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Improving Broiler Chicken Performance

The efficacy of organic acids, prebiotics and enzymes in controlling necrotic enteritis

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Foreword

Increased consumer pressure has forced the livestock industry to reduce the amount of antibiotic growth promoters used in animal feed. Following the European lead, the Australian Government appointed a Committee (The Joint Expert Technical Advisory Committee on Antibiotic Resistance) in April 1998 to review the scientific evidence on the link between the use of antibiotics in the livestock industries and the emergence and selection of antibiotic-resistant bacteria and their spread to humans. The Committee has presented evidence of such a link and outlined 22 recommendations, highlighting the need to have programs in place to reduce the overall use of antibiotics.

The aim of the project was to examine the efficacy of organic acids, prebiotics and enzymes: (1) in maintenance and improvement of bird performance; and (2) as a replacement for antibiotic growth promoters in the control of necrotic enteritis in broiler chickens.

Thirteen experiments were conducted as part of this study. Several models for the experimental introduction of clostridial enteritis were tested and a final model for the successful introduction of a subclinical necrotic enteritis infection is presented. Several alternative feed additives to the combination of zinc-bacitracin and monensin were tested.

Results indicate that the use of some feed enzymes, in particular the use of xylanases, have a profound inhibitory impact on the growth of *C. perfringens* in the intestine of broiler chickens. However, the effectiveness of enzymes as a replacement for antibiotic growth promoters has to be further evaluated using the model described in this report.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report, an addition to RIRDC's diverse range of over 1800 research publications, forms part of our Chicken Meat R&D program and aims to support increased sustainability and profitability in the chicken meat industry through carefully focused R&D.

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Peter O'Brien
Managing Director
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The establishment of the successful model would not have been possible without the leadership and dedication of Dr Vivien Kite of the RIRDC Chicken Meat Program. Dr Kite organised numerous industry meetings and workshops to exchange ideas and experience amongst our colleagues in the industry and research organisations. The result was a well-fascilitated, Australia-wide approach to the research issue – the establishment of a repeatable and reliable model for NE.

We would also like to thank Drs Hai Meng Tan, Alex Teo (Kemin Asia, Singapore) and Rick Carter (Kemin Australia) for their professionalism, continuous interest in the work, and stimulating discussions and ideas on the progress of the project.

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Abbreviations

AGP	Antibiotic growth promoter
AME	Apparent metabolisable energy
BW	bodyweight
CFU	Colony forming unit
CP / *C. perfringens*	Clostridium perfringens
d.p.i.	days post infection
DM	Dry matter
EM	Excreta moisture
FCR	Feed conversion ratio
FOS	Fructo-oligosaccharides
FDA	Food and drug administration (of USA)
GE	Gross energy
GIT	Gastrointestinal tract
LAB	Lactic acid bacteria
MOS	Manno-oligosaccharides
NE	Necrotic enteritis
NSP	Non-starch polysaccharides
PIS	Porcine intestinal spirochaetosis
RNA	Ribonucleic acid

SBM	Soybean meal
SD	Swine dysentery
TOS	Transgalacto-oligosaccharides
UNE	University of New England
VFA	Volatile fatty acids
VRE	Vancomycin resistant enterococci

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Executive Summary

What the report is about

This report is about the efficacy of organic acids, prebiotics and enzymes in maintaining and improving bird performance, and as a replacement for antibiotic growth promoters in the control of necrotic enteritis in broiler chickens.

Background

The imminent ban of antibiotic growth promoters (AGPs) in the European Union in January 2006 has forced Australia to scrutinise the use of AGPs in animal feed. In 1999, the Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) released its findings on the future use of AGPs in Australia. The report included the recommendation to develop alternative methods of controlling necrotic enteritis (NE) in broiler chickens.

Necrotic enteritis is one of the most common and financially devastating bacterial diseases in modern broiler flocks. The disease is caused by the α-toxin of the gram-positive pathogen *Clostridium perfringens* types A and C. Damage to the intestinal mucosa through *Eimeria* infection or a change in the normal intestinal microflora because of a dietary change can predispose birds to the rapid proliferation of *C. perfringens*. It has been estimated that the global cost of clinical and subclinical NE can be as high as US$220 million per annum. Today, NE can be effectively controlled, or clinical NE outbreaks treated, with antibiotics such as virginiamycin, bacitracin, avilamycin or flavomycin.

Included at sub-therapeutical dosages AGPs selectively modify the gut flora, suppress bacterial catabolism and reduce bacterial fermentation, all these changes leading to increased nutrient availability for the animal and improved growth performance. The biggest concern regarding the use of AGPs is the occurrence of resistance to these AGPs as well as the occurrence of resistance to antibiotics used in human medicine and the development of so-called superbugs. Although there is still considerable controversy among leading scientists as to whether the ban of AGPs in feed is justified on the basis of increasing resistance, consumer pressure overseas as well as in Australia has forced the industry to look for alternative feed additives to control NE.

Aims/objectives

The aim of the current project is to examine the efficacy of organic acids, prebiotics and enzymes: (1) in maintenance and improvement of bird performance; and (2) as a replacement for antibiotic growth promoters in the control of necrotic enteritis in broiler chickens.

Methods used

This project examined the possibility of controlling the occurrence of a subclinical NE infection with natural feed additives. The key for the successful test of such alternative feed additives was the establishment of a reliable and reproducible model for the experimental introduction of a subclinical NE infection. Thirteen experiments were conducted during this project. Initial work concentrated solely on the development of such a working model whereas the second part of the project tested a range of possible alternative feed additives to control the occurrence of *C. perfringens* and related diseases using the successful model.

Results/key findings

Preliminary studies showed that both a dual infection with *Eimeria* (7000 sporulated oocysts each of *E. acervulina* and *E. brunetti*) and *C. perfringens* (10^8 colony forming units (CFU) per ml over three consecutive days) and a single challenge with *C. perfringens* only resulted in reduced growth performance compared to the unchallenged control. Results further suggested that it is difficult to

attribute the loss in performance to either the *Eimeria* challenge or the challenge with *C. perfringens*. The most reliable indicator of a bacterial overgrowth because of the challenge with *C. perfringens* is the reduction in feed efficiency in challenged birds. In four experiments when birds were challenged with *C. perfringens* alone, feed conversion ratio (FCR) was reduced by between 3–5%.

There are a large number of commercial feed additives used to improve broiler growth performance. However, to date only antibiotic growth promoters are effective in controlling a subclinical clostridial infection. In four separate experiments the effects of enzymes, organic acids, prebiotics and probiotics were tested on their effectiveness in controlling the proliferation of *C. perfringens* in the small intestine. All four experiments failed to show any significant differences between the unsupplemented control group (*C. perfringens* control) and diets containing alternatives to AGPs. However, numerical differences in growth performance between treatments and the change in secondary indicators of a disease (lesion score and occurrence of *C. perfringens*) would indicate that some of the tested products, such as enzymes, may be able to effectively maintain performance of birds experimentally challenged with *C. perfringens*.

After numerous trials, a reliable model (UNE NE Model) for the experimental reproduction NE for broiler chickens was established. Using the UNE Model, it was possible to induce a subclinical NE infection with lesion scores ranging from 1-4. Tested under the model, it appears that some feed enzymes and organic acids can control the proliferation of *C. perfringens*. However, only the addition of some feed enzymes (e.g., xylanases for wheat-based diets) will translate into improved growth performance and reduced FCR.

In addition to the use of feed additives to alleviate the effect of necrotic enteritis, the current studies showed that birds with early access to feed and water post hatch exhibited superior disease resistance and growth performance compared to those with delay access to feed and water.

Recommendations

In conclusion, the success in developing an alternative strategy to the use of AGPs depends largely on the success of the model used to induce the disease experimentally. Work described in this report suggests that the use of feed enzymes may have a role in reducing the negative impact on growth performance and feed utilisation after challenge with *C. perfringens*. Further work is needed to test a wider range of commercially available feed additives before giving out substantial recommendations to poultry producers in Australia.

1. Introduction

The most common and financially devastating bacterial disease in modern broiler flocks is necrotic enteritis (NE). Necrotic enteritis was first described by Parish (1961) and is believed to be caused by the α-toxin of *Clostridium perfringens* (*C. perfringens*) types A and C. *Clostridium perfringens* is a gram positive, anaerobic bacterium that can be found in soil, litter, dust and at low levels in the intestine of the healthy bird. Damage to the intestinal mucosa through *Eimeria* infection or a change in the normal intestinal microflora as a result of a change in diet can predispose birds to the rapid proliferation of *C. perfringens* (Ficken & Wages 1997). The intestine of infected birds is friable and distended with gas, and gross lesions caused by the α-toxin are usually found in the small intestine. In its acute form, birds often die without clinical signs. However, in its subclinical form the disease is much more financially damaging for the producer. The commonly observed symptoms of the disease vary with the age of the birds (van der Sluis 2000b) and early signs of an NE outbreak such as wet litter, diarrhoea and a small increase in mortality of less than 1% are often overlooked. However damage to the intestine and the subsequent reduction in digestion and absorption can reduce weight gain by more than 200 g (van der Sluis 2000a) and the feed conversion ratio (FCR) at 35 days of age up to 10 conversion points (Kaldhusdal & Løvland 2000). Furthermore, increased condemnations at processing due to liver lesions associated with subclinical NE can occur (Kaldhusdal & Løvland 2000). It has been estimated that the total cost of clinical and subclinical NE can be as high as US$0.05 cents per bird (van der Sluis 2000b) or a staggering US$220 million based on the global production of chicken meat in 2002 (FAOSTAT 2002).

