of protein. This may indicate that the NSPs in wheat exacerbated the amount of excessive protein escaped to the hindgut where it is fermented resulting in cecal pH reduction. Nordgaard et al. (1995) indeed reported that protein escaped from digestion and absorption in the small intestine produced SCFAs and branched chain fatty acids. Protein fermentation leads to unfavorable outcomes thorugh production of toxic substances, such as phenols, thiols, amines, ammonia and indoles, which may have negative effects on broiler performance.

6.5 Conclusion

Our results indicate that the inclusion of encapsulated SB at 1 g/kg did not substantially improved growth performance, ileal and cecal pH and SCFAs, but increasing inclusion rate to 2g/kg improved bird performance.

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(To appear at the end of each thesis chapter submitted as an article/paper)

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 discussion and conclusion

7.1 Introduction

The performance of broiler chickens is closely related to the growth, development, and health of the digestive tract. The gastrointestinal tract (GIT) is a barrier between the tissues of the bird and its luminal environment (Yegani and Korver, 2008), and its function is to digest feed and absorb nutrients that are required for maintenance, growth, and reproduction. The health status and homeostasis of the GIT plays a significant role in achieving optimum productivity and welfare in poultry production. The GIT affects feed digestion, protein and energy utilization, nutrient absorption and metabolism, disease resistance and immune response (Kelly and Conway, 2001; Yegani and Korver, 2008). Gut health itself is a dynamic equilibrium of complex interactions between diet quality, microfloral balance, macro-and micro-structural integrity and immune system homeostasis (Choct, 2009). Slight perturbations in equilibrium can cause disruption of gut health and thus overall performance. The disturbances of these processes can result in gastrointestinal problems (Dekich, 1998) such as diarrhea, wet droppings, dysbacteriosis, intestinal colibacillosis, malabsorption syndrome, coccidiosis and necrotic enteritis (NE). To address the issues, antibiotics have been used for many years to maintain gut health, improve growth performance and feed efficiency, and to control enteric disease outbreaks (Kim et al., 2011).

The restriction of in-feed antibiotics either by government regulation or voluntary actions has had both expected and unexpected effects. The absence of antimicrobials in animal feed has resulted in reduced feed efficiency (Feighner and Dashkevicz, 1987) which has resulted in consequential economic losses to livestock producers (Van Immerseel et al., 2008). More worryingly, the incidences of some diseases such as NE have become more widespread in poultry farms (Van Immerseel et al., 2004). Therefore, a large amount of multidisciplinary research has been conducted to explore alternative strategies to alleviate the problems associated with antibiotic withdrawal from poultry diets. The major focus of this thesis was to investigate the efficacy of selected feed additives on performance, gut health and reduction of the severity of NE in broilers under experimental disease challenge.

7.2 Necrotic enteritis challenge

The birds in this study were inoculated with *Eimeria* sp. and *C. perfringens* to reproduce clinical and sub-clinical signs of NE experimentally (Chapters 3, 4 and 5). The reason for reproducing the disease was to investigate the efficacy of yeast cell wall extract and acylated starch as a replacement for antibiotics (zinc bacitracin and salinomycin) in ameliorating the severity of NE. The data from these experiments showed that challenged birds had decreased weight gain (WG), feed intake (FI) and livability (LV) as expected based on the reports by other researchers (Mikkelsen et al., 2009; Jayaraman et al., 2013). The clinical signs of affected birds included ruffled feathers, closed eyes, relative immobility, depression, diarrhea, decreased appetite and sudden death. Post-mortem examination revealed that the small intestines of infected birds were thin walled, filled with gas and showed gross lesions over the duodenum, jejunum and ileum; these signs were similar to those described by Broussard et al. (1986); Olkowski et al. (2006); Timbermont et al. (2011). Microscopic examination (Chapters 3 and 4) showed that the challenged birds exhibited increased crypt depth and decreased villus height to crypt depth ratios compared to unchallenged birds, which was in agreement with Collier et al. (2008) who proved that a severely impaired intestinal morphology results from an *Eimeria* and *C. perfringens* co-challenge. Based on the evidence above, it is reasonable to conclude that NE challenge was successfully induced and it had a profound effect on overall performance and gut health.

Interestingly, challenged birds showed significantly higher relative weights of liver and spleen compared with unchallenged birds (Chapter 4). Enlarged livers can be a subclinical sign of *C. perfringens* infection. Ibitoye et al. (2012) reported that heavier livers could be a result of toxins causing inflammation. This was supported by Løvland and Kaldhusdal (1999) who postulated that in cases of subclinical necrotic enteritis, due to the high number of *C. perfringens* residing in the small intestine and the intestinal damage, some organisms can enter the biliary ducts and portal blood stream to reach the liver. The higher spleen weights may reflect an immune challenge faced by the birds. There is a positive relationship between spleen mass and immune competence, and a large spleen is more effective at producing an immune response for a particular size of bird (Moller and Erritzoe, 2000).