Outbreaks of NE can be effectively treated with antibiotics such as virginiamycin, bacitracin, avilamycin, flavomycin or the now banned avoparcin (Watkins et al. 1997). When used as so-called antibacterial growth promoters (AGPs) the same agents can be very effective in controlling and preventing NE. Included at sub-therapeutical dosages such products selectively modify the gut flora, suppress bacterial catabolism and reduce bacterial fermentation, all these changes leading to increased nutrient availability for the animal and increased growth performance (Corpet 1999). Mucosal damage caused by *Eimeria* infection can predispose birds to rapid proliferation of *C. perfringens*. The effective control of *Eimeria* infection can therefore greatly reduce the risk of NE. The inclusion of anti-coccidials of the ionophore type has shown to be effective in reducing the level of *C. perfringens* in chickens (Elwinger et al. 1998).

Reports of the occurrence of vancomycin resistant enterococci (VRE) in hospitals, and the speculated link with the use of the now banned AGP avoparcin (Collignon 1999) and general fears concerning the possible development of antibiotic resistant “super-bacteria” have led to reviews of the registration status of AGPs in animal feeds. In 1999, the European Union (EU) placed a partial ban on the use of AGPs that will be replaced in 2006 by the general ban of all AGPs (including ionophore anti-coccidials) in animal feed. It was shown that the removal of AGPs led to a massive increase in NE outbreaks in many European countries and the widespread occurrence of ill-defined intestinal dysbacteriosis (Kaldhusdal & Løvland 2000; Pattison 2002). Although in Australia there are still several antibiotic products registered with growth promotion or NE control claims or both, it is widely anticipated that Australia will follow the European lead in placing increasing restrictions on the use of such products. Alternative methods to control NE will need to be developed.

Data from Europe have shown that strict management of hygiene, climate of the buildings and feed composition are possible alternatives to AGPs and can help to maintain broiler performance and control the occurrence of NE (Inborr 2000). Nutritional management such as lowering the inclusion rate of fishmeal, wheat or barley in the diet may prevent NE (Ficken & Wages 1997). It has been recognised that modulation of the natural bacterial populations of the intestine in broilers through nutritional manipulation, such as the selection of feed ingredients or the use of feed supplements, can be effective tools in controlling NE and other *C. perfringens* related diseases.

2. Background

2.1 The use of antibiotic growth promoters – advantages and disadvantages

The use of antibiotics in animal feed has a wide range of benefits. Undoubtedly, AGPs are an effective tool to improve growth performance in farm animals. In a review of over 12 000 studies, Rosen (1995) concluded that antibiotics will improve growth and FCR by 2-3 % at 72% of the time. However, the use of AGP has wider implications than just improving performance. Antibiotic growth promoters selectively modify the gut flora, suppress bacterial catabolism, reduce bacterial fermentation and reduce the intestinal wall thickness, all these changes leading to increased health, increased nutrient availability for the animal and subsequently increased growth performance (Carlson & Fangman 2000). Improved feed utilisation means that feed resources will last longer. This is of particular relevance when feed ingredients are limited due to extreme weather conditions and poor crop yield. The more efficient use of nutrients through the use of AGPs results in a significant reduction of nutrients that are excreted into the environment (Cromwell 2000). Furthermore, the selective use of AGPs has a major impact on overall animal health and welfare. One of the main reasons AGPs are still used at present is to protect animals against subclinical clostridial infections (such as necrotic enteritis), *E. coli* infections (such as post-weaning diarrhoea in piglets) or *Eimeria* infection.

The main concern with respect to the use of AGPs is the occurrence of resistance to these AGPs as well as the occurrence of resistance to antibiotics used in human medicine. There is considerable debate amongst leading scientists as to whether a ban on the use of AGPs in feed is justified because of increased resistance. Veterinarians defend the use of AGPs on the basis that there is no link between the use of AGPs in feed and any resistance pattern in human medicine (Cummings 2004; Schaffer 2004). In addition, a ban on AGPs that are not used to treat human diseases has led to an increase in the use of therapeutical antibiotics for animals that are also used to treat human disease. As a result there is now a trend towards increased resistance of human pathogens such as *Salmonella typhimurium*, *E. coli* or *C. jejuni* to these therapeutics (DANMAP 2002, 2003). There is also considerable doubt whether a simple ban on the use of AGPs will reduce or eliminate resistance. A study at the University of Kentucky showed that even after the complete withdrawal of all antibiotics, populations of antibiotic resistant bacteria can survive in a pig herd for decades (Newman 2003). This is in direct contrast to reports from Denmark. The Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) has reported that after the ban of AGPs in Denmark the occurrence of resistance in *E. faecium* has dropped significantly, for example cessation of avilamycin use resulted in a decrease in avilamycin resistant *E. faecium* from broilers (DANMAP 2002, 2003) (Figure 1).

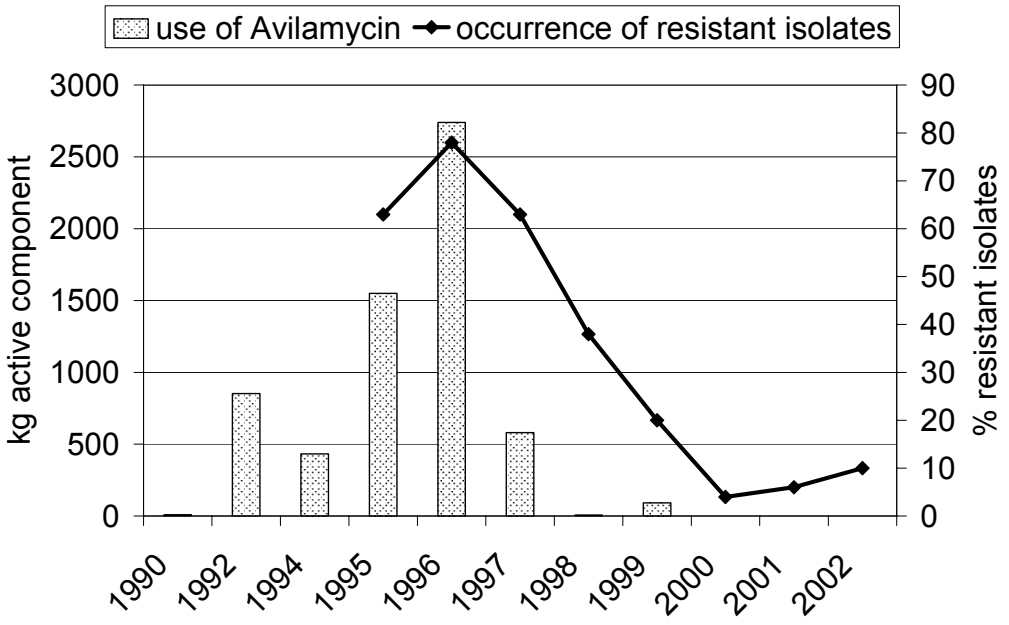


Figure 1. Trends in avilamycin resistance among *Enterococcus faecium* from broilers, and the consumption of the growth promoter avilamycin in animals, Denmark (DANMAP 2002, 2003).

Possibly the most commonly suggested link between the use of AGPs in livestock and the increase in resistance in human pathogens is the occurrence of vancomycin resistant enterococci (VRE) in hospitals and in the general population (Collignon 1999; Revington 2002). Several reports have demonstrated antibiotic resistance of these organisms to commonly used AGPs (Barton & Wilkins 2001; Mathew 2003).

Despite this apparent controversy on the benefits of a total ban on the use of AGPs in animal feed, it seems to be highly unlikely that any banned antibiotic will be reintroduced.

2.2 The ban of antibiotic growth promoters

The animal feed industry worldwide has been using antibiotics for over 50 years. In 1946 it was reported that the inclusion of antibiotics in chicken feed resulted in increased weight gain (Moore et al. 1946). Soon after, the advantages of antibiotics in animal feed were officially recognised by the US Food and Drug Administration (FDA) and in 1951 the FDA approved the use of antibiotics in animal feed without veterinary prescription (Jones & Ricke 2003). Antibiotics may still be used in farm animals at therapeutical levels to control actual disease and at subtherapeutical levels to promote growth and feed efficiency. The first step towards re-evaluating the role of antibiotics as growth promoters was made by the Swann Committee in 1969 (Swann Committee 1969). This Committee initiated restrictions on the use of AGPs without veterinary prescriptions. More recently, the use, and possible over-use, of antibiotics in animal feed has been widely discussed in scientific literature, at scientific meetings and in the general press. The main concern is the possibility of the emergence of so-called superbugs, antibiotic resistant human pathogens, after the prolonged use of antibiotics in animal feed (Phillips 1999).

As a result of increased pressure from consumer groups to further reduce AGPs in animal feed, Sweden was the first country to implement a partial ban on the use of AGPs in farm animals in 1986. Sweden was joined by the European Union (EU), which placed a partial ban on the use of AGPs in 1997. This will be replaced in 2006 by the general ban on the use of all AGPs (including ionophore anti-coccidials) in all animal feed. In the US one of the largest purchasers of meat, McDonald's Corporation, has announced that it will only use meat produced by retailers or fast food chains that

have adopted a policy that prohibits its supplier from using medically important antibiotics as growth promoters (McDonald's Corporation 2004). Some groups heavily criticise the total ban on AGPs since the ban itself follows a “precautionary principle” rather than scientific fact (Schaffer 2004). Despite this, it appears to be inevitable that we will face increasing restrictions on the use of antibiotics as growth promoters in the future. In advance of this, the animal industries have initiated programs to develop alternate strategies to maintain current standards of animal production, health and welfare.

2.3 Intestinal flora in broiler chickens

The intestinal tracts of a newly hatched chick are sterile but within a few hours of hatching the first organisms appear. Interestingly the first organisms that are present include *E. coli, C. perfringens* and *Streptococci* depending on the cleanliness of the environment (Woolcock 1979). Compete colonisation of the intestine can take up to six weeks and is a fine balance between all the organisms present (Barnes et al. 1972). Conditions in the intestine range from a relatively aerobic environment in the crop and duodenum to strictly anaerobic in the caeca. Initially the small intestine is colonised by *E. coli, Lactobacillus, Streptococcus* and *Enterococcus* (Mead 2000). Although obligate anaerobes (anaerobic cocci, *Clostridium*) may also be present, in the first weeks the oxygen concentration is generally too high for their rapid proliferation. This may explain the fact that the occurrence of NE in birds younger than two weeks is rare. In the presence of these facultative anaerobes, conditions become more anaerobic, which makes it possible for *C. perfringens* to proliferate. The bacterial populations in the intestine reach a stable balance within two to three weeks after hatching. In contrast, the development of the microflora in the caeca can take up to 30 days and changes can occur up to six weeks after hatching (Barnes et al. 1972). The caeca are the main place of bacterial fermentation in the chicken. Dominant bacteria in caeca are obligate anaerobes and can reach up to 10^{11} organisms/g of caecal contents (Barnes et al. 1972). One of the biggest problems in evaluating the composition of the microflora of broilers is the fact that most bacterial species cannot be cultivated outside the intestine on conventional growth media. Recent advances in ribosomal ribonucleic acid (RNA) molecular techniques have made it possible to detect a wide range of bacteria.

The microfloral populations depend very much on the balance between communities of organisms and the diet composition, the main factor influencing the intestinal microflora. Because only between 10% and 60% of the intestinal bacteria can be grown in culture it is impossible to link changes in the bacterial populations with a specific dietary ingredient (Apajalahti 1999). Despite it is almost impossible to comprehensively measure the changes in the microflora, it is possible to measure changes in the products of bacterial metabolism or changes in the number of the culturable and identified isolates. For example, Wagner and Thomas (1978) reported that the inclusion of rye significantly increased butyric acid concentration and gas production in the small intestine. However, when an antibiotic such as penicillin was added to the diet the butyric acid and gas-producing components were removed. The authors concluded that the penicillin sensitive, gas and butyric acid-producing bacterium is most likely a *Clostridium*.

2.4 Manipulation of the intestinal microflora without antibiotic growth promoters

Antibiotic growth promoters work primarily by reducing the microbial load in the intestine. In the absence of microflora, the demand on nutrients to maintain intestinal tissue and the immune system is reduced, and hence more nutrients are available to the bird for growth and production. It is known that germ-free animals have increased performance parameters compared to ‘conventional’ animals (Muramatsu et al. 1994; van Kessel 2004). The key to successful animal production without AGPs is clearly the control and maintenance of a healthy and diverse gut microflora. Reports in the literature emphasise that the incidence of clostridial infections is significantly higher in birds fed diets based on wheat, barley, oats or rye containing high levels of indigestible soluble non-starch polysaccharides (NSP) which lead to increased digesta viscosity and decreased digesta passage rate and nutrient digestibility (Choct et al. 1996). A highly viscous intestinal environment will increase the proliferation

of facultative anaerobes like gram-positive cocci and enterobacteria (Vahjen et al. 1998). Larger amounts of undigested material in the small intestine together with a slower flow of digesta increases the chances of rapid bacterial colonisation. Pluske (2001) showed that the incidence of porcine intestinal spirochaetosis (PIS), swine dysentery (SD) and post weaning diarrhoea is closely related to the amount of indigestible starch and NSP in the diet and the proliferation of pathogenic bacteria in the intestine. Similarly, the use of poorly digestible protein sources alters the microflora and creates favourable conditions in the intestine for the proliferation of pathogens. The bacterial populations depend very much on the balance between communities of organisms and the diet composition as the source of available substrates for microorganisms. Thus, it is often the case that the colonisation of the gut by potential pathogens is greatly reduced when animals are fed a highly digestible and balanced diet according to their nutrient needs.

2.5 Effects of dietary ingredients on the proliferation of *C. perfringens* in the intestine and the occurrence of necrotic enteritis

Knowing that specific feed ingredients can influence the intestinal microflora is a powerful tool in the formulation of feed rations without AGPs. In addition, a number of possible feed supplements have been identified that specifically alter the intestinal microflora and eliminate potential pathogens.

The use of different feed ingredients will affect the environment in the intestine and subsequently influence the gut microflora. In order to find successful nutritional strategies to reduce the risk of NE a broad understanding of the composition of feed ingredients and their effect on the microbial populations, in particular the growth of *C. perfringens*, is necessary.

2.5.1 Maize

Maize is considered an excellent ingredient in broiler diets due to its high-energy content and high nutrient availability. Broiler diets high in maize may also help reduce the incidence of NE in comparison to diets based on wheat or barley (Kaldhusdal & Løvland 2000). In an observational study attempting to link the incidence of NE with the cereal content of broiler diets in Norway, Kaldhusdal and Skjerve (1996) found a close association between the ratio of wheat and barley to maize and the outbreak of NE. These authors showed that in periods with higher inclusion of maize over wheat or barley the occurrence of NE was reduced. Interestingly, the inclusion of maize in broiler diets was also linked to a reduced susceptibility to coccidiosis (Williams 1992).

2.5.2 Wheat, barley and rye

Current research emphasises that the incidence of NE is significantly higher in broilers fed diets based on wheat, barley, oats or rye compared to diets based on maize (Kaldhusdal & Hofshagen 1992; Riddell & Kong 1992). Data by Choct and Sinlae (2000) confirmed that in three-week old broilers one day after the inclusion of a wheat-based diet the numbers of total anaerobes and in particular, the number of *C. perfringens* significantly increased. It is well known that these cereal grains contain high levels of indigestible water-soluble NSP (Annison & Choct 1991). The ingestion of high levels of soluble NSP leads to increased digesta viscosity and decreased digesta passage rate and nutrient digestibility (Choct et al. 1996; Hesselman & Åman 1986). A highly viscous intestinal environment will increase the proliferation of facultative anaerobes like gram-positive cocci and enterobacteria (Vahjen et al. 1998) and, in turn, create an environment that can support obligate anaerobes such as *C. perfringens*. The change in the microbial balance in the small intestine will lead to dysbacteriosis, which was defined by Tice (2000) as “the presence of a qualitatively and/or quantitatively abnormal flora in the intestine”. Birds affected with dysbacteriosis have substantially higher numbers of bacteria in the small intestine, in particular, *Clostridium* spp. compared to normal birds (Panneman 2000). Excessive proliferation of *C. perfringens* could lead to an outbreak of subclinical or clinical NE.

2.5.3 Animal protein ingredients

Animal protein ingredients such as fishmeal or meat and bone meal are often associated with an increased risk of NE (Ficken & Wages 1997; Ross breeders 1999). Smith (1965) showed that the increase in the number of *C. perfringens* in the intestinal tract of chickens is directly related to the proportion of meat and bone meal in wheat-based diets. Most current models designed to reproduce NE under experimental conditions are based on diets containing in excess of 25% fishmeal or meat and bone meal or both prior to a challenge with *C. perfringens* (Cowen et al. 1987; Prescott 1979; Truscott & Al-Sheikhly 1977). Challenge models without the inclusion of fishmeal are less successful in reproducing the disease. It can be speculated that the high nutrient density, in particular a high level of protein, alters the microflora and creates a favourable condition in the intestine for the proliferation of *C. perfringens*. Kaldhusdal and Skjerve (1996) found in their survey that the level of animal protein modified the association between cereal type and incidence of NE, as described in the previous section. It is important to note that the inclusion of increased levels of fishmeal alone is no guarantee for the proliferation of *C. perfringens*. The inclusion of 25% fishmeal in a wheat-based diet compared to only 9% fishmeal had no effect on the number or incidence of *C. perfringens* in the period from two to four-and-a-half weeks of age in birds (Barnes et al. 1972). Unfortunately, these authors did not report the crude protein content of the feed. Considering that a marked increase in performance was observed when more fishmeal was added, it is likely that the protein level in the diet with only 9% fishmeal was below the requirement, hence the excess protein was in fact utilised by the chicken and did not change nutrient availability for the microflora.

2.5.4 Vegetable protein ingredients

The association of animal protein ingredients with the increased occurrence of NE together with the general ban on protein sources of animal origin in Europe (Adams 2000) placed great emphasis on vegetable proteins in feedstuffs. Studies *in vitro* showed that in modified growth media some isolated soy protein stimulated the growth of *C. perfringens* (Busta & Schroder 1971). However, there are no reports in the literature that link the occurrence of NE with the inclusion of soybean meal or any other vegetable proteins.

It is known that low molecular weight carbohydrates such as α-galactoside oligosaccharides in vegetable proteins cannot be digested in the small intestine of monogastric animals due to the absence of endogenous α-galactosidase and are subsequently broken down by microbial fermentation in the caeca (Carré et al. 1990). Bacterial degradation of α-galactosidase can lead to increased hydrogen production, impaired utilisation of nutrients and subsequently reduced performance (Saini 1989). Despite the apparent abundance of nutrients for the intestinal microflora, there appear to be no changes in the composition of the microflora (including *C. perfringens*) when birds were fed diets containing up to 80% peas compared to a corn/soy control diet (Brenes et al. 1989).

2.5.5 Physical form of the diet

Only a few studies have investigated the effects of grinding or pelleting on the microbial composition of the intestine. The mortality attributed to NE was significantly higher in birds fed crumbles with hammer-milled wheat compared to roller-milled wheat (Branton et al. 1987). In contrast, Engberg et al. (2002) found no difference in the numbers of *C. perfringens* in the small intestine between diets with hammer milled (fine) or roller milled (coarse) wheat. The same study, however, found that birds fed mash diets had higher counts of *C. perfringens*, total anaerobes and lactobacilli compared to birds fed pellets. These authors concluded that pelleting improved nutrient digestibility hence reduced the amount of available substrate for the microflora.

2.6 Effects of alternatives to antibiotic growth promoters on the proliferation of *C. perfringens* in the intestine and the occurrence of necrotic enteritis

The number of publications on the efficacy of possible replacements of AGPs to control the occurrence of NE has been steadily growing. The mode of action of these supplements can be divided into four basic groups with distinct strategies: (1) improvement of nutrient utilisation by the host (exogenous feed enzymes); (2) stimulation/modulation of the immune system (cytokines, vaccines, gluco- (GOS) and manno-oligosaccharides (MOS)); (3) stimulation or introduction of beneficial bacteria (probiotics or direct fed microbials, fructo-oligosaccharides (FOS)) and (4) direct reduction of pathogens (MOS, organic acids, botanicals and herbs, bacteriocins, antimicrobial peptides, bacteriophages). Within these general categories, there are hundreds of commercial products available claiming to be effective in improving growth performance and animal health. Rosen (2004) proposed a seven-question test with producers that can be used to assess the potential value of an alternative. Two of the central questions in this test are the number of feeding tests conducted and the frequency of positive responses. Many replacement products have only been recently developed and therefore have not been tested under a wide range of conditions.

2.6.1 Enzymes

Today the use of exogenous enzymes has become standard in all poultry feed and their benefits in terms of improved growth performance have been established in over 2500 publications (Rosen 2003). It is well documented that the addition of feed enzymes to diets based on wheat, barley, oat or rye significantly decreases viscosity in the small intestine by partially depolymerising the soluble NSP (Annison & Choct 1993; Bedford & Classen 1992). It has also been demonstrated that the inclusion of xylanase in wheat based diets significantly reduced bacterial populations in the small intestine (Apajalahti 1999; Choct et al. 1999) and in particular the numbers of *C. perfringens* (Choct & Sinlae 2000). The addition of enzyme reduced digesta viscosity in the small intestine and increased nutrient digestion and digesta flow rate which effectively reduced the amount of available nutrients to the microflora (Choct et al. 1999). Despite the profound changes in the intestinal microflora and the apparent reduction in the numbers of *C. perfringens*, the addition of feed enzymes alone cannot provide complete protection against NE (Elwinger & Teglöf 1991; Riddell & Kong 1992). Enzymes will only change the conditions in the intestine but have no direct effect on the growth of *C. perfringens*. Apajalahti and Bedford (2000) suggested further that the depolymerisation of larger arabinoxylans in wheat with xylanase produced xylo-oligomers and xylose that could only be partially utilised by the microflora. Subsequently the total number of bacteria in the ileum was reduced by 60%. However, it has to be mentioned that the inclusion of exogenous enzymes is only useful if the diets contain the specific substrate for the enzyme to work on.

2.6.2 Prebiotics

Unlike exogenous enzymes, prebiotics have little influence on nutrient utilisation. By definition, prebiotics are short chain carbohydrates that are neither hydrolysed nor are they absorbed by the host and therefore are available to the microflora in the intestine (Gibson & Roberfroid 1995). The inclusion of substrates like FOS, TOS or inulin can selectively stimulate the growth of beneficial micro-organisms (bifidobacteria, *Lactobacillus* spp.) in the intestine (Bielecka et al. 2002). Pathogens like *E. coli* or *C. perfringens* are unable to use FOS as an energy source; hence, the number of FOS fermenters will increase. The increase in numbers of these bacteria not only reduces the amount of available substrate to potential pathogens but also decreases pH in the intestine due to increased fermentation and production of volatile fatty acids.

The growth promoting effect of MOS is believed to be based primarily on inhibiting colonisation of pathogenic bacteria by blocking type-1 fimbriae on the bacterial surface (Dawson & Pirvulescu 1999) and improving overall intestinal health by improving gut integrity and modulating the immune system (Davis et al. 2004; Iji et al. 2001). It has also been reported that MOS has a direct influence on nutrient utilisation in the intestine. An increase in specific populations of microbes with enhanced fibre fermentation capacity has been reported in birds fed diets containing MOS (Ferket et al. 2002). The relationship between the modes of action of MOS and its effects on performance and health of animals under a range of conditions has been speculated in a number of recent publications (Hooge 2004a; Hooge 2004b).

2.6.3 Probiotics

In contrast to prebiotics, which stimulate the growth of beneficial bacteria within the host, the concept of probiotics is based on the direct use of live cultures of these bacteria. Micro-organisms used as probiotics in animal nutrition are *Enterococcus* spp., *Saccharomyces* yeast, spore-forming *Bacillus* spp., and to a lesser extent, *Lactobacillus* spp. (Simon et al. 2001). The functional benefits of probiotics include modification of the microflora, influencing of the mucosa permeability, prevention of binding of potential pathogens to intestinal mucosa by blocking binding sides, modulation of the immune system and production of bacteriocins (reviewed by Simon et al. 2001). *In vitro* assays showed that the adhesion of *C. perfringens* could be reduced by lactic acid bacteria (LAB) (Rinkinen et al. 2003). Data on the effects of probiotics in poultry diets are limited, as probiotic research has been targeted primarily for the human food supplement market. Growth studies without disease challenge showed that the inclusion of *Bacillus* coagulans or *Lactobacillus* cultures in broiler diets significantly improved growth performance (Cavazzoni et al. 1998; Jin et al. 1998), and challenge studies with *C. perfringens* and a commercially available probiotic significantly reduced the severity of NE (Hofacre et al. 1998).

Feed ingredients consist of numerous compounds and it is often impossible to directly link a single compound with the overall changes in the microflora. Some Reports from Europe show that it is possible to control the occurrence of NE through nutritional manipulation. However, there are still gaps in understanding how and under what conditions these ingredients will alter the numbers of *C. perfringens*. Necrotic enteritis is a complex multifactorial disease with many unknown factors and future research has to focus on understanding the disease itself and the development of reliable and repeatable disease models to investigate nutritional manipulation in detail.

3. Reproduction of necrotic enteritis under experimental conditions

The experimental reproduction of a potentially fatal disease requires a high level of ethical standards and special animal care. The Animal Ethics Committee of the University of New England approved all experiments covered in this report. Health and husbandry practices complied with the Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council 1990) and the Australian Model Code of Practice for the Welfare of Animals (Standing Committee on Agriculture and Resource Management 1995).

3.1 Influence of *Eimeria* infection on broiler performance and the occurrence of lesions in the small intestine

Two experiments were conducted to examine the possibility of successfully introducing a subclinical *Eimeria* infection in broiler chickens. The objective of the first experiment was to establish to what extent environmental factors such as cleanliness, feed treatment and handling procedures affect the introduction of an experimental *Eimeria* infection. The second experiment examined the necessary

dosage of sporulated oocysts to induce a sub-clinical *Eimeria*l infection resulting in a drop in performance or visible lesions.

3.1.1 Birds, housing and management

Climate controlled rooms located at the University of New England (UNE) were thoroughly cleaned and disinfected with Divason Q-cide to ensure a completely *Eimeria* free environment. Cobb broilers were obtained from a local hatchery (Baiada hatchery, Kootingal) on 2 January 2001 and 30 January 2001, respectively. Chickens were raised from hatch to the start of the experiment in brooders located in climate-controlled rooms at UNE. Access to birds was restricted to nominated personnel to ensure complete bio-security.

The birds were fed commercial starter crumbles (Ridley AgriProducts Tamworth NSW). According to the manufacturer, the crumbles included the commercially available coccidiostats monensin or salinomycin. Prior to usage, the feed was kept in a freezer at –20ºC for a minimum of 72 hours to kill possible occycts in the feed. The brooding temperatures were gradually decreased from approximately 30°C at day-old to 23–25°C at three weeks of age. Water and feed were provided *ad libitum*.

On day 13, chickens were relocated to single bird metabolisable energy cages located in an adjacent room and were given an experimental diet based on corn and soyabean meal (SBM) without added coccidiostat. Feed intake and weight gain were measured over a seven-day experimental period. On days seven and 14 (prior to the *Eimeria* challenge) and on days 21/22 and 23 (after the *Eimeria* challenge) sub-samples of fresh excreta were collected and examined for the presence of oocysts. On day 23, half of the control group and all *Eimeria*-infected birds were killed by cervical dislocation and each intestine examined for possible damage due to the *Eimeria* infection.