7.3 Yeast cell wall extract

In the current study, dietary yeast cell wall extract (YCW) derived from *Saccharomyces cerevisiae* was as effective as zinc bacitracin (ZB) and salinomycin (SM) in preventing performance decline due to NE (Chapter 3). The supplementation of YCW, ZB or SM exerted a greater positive impact on the performance of birds challenged with NE. Birds fed ZB, YCW or SM had higher WG, FI and LV relative to birds fed no additive when challenged with NE, whereas no difference was observed between unchallenged birds. Furthermore, challenged birds fed YCW exhibited decreased crypt depth, increased villus height and increased villus to crypt ratio.

The exact mode of action of YCW in reducing the severity of NE is unclear. It is believed that mannanoligosaccharides (MOS) , which is the major constituent of YCW extract, complete colonizing sites with organisms that possess type-1 fimbriae, thereby displacing them from the intestinal wall (Yang et al., 2009). However, Clostridia are not known to express type-1 fimbriae, and thus the mode of action of MOS in reducing clostridial counts may be more complex than competition with binding sites in the gut. Possibly, immune or mucin expression modulations by YCW are the reasons underlying such effect on the control of *C. perfringens* in the intestinal tract. Data from Sims et al. (2004) and Kim et al. (2011) has revealed that dietary MOS has the ability to reduce *C. perfringens* populations in the gastrointestinal tracts of birds. It has been reported that β-glucans from YCW act as microbial recognition receptors of the innate immune system (Gantner et al., 2003) and both mannans and β-glucans structures stimulate the immune system (Spring et al., 2000). Earlier reports have indicated that MOS enhance macrophage response in animals (Che et al., 2012) and increases macrophage nitric oxide production (Lillehoj et al., 2005). Therefore, it can be expected that the improved performance of challenged birds fed YCW might be related to pathogen reduction, immune response modulation or improved gut integrity through increasing villus height and villus to crypt ratio.

7.4 Acylated starch

The current study demonstrated that although acylated starch products did not have the same effect as antibiotics in terms of reducing lesions or preventing mortality,

they had positive effects on gut health and bird performance (Chapter 4). Challenged birds fed acylated high amylose maize starch (acylated starch A) and butyralated high amylose maize starch (acylated starch B) had significantly higher WG compared with those fed the control diet. The improved body weight gain is probably due to the beneficial effect of acetate and butyrate on gut health. The current results showed that acylated starch A and acylated starch B increased acetate and butyrate concentrations in the ileum and caecum. Both butyrate and acetate are known to have a positive effect on energy metabolism and gut health (Topping and Clifton, 2001). It has been reported that SCFAs have a direct stimulatory effect on gastrointestinal cell proliferation (Tappenden and Mcburney, 1998). In the current study, acylated starch improved the villus to crypt ratio. Thus, increased intestinal surface area, and hence nutrient absorption, may be the underlying mechanisms for the improved WG. Although the challenged birds fed acylated starch A and acylated starch B had higher WG compared to the birds receiving the control diet, the challenged and unchallenged birds fed acylated starch A and acylated starch B had higher feed intake. This indicates that these additives were not digestible, and hence the birds increased intake to compensate for lower nutrients compared to other treatments. This is plausible because resistant starch behaves like fibre in monogastric animals (Annison and Topping, 1994).

An interesting finding from the current study was that the greatest increase in intestinal SCFA content was observed in birds fed the esterified starches. The increase in ileal and caecal digesta acetate and butyrate concentrations in birds fed acylated starch A and acylated starch B, respectively, is likely to be the result of the release of esterified acetate and butyrate by bacterial enzymes, rather than fermentation. This may indicate the ability of acylated starch to deliver specific acids that had been esterified in the ileum and caecum in significantly greater amounts. This is consistent with data that have been reported for rats (Annison et al., 2003; Bajka et al., 2006), where butyralated starch increased caecal butyrate concentration.