3.1.2 Experiment 1 – experimental design

On day 17, all birds were weighed and half (n = 10) were orally infected with 4000 sporulated occysts (*E. acervulina* and *E. brunetti*). All *Eimeria* species for this experiment and all subsequent experiments were kindly prepared by Dr. Glenn Anderson, Queensland Department of Primary Industries and Fisheries, Animal Research Institute, Yeerongpilly, Qld. The control group consisted of 10 birds located in the same room and fed the same experimental diet without a commercial coccidiostat.

3.1.3 Experiment 2 – experimental design

On day 17, all birds were weighed and divided into four treatment groups (control (given AGPs), low, medium, and high). Thirty-six birds were orally infected with three different dosages of sporulated oocysts (*E. acervulina* and *E. brunetti*) – low (4000 sporulated occysts of each), medium (7000 oocysts of each) and high (10 000 occysts of each). The control group consisted of 12 birds located in the same room and fed the same experimental diet without a commercial coccidiostat.

3.1.4 Results

Although there were no significant differences in growth performance measured over a seven-day period, inoculation with 4000 sporulated oocysts each of *E. acervulina* and *E. brunetti* resulted in a subclinical *Eimeria*l infection. All birds challenged with *Eimeria* had medium to heavy shedding of oocysts five to seven days post-infection. Despite the close proximity between the challenged birds and the control, no oocysts were found in any of the control birds.

The challenge of three-week old broiler chickens with 10 000 sporulated oocysts of *E. acervulina* and *E. brunetti* each resulted in a significant drop in liveweight gain, increased feed intake and subsequently a reduced FCR (Table 1). The challenge with lower numbers of oocysts had no effect on weight gain or feed intake, however, FCR was almost linearly affected by the challenge dosage.

Examination of the intestine showed that the increase in inoculated oocysts caused more visible and more severe lesions throughout the intestine.

It was concluded that infection with a dosage of 7000 sporulated oocysts of *E. acervulina* and *E. brunetti* is the desired dosage to cause a subclinical *Eimeria*l infection with visible signs of lesions in the upper and lower intestine but only a small reduction in growth performance.

Table 1. Performance data of birds inoculated with three levels of sporulated oocysts

	Liveweight	Feed	Growth	FCR	Lesions	
	g/bird	g/bird/week	g/bird/week		jejunum	ileum
AGP control	952.3 ± 110.1[a]	812.7 ± 79.7[a]	514.4 ± 81.5[a]	1.600 ± 0.183[c]	-	--+
4000	899.5 ± 76.1[a]	794.6 ± 90.9[a]	455.6 ± 54.1[b]	1.755 ± 0.207[b] [c]	+	-+
7000	925.8 ± 100.5[a]	829.3 ± 64.9[a]	465.5 ± 61.8[ab]	1.799 ± 0.179[b]	++	+
10 000	781.1 ± 85.0[b]	707.9 ± 72.1[b]	350.7 ± 64.9[c]	2.053 ± 0.236[a]	++	++
Mean	889.3	786.7	445.7	1.805		
Source of variance	Probability of greater *F* value in analysis of variance					
Cocci	***	***	***	***		

*P<0.05, **P<0.01, ***P<0.001.

a, b, c Values with different superscripts differ significantly (*P*<0.05).

3.2 Influence of a dual infection with *Eimeria* and *C. perfringens* on broiler performance and occurrence of necrotic lesions

The occurrence of NE is strongly correlated with diet composition. In a survey conducted in Norway Kaldhusdal & Skjerve (1996) found that the incidence of NE between 1969 and 1989 was closely related to the proportion of cereal grains included in the diet, which increased digesta viscosity (wheat, wheat by-products or barley). The same study also found that the incidence of NE was linked to high levels of animal protein in the diet. It is thought that increased digesta viscosity and high levels of protein will alter the microflora and create conditions in the intestine, which allow the proliferation of *C. perfringens*. A more detailed discussion on the effects of dietary ingredients on the occurrence and evidence of proliferation of *C. perfringens* in the intestine is given in Section 2.5 of this report.

The actual cause of NE, however, is not the bacterium itself. It is, rather, believed to be caused by the α-toxin, a phospholipase C, produced by *C. perfringens*. It was found that for *C. perfringens* to produce α-toxin, it requires the presence of zinc in the growth medium (Sato & Murata 1973) and Baba et al. (1992) showed that the highest concentration of phospholipase C was found in a growth medium containing 100 ppm of zinc. However, phospholipase C activity rapidly decreased when the digestive enzyme trypsin was added to the medium. Trypsin activity was partially blocked when zinc was present in the medium at levels higher than 800 ppm. *In vivo* trials on the effect of a dual infection with *C. perfringens* and *E. brunetti* in feed supplemented with zinc at a level of 1000 ppm in the form of $ZnSO_4$ showed that the average lesion score and mortality rate were significantly increased compared to the control diet without added zinc. These findings can be explained by the protective action of zinc on the α-toxin as well as the finding that α-toxin is a zinc metalloenzyme (Titball et al. 1999).

In order to maximise the possibility of successfully establishing an experimentally introduced NE infection, a specific “NE” diet was formulated. The diet was based on high levels of wheat and wheat by-products and high levels of animal protein, in particular fishmeal, to maximise the growth of *C. perfringens* in the small intestine. The inclusion of 1000 ppm of $ZnSo_4$ was designed to maximise toxin production and survival, and hence increase the likelihood of the occurrence of NE lesions. The composition of this diet is shown in Table 2.

A series of experiments were conducted to test the hypothesis that an oral infection with *Eimeria acervulina* and *E. brunetti* (7000 sporulated oocysts each) and the challenge with *C. perfringens* of broiler chickens fed a diet that creates optimal growth conditions for *C. perfringens* and the production of α-toxin will result in the occurrence of clinical or subclinical NE.

3.2.1 Birds, housing and management

Climate controlled rooms located at UNE were thoroughly cleaned and disinfected with Divason Q-cide to ensure a completely *Eimeria* free environment. Cobb broilers were purchased from a local hatchery (Baiada hatchery, Kootingal) between April and September 2001. Chickens were raised from hatch to the start of the experiment in brooders located in climate-controlled rooms. Access to birds was restricted to nominated personnel to ensure complete biosecurity.

Table 2. Composition of diet to maximise growth and toxin production of *C. perfringens*

Main component	Ingredients	%
Cereal component:	Wheat	73.0
	Millrun	2.0
Protein component:	SBM 48	15.0
	Sunflower meal	3.0
	Fishmeal	5.0
Minerals:	Lime	0.5
	Dicalcphosohate	0.6
	Salt	0.1
	Methionine	0.2
	Lysine	0.3
	Premix*	0.2
	Choline Chloride	0.1
	$ZnSO_4$	0.1
Calculated Composition:		
	Crude Protein%	22.2
	Metabolisable Energy MJ/kg	12.2
	Methionine + Cystine %	0.9
	Lysine %	1.1
	Calcium %	0.9
	Available Phosphorous %	0.4

*The active ingredients contained in each kg premix were as follows: retinol 3.03 mg, cholecalciferol 0.09 mg, all-*rac*-α-tocopherol acetate 20 mg, menadione 6.3 mg, riboflavin 8 mg, pyridoxine hydrochloride 5 mg, biotin 0.01 mg, niacin 30 mg, thiamine 1.5 mg, D-calcium pantothenate 15 mg, folic acid 2 mg, ethoxyquin 125 mg, Mn 75 mg, Fe 20 mg, Cu 5mg, I 1 mg, Co 0.3 mg, Se 0.5 mg, Mo 0.16 mg cyanocobalamin 0.15 mg.

To ensure that the diet was free of any *C. perfringens*, the meat and bone meal and the fishmeal were irradiated prior to feed mixing using gamma irradiation at 25 Grays (Steritech, Dandenong).

The birds were given commercial starter crumbles (Ridley AgriProducts, Tamworth NSW). According to the manufacturer's specification, the crumbles included the commercially available coccidiostats monensin or salinomycin. Prior to usage the feed was be kept at -20ºC for a minimum of 72 hours to kill possible oocycts in the feed. The brooding temperature was gradually decreased from approximately 30°C at day-old to 23–25°C at three weeks of age. Water and feed were provided *ad libitum*.

3.2.2 Experiment 3 – experimental design

Thirty six birds were used for this experiment. Birds were raised in multi-tier brooders in a climate-controlled room. On day, 14 all birds were weighed and transferred to 36 single bird metabolisable energy cages located in the same temperature-controlled room.

Birds were allocated to the following three treatments:

1. Control.
2. Eimeria.
3. *Eimeria* + *C. perfringens* (UNE 8–4 isolate).

On day 17, birds in groups two and three were orally infected with 1 ml of solution containing 7000 sporulated occysts each of *E. acervulina* and *E. brunetti* using a commercially available crop needle (size 12). The control group was inoculated with water only. On days 21, 22 and 23 birds in group three were orally infected with 1 ml *C. perfringens* broth culture. Birds in groups one and two received 1 ml of broth without the bacterium.

The strain of *C. perfringens* used in this experiment was isolated from a subclinical case of NE from a previous experiment at the UNE (Choct & Sinlae 2000). Bacterial cultures were suspended in a cryoprotectant storage medium and were stored in a deep freezer (-40°C). On days 20, 21 and 22 (one day before the inoculation) 100 ml of a broth culture (basal medium 10 with 0.5% glucose) containing approximately 10^6–10^8 colony forming units (CFU) of *C. perfringens* was prepared.

3.2.3 Experiment 4 – experimental design

A total of 40 birds were used for this experiment. The management of birds and the challenge procedures for this experiment are described in Section 3.2.2.

On day 17, birds in groups two and three were orally infected with 1 ml of solution containing 7000 sporulated oocysts each of *E. acervulina* and *E. brunetti* using a commercially available crop needle (size 12). The control group was inoculated with water only. On days 21, 22 and 23 birds in group three were orally infected with 1 ml of *C. perfringens* broth culture. Birds in groups one and two received 1 ml of broth without bacteria.

Chickens were allocated to six treatment groups:

4. Control.
5. *C. perfringens* (UNE 8-4 isolate).
6. *C. perfringens* (CSIRO R61 isolate).
7. Eimeria.
8. *Eimeria* + (UNE 8-4 isolate)
9. *Eimeria* + (CSIRO R61 isolate).

One strain of *C. perfringens* (UNE 8-4 isolate) used in this experiment was from the same stock culture as in the previous experiment (3.2.2). The strain *C. perfringens* CSIRO R61 was obtained from the CSIRO Australian Animal Health Laboratory at Geelong. The strain was isolated from a clinical field case of NE in Victoria in 1999. Previously this strain had been used successfully to introduce a clinical form of NE under experimental conditions (Chang, personal communication).

Strain UNE 8-4 was transferred several times (exact number unrecorded) between the actual isolation from the infected bird and the preparation of the storage medium whereas strain CSIRO R61 was transferred only once after the isolation from an infected bird that had a NE lesion score of 4. Bacterial

broth cultures for both strains were prepared using a commercially available growth medium (Thioglycollate broth USP – Alternative CM0391, Oxoid, Heidelberg, Victoria, Australia).

3.2.4 Results and discussion

It is widely recognised that coccidiosis is a major disease problem worldwide for the poultry industry. Damage to the intestine and reduced feed efficiency can cost the poultry industry in excess of US$1.5 billion per annum (Lillehoj 2000). Furthermore, it is also well established that damage to the intestine initiated by coccidial infections will increase the risk of the occurrence of necrotic enteritis. Baba et al. (1992, 1997) found that birds infected with *E. brunetti* or *E. necatrix* are more likely to have increased numbers of *C. perfringens* in the intestine compared to birds free of coccidiosis.

Due to technical difficulties in the cultivation of *C. perfringens*, it was impossible to enumerate *C. perfringens* in the intestine of birds. It appears that the infection with 7000 sporulated oocysts each of *E. acervulina* and *E. brunetti*, in combination with an oral challenge with *C. perfringens*, results in a significant reduction in weight gain and feed efficiency ($P<0.05$; Tables 3 and 4). The loss in growth performance was more profound when birds were challenged with *C. perfringens* isolated from a clinical case of NE (CSIRO R61), rather than with a strain isolated from a subclinical case of NE (UNE8-4). These findings tend to suggest that the type of *C. perfringens* used under experimental conditions has a major impact on the growth performance of infected broilers and the reproducibility of the disease. The challenge of three week old broilers with *C. perfringens* without a prior *Eimeria* challenge also resulted in a significant reduction in growth performance ($P<0.05$). Although there was no direct evidence that the depression in growth performance is linked to the proliferation of *C. perfringens*, it has to be assumed that the experimental challenge with *C. perfringens* has changed the overall microflora. It is known that disturbances caused by bacterial challenge, toxins or feed factors can lead to the development of the disbacteriosis complex (Mortimer 2002). This complex is defined as the presence of an abnormal and unhealthy microflora in the small intestine resulting in depressed growth and feed efficiency.

Despite the specially formulated diet designed to maximise the potential for the colonisation of *C. perfringens* as well as the production of α-toxin, no bird died as a result of NE. Although there are a number of procedures for the experimental introduction of NE described in the literature, it is widely acknowledged that other factors, such as the physical form of the diet, the temperature or intestinal viscosity, will contribute to the occurrence of the disease.

Table 3. Growth performance data of birds experimentally infected with *Eimeria* and *C. perfringens*

	Weight start day 17 g/bird			Weight before CP infection day 21 g/bird			Weight end of experiment g/bird			Feed intake g/bird			FCR		
AGP control	441.1	±	19.1	790.2	±	41.4	1369.2	±	98.4[a]	1452.2	±	99.5	1.575	±	0.096[a]
Eimeria	442.2	±	32.1	786.9	±	48.4	1297.0	±	120.3[ab]	1382.9	±	157.9	1.641	±	0.153[ab]
UNE8-4	444.2	±	29.5	777.7	±	61.5	1195.0	±	77.6[b]	1326.4	±	64.1	1.779	±	0.133[b]
Mean	442.5			784.9			1287.0			1384.4			1.670		
Source of variance	Probability of greater *F* value in analysis of variance														
Diet	NS			NS			**			NS			**		

*P<0.05, **P<0.01,***P<0.001.

a, b Values with different superscripts differ significantly (*P*<0.05).

Table 4. Growth performance data of birds experimentally infected with *Eimeria* and two strains of *C. perfringens*

	Weight start day 17	Weight before CP infection day 21	Weight end of experiment	Feed intake	FCR
	g/bird	g/bird	g/bird	g/bird	
AGP control	379.7 ± 41.1	689.2 ± 72.7	1200.4 ± 111.0[a]	1507.1 ± 159.1	1.837 ± 0.127[c]
CP UNE 8-4	365.3 ± 27.8	661.5 ± 51.1	1150.9 ± 56.1[ab]	1427.2 ± 60.8	1.821 ± 0.098[c]
CP CSIRO R61	375.4 ± 29.2	668.1 ± 51.4	1128.2 ± 80.2[ab]	1449.0 ± 139.3	1.930 ± 0.133[bc]
Eimeria	379.9 ± 45.1	697.0 ± 62.5	1076.2 ± 77.5[b]	1494.1 ± 216.8	2.142 ± 0.185[b]
Eimeria + 8-4	395.2 ± 28.8	687.2 ± 35.4	1080.9 ± 67.1[b]	1420.8 ± 90.6	2.087 ± 0.243[b]
Eimeria + R61	389.1 ± 40.2	678.4 ± 59.3	924.3 ± 87.0[c]	1289.6 ± 131.3	2.438 ± 0.257[a]
Mean	380.6	678.9	1087.9	1423.4	2.050
Source of variance	Probability of greater *F* value in analysis of variance				
Eimeria	NS	NS	***	NS	***
CP	NS	NS	*	NS	**
Eimeria × CP	NS	NS	NS	NS	NS

*P<0.05, **P<0.01,***P<0.001.

a, b, c Values with different superscripts differ significantly (P<0.05).

3.3 Experimental model for inducing clostridial enteritis in broilers

Experiments described in the previous sections investigated a number of possible predisposing factors for the experimental reproduction of a sub-clinical clostridial infection. Although it has been established that the challenge with two strains of *Eimeria* will result in the occurrence of lesions in the small intestine, thus far no reliable model for the successful and repeatable introduction of a sub-clinical clostridial infection has been found. One of the key factors in any experimental challenge model is the subtype of *C. perfringens.* It has been shown that healthy broilers can carry two to three different subtypes of *C. perfringens* whereas *C. perfringens* isolated from diseased birds belongs to a specific subtype (Engström et al. 2003). In addition, anecdotal evidence suggests that a mild coccidial infection can, in fact, prevent the rapid proliferation of *C. perfringens* (Williams 1999).

Although there is no doubt that severe coccidiosis is a strong precursor for the development of clinical NE, the role of *Eimeria* as a predisposing factor to experimental reproduction of subclinical NE infection needs further examination.

Two experiments examined the role of a dual infection model with *Eimeria* spp. and *C. perfringens* in the successful reproduction of subclinical NE. The objective of these experiments was to find a suitable working model for the subsequent test of alternative feed additives to control the proliferation of *C. perfringens* in the intestine of broiler chickens.

3.3.1 Birds, housing and management

Climate controlled rooms located at UNE were thoroughly cleaned and disinfected with Divason Q-cide to ensure a completely *Eimeria* free environment. Single sex (male) or mixed sex Cobb broiler chickens were purchased from a local hatchery (Baiada hatchery, Kootingal) between October 2001 and April 2002. Chickens were raised from hatch to the start of the experiment in small multi-tier brooders located in climate-controlled rooms at UNE and fed a commercial broiler starter diet (Ridley AgriProducts, Tamworth NSW). Access to birds was restricted to nominated personnel to ensure complete biosecurity.

3.3.2 Experiment 5 – experimental design

A total of 144 male Cobb broiler chicks were used in this experiment. On day five all birds were weighed and diets were changed to the experimental NE diet (described in Section 3.2.1).

A 2 × 2 factorial design was used to establish the effect of a *Eimeria*l infection prior to the infection with *C. perfringens* on the severity of the NE infection. Chickens were allocated to the following four experimental treatment groups:

1. Control = C.
2. Control plus *C. perfringens* strain R61 = C +CP61.
3. *Eimeria* control (infection with 10000 sporulated oocysts each of *E. acervulina* and *E. brunetti*) = Cocci Control.
4. *Eimeria* plus *C. perfringens* strain R61 = Cocci + CP61.

On day eight, treatment groups three and four were orally infected with 1 ml diluted dicalcium chromate solution containing 10000 sporulated oocysts each of *E. acervulina* and *E. brunetti*. Treatment groups one and two were administered with 1 ml of diluted dicalcium chromate solution.

On days 11, 12 and 13, treatment groups two and four were orally infected with 1 ml of a *C. perfringens* culture (approx. 10^9 CFU/ml). Two days prior to the inoculation of the birds (day 9) a master culture of *C. perfringens* broth was prepared using 0.1 ml of a bacterial stock culture (stored at -40°C). The stock culture was incubated for 16 hours at 37°C and stored in the fridge (4°C) for the duration of the experiment. On days 10, 11 and 12, respectively, 5 ml of *C. perfringens* broth from the master culture was used to prepare 500 ml of fresh thioglycollate broth. Each batch was grown at 37°C for 20 hours and contained approximately 10^8 CFU of *C. perfringens.*

On day 13, birds were relocated to 72 metabolisable energy cages (two birds per cage) located in three adjacent rooms. At this point the number of replicates was increased to 18 per treatment. Treatment groups were distributed throughout the three rooms using a randomised block design. On days 22, 27 and 32, eight birds from each treatment group (four replicates each) were killed by cervical dislocation. Swab samples from the duodenum/jejunum junction were taken and plated onto commercially available agar specific for the growth of *C. perfringens* (OPSP agar, CM543, Oxoid, Heidelberg). The occurrence of *C. perfringens* in the upper intestinal tract was recorded using a scale of 0–3. Score 3 indicates massive bacterial growth (excess of 10^4 CFU/plate), whereas score 0 indicates no growth. Jejunum and ileum were incised longitudinally and examined for evidence of necrotic lesions and inflammation of the intestine (0 = no lesions, no inflammation, 1 = slight redness of the intestine, 2 = small lesions, red intestine, 3 = visible gross lesions).

Between days 22 and 26, apparent metabolisable energy (AME) of the experimental diets was determined using 40 cages (10 replicates/treatment). Feed intake was measured and all excreta voided were collected daily. The excreta were dried in a fan-forced oven at 80ºC for 36 hours and excreta from each replicate were pooled over the collection period for determination of gross energy (GE). The moisture content of the excreta voided was measured and AME value of the diets was calculated using the following equation:

$$\text{AME diet} = \frac{(\text{g feed eaten x GE feed}) - (\text{g excreta voided x GE excreta})}{\text{g feed eaten}}$$

3.3.3 Experiment 6 – experimental design

Sixty four male and 64 female Cobb broiler chicks were used in this experiment. The same design was used as in the previous experiment to establish the effect of a coccidial infection prior to the infection with *C. perfringens* on the severity of the NE infection (Section 3.3.2). However, the sex of the birds was included as an additional factor to establish differences in the susceptibility to experimentally introduced NE infections of male versus female birds.

On day five, all birds were weighed and feed was changed to the experimental NE diets. Birds were relocated to ‘single-bird’ metabolisable energy cages fitted with special inserts for small birds (two birds per cage) and were allocated to four experimental treatments (eight replicates each of males females/treatment). Each treatment group was located in a separate room in order to minimise the likelihood of cross-contamination between the four treatments.

The same experimental challenge procedure was used as described in Section 3.3.2.

On days 17, 21 and 28, eight birds from each treatment group (four replicates; two replicates each of male and female) were killed by cervical dislocation. Tissue samples from the mid section of the jejunum (2 cm above the Meckel’s diverticulum) were collected and placed in 10% buffered formalin for histology examination. Histological examinations were carried out at the Flinders University under the supervision of Dr. Chris Lunam (Sensory Nervous System Laboratory, Anatomy and Histology, Bedford Park, South Australia) using standard procedures for the preparation of histological cuts with haematoxylin and eosin stains. Swab samples from the duodenum/jejunum junction were taken and plated onto commercially available agar specific for the growth of *C. perfringens* (OPSP agar, CM543, Oxoid, Heidelberg). The occurrence of *C. perfringens* in the upper intestinal tract was recorded using a

scale of 0–3. Score 3 indicates massive bacterial growth (in excess of 10^4 CFU/plate), whereas score 0 indicates no growth. The jejunum and ileum were incised longitudinally and examined for evidence of necrotic lesions and inflammation of the intestine (0 = no lesions, no inflammation, 1 = slight redness of the intestine, 2 = small lesions, red intestine, 3 = visible gross lesions). Digesta samples from the jejunum (day 21 only) were collected for the determination of intestinal viscosity. Digesta were kept on ice prior to centrifugation (12000 *g*, 10 minutes, 4°C). Supernatant and pellets from each pooled sample were frozen and stored at -20ºC pending analyses. Viscosity of thawed supernatants was measured with a Brookfield DVIII viscometer at 25ºC with a CP40 cone and shear rate of 5-500 s^{-1}. The samples did not exhibit shear thinning at these shear rates. The samples were re-constituted, freeze-dried, and finely ground.

On day 21 excreta were collected and the moisture content of the excreta voided was determined.

3.3.4 Results

Growth performance of broiler chicks challenged with 7000 sporulated oocysts each of *E. acervulina* and *E. brunetti* alone or in combination with an oral challenge of *C. perfringens* resulted in a marked reduction in weight gain and feed efficiency (Figures 2–5 and Tables 5 and 8). Growth performance and severity of the experimental challenge were not influenced by the sex of the birds. Despite the challenge with either *C. perfringens* alone or the combination of *C. perfringens* and *Eimeria*, no bird died as a result of necrotic enteritis. Furthermore, no treatment resulted in the occurrence of visible gross lesions in the intestine of birds. However, the intestine of birds infected with *Eimeria* alone or in combination with *C. perfringens* had increased mucosal secretion and redness compared to that of the control birds (Tables 7 and 10). The inoculation with *Eimeria* or *C. perfringens* had no significant effect on excreta moisture, AME or intestinal viscosity (Tables 6 and 9). Histological examination of the small intestine showed minor differences between unchallenged and challenged birds (Figure 6). Birds challenged with *C. perfringens* had a greater occurrence of sloughing of the epithelium near tips of villi and inflammatory cells. The *Eimeria* alone did not affect bird performance.

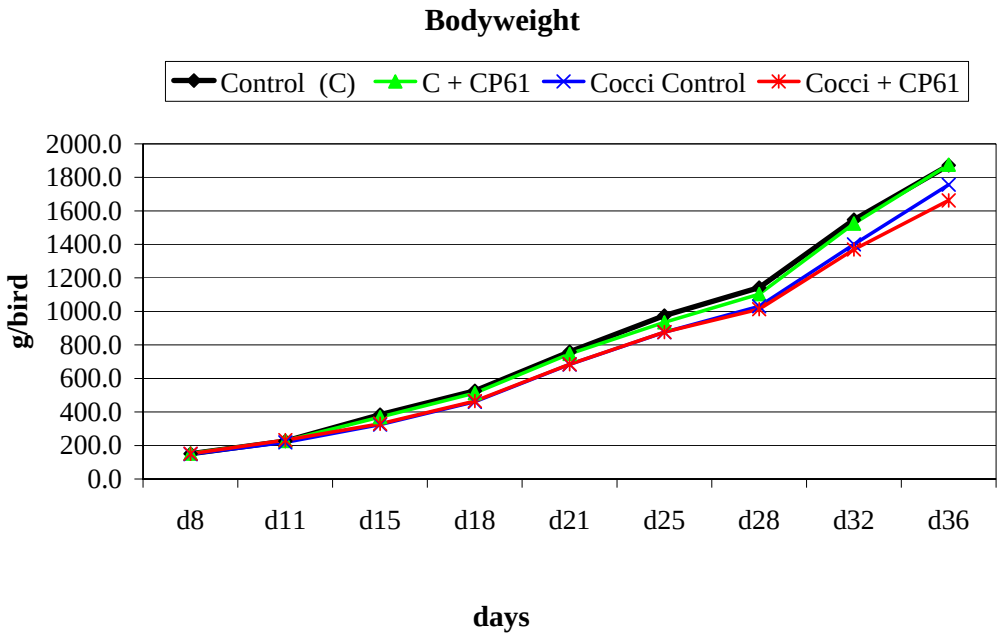


Figure 2. Development of bodyweight of birds experimentally infected with *Eimeria* and *C. perfringens* (CP)

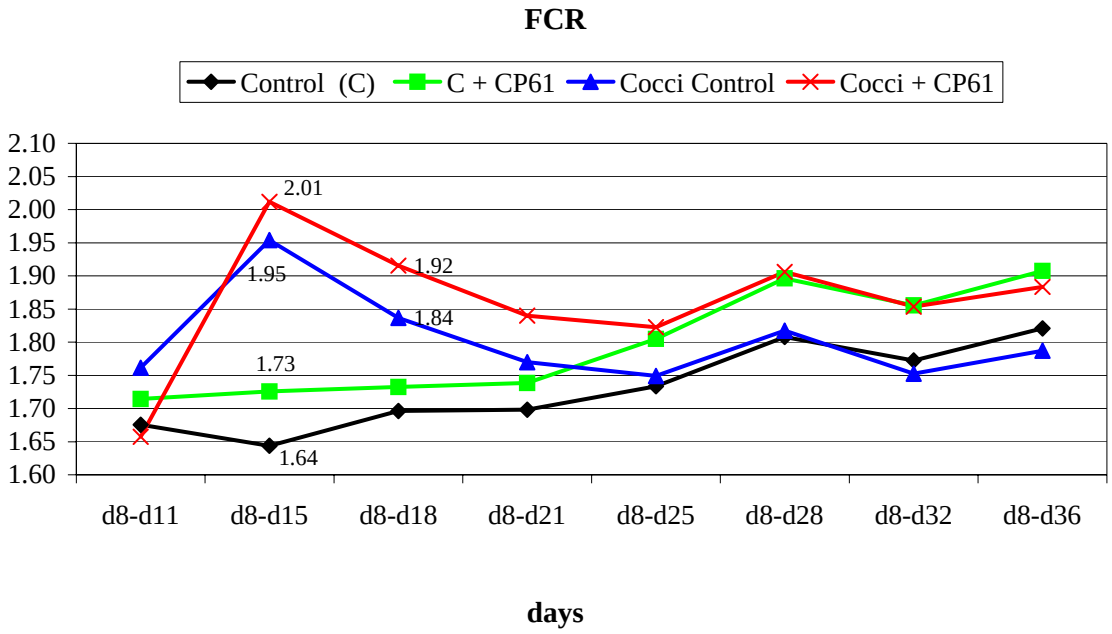


Figure 3. Change in feed conversion ratio (8–36 days) of birds experimentally infected with *Eimeria* and *C. perfringens* (CP)

Table 5. Growth performance data of birds experimentally infected with *Eimeria* and *C. perfringens* (CP)

Diet	Weight after CP infection day 13 g/bird	Weight at end of experiment day 32 g/bird	Feed intake day 5–day 32 g/bird	FCR day 5–day 32
AGP control	385 ± 25[a]	1548 ± 83[a]	2415 ± 175[a]	1.77 ± 0.09
CP61	370 ± 23[a]	1524 ± 83[a]	2497 ± 97[a]	1.86 ± 0.09
Cocci control	324 ± 23[b]	1400 ± 93[b]	2129 ± 107[b]	1.75 ± 0.03
Cocci + CP61	330 ± 21[b]	1369 ± 98[b]	2201 ± 104[b]	1.85 ± 0.10
Mean				
Source of variance	Probability of greater *F* value in analysis of variance			
Diet	***	***	*	NS
Cocci	***	***	**	NS
CP	NS	NS	NS	NS
Cocci × CP	0.07	NS	NS	NS

*P<0.05, **P<0.01,***P<0.001.

a, b Values with different superscripts differ significantly (P<0.05).

Table 6. Apparent metabolisable energy of diets and excreta moisture in birds experimentally infected with *Eimeria* and *C. perfringens* (CP*)*

Diet	Excreta moisture %	AME MJ/kg DM
AGP control	67.79 ± 2.96	10.62 ± 0.46
CP61	66.58 ± 4.16	10.82 ± 0.47
Cocci control	66.42 ± 3.78	11.00 ± 0.27
Cocci + CP61	65.90 ± 3.24	10.70 ± 0.54
Mean	67.18	10.78

Table 7. Lesion score and occurrence of *C. perfringens* in birds experimentally infected with *Eimeria* and *C. perfringens* (CP)

Diet	Lesion score[1]		Occurrence of *C. perfringens**
	Jejunum	Ileum	Jejunum
AGP control	0.59 ± 0.62	0.61 ± 0.49	0.14 ± 0.18
CP61	0.74 ± 0.56	0.88 ± 0.51	0.18 ± 0.25
Cocci control	0.74 ± 0.47	0.89 ± 0.49	0.10 ± 0.00
Cocci + CP61	1.10 ± 0.51	0.92 ± 0.40	0.25 ± 0.34
Mean	0.79	0.82	0.17

1. Lesion scoring: 0 = no lesions, no inflammation, 1 = slight redness of the intestine, 2 = small lesions, red intestine, 3 = visible gross lesions.

* The occurrence of CP was determined using a scale 0-3 with 0 being no growth to 3 being massive growth of CP.

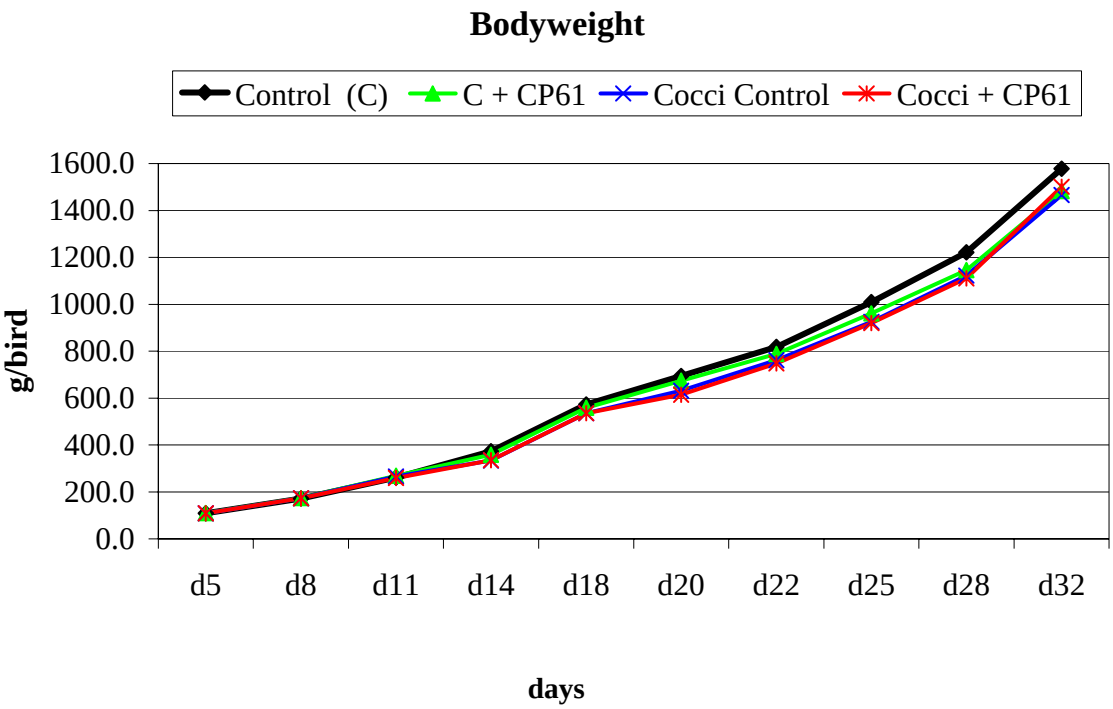


Figure 4. **Development of bodyweight of birds experimentally infected with Eimeria and C. perfringens (CP)**

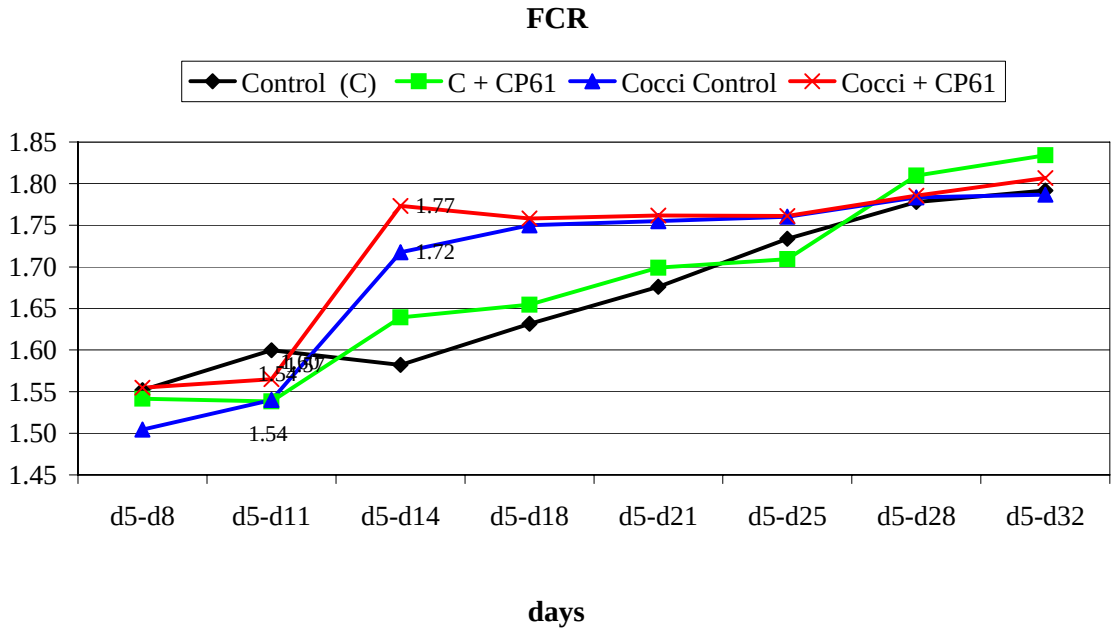


Figure 5. **Change in feed conversion ratio (8–36 days) of birds experimentally infected with *Eimeria* and *C. perfringens* (CP)**

Table 8. Growth performance data of birds experimentally infected with *Eimeria* and *C. perfringens* (CP)

	Sex	Weight after CP infection day 13 g/bird	Weight at end of experiment day 32 g/bird	Feed intake day 5–day 32 g/bird	FCR day 5–day 32
AGP control	M	385.8 ± 19.4	1664.7 ± 88.9	2804.9 ± 113.4	1.829 ± 0.064
AGP control	F	360.2 ± 24.2	1447.7 ± 26.2	2405.2 ± 