Supplemental acylated starches were not effective as antibiotics in controlling performance decline, heat production and energy balance and efficiency during an NE outbreak (Chapter 5). Birds fed control, acylated starch A and acylated starch B diets continually decreased body weight gain and feed intake after 19 and 42 h post infection compared to those fed antibiotics. This is likely due to the spectrum of

activity of Zn bacitracin against Gram-positive *C. perfringens* and salinomycin affecting ion transport in *Eimeria* as compared to the bacteriostatic action of organic acids acylated to resistant starch. In contrast, acylated starches may act by enhancing immunity and shifting the gut microflora to reduce the damaging effects of clostridia. The current study also demonstrates that acute challenge with *Eimeria* and *C. perfringens* reduces heat production, respiratory quotient, heat increment, metabolisable energy, and metabolisable energy intake as compared to birds given protective antibiotics. This may indicate that birds fed control, acylated starch A and acylated starch B diets had metabolic disorders due to the challenge. It has been reported that the infectious agent resulted in malabsorption or maldigestion (Shapiro and Nir, 1995). Of course, a near shut down of the metabolic functions and body systems of the body would have reduced the energy need for maintenance, such as that required by the sodium pump for instance.

7.5 Microencapsulated sodium butyrate

Several studies have suggested that dietary sodium butyrate and its salts improve bird performance (Antongiovanni et al., 2007; Taherpour et al., 2009). However, the current study demonstrated that inclusion of microencapsulated sodium butyrate at the recommended dose of 1 g/kg in wheat or corn based diets had no effect on broiler performance, levels of caecal and ileal short chain fatty acids (SCFAs) and intestinal pH (Chapter 6). These results are in agreement with those of Smulikowska et al. (2009) and Czerwiński et al. (2012), who reported that dietary sodium butyrate did not have any significant effect on growth performance, intestinal acidity or SCFA content. On the other hand, the addition of sodium butyrate in high protein wheat-based diets showed that birds fed 2 g/kg sodium butyrate in starter diets and 1 g/kg in grower diets were heavier compared with those fed 1 g/kg in starter diets and 0.5 g/kg in grower diets, or those without any sodium butyrate. This indicates that at high levels of inclusion, sodium butyrate is effective in improving the intestinal environment, and thus protein digestion. Improved protein and amino acid digestibility by other organic acids was reported by Afsharmanesh and Pourreza (2005). Thus, the dosage of addition may be related to the improved peroformance.

The current study also showed that grain type and high dietary protein levels had a clear effect on bird performance and intestinal acidity. The body weight gain and

intestinal pH of birds fed wheat based diets or high dietary protein levels were significantly lower compared to those fed corn based diets and normal dietary protein levels, respectively. This is not surprising as wheat contains a considerable amount of non-starch polysaccharides (NSPs) which are resistant to enzymatic digestion and can allow more substrates to become available in the lower part of the gut for fermentation by microorganisms. Furthermore, the reduction of caecal pH in birds fed high levels of dietary protein may indicate that the NSPs in wheat exacerbated the amount of excessive protein escaping to the hindgut, where it is fermented.

7.6 Conclusions and recommendations for further studies

Despite the tremendous benefits of antibiotics in animal feed, concern over the use of in-feed antibiotics and the emergence of antibiotic-resistant "superbugs" has led many countries to ban the use of dietary antimicrobials. Phasing in-feed antibiotics out from poultry feed in Europe and recent removal or reduction of these compounds in other parts of the world pose a significant challenge to the poultry industry.

This thesis confirms that the inclusion of encapsulated sodium butyrate does not substantially improve growth performance, ileal and caecal pH and levels of SCFAs. However, YCW and acylated starches are effective in controlling performance decline under a challenge environment, possibly through improving gut integrity by increasing villus to crypt ratio and/or through influencing luminal pH values and SCFAs content. Dietary YCW was effective in improving gut integrity by increasing villus height and villus to crypt ratio as demonstrated. More interestingly, the data of this thesis also demonstrate that acylated starch consumption significantly influences luminal pH values and offers a degree of specificity in SCFA delivery. Also, dietary acylated starch B was effective in improving gut integrity by increasing the villus to crypt ratio. However, acylated starches were not effective in controlling performance decline during the first three days of an NE outbreak. An outbreak of NE reduces heat production, respiratory quotient, heat increment, metabolisable energy, and metabolisable energy intake of birds fed the control or acylated starches. Perhaps it is important to remember that antibiotics have a direct effect on the organisms themselves. In contrast, acylated starches may act by enhancing immunity and shifting the gut microflora to reduce the damaging effect of clostridia. In view of the above results, it is reasonable to conclude that YCW and acylated starches improve

gut health and that they can be used as a tool for reducing the severity of NE outbreaks.

Further studies on the use of these YCW to control NE are required, particularly studies on the efficacy and mode of action of YCW on the immune status and gut microflora of broilers. Furthermore, the results in this thesis have confirmed the ability of acylated starches to selectively increase certain types of SCFAs in the gastrointestinal tract. Further work is required to increase the understanding of the efficacy of specific SCFAs on gut health and microflora.

There is also a need to investigate the role of microencapsulated organic acids on performance and gut health in NE challenged broilers.

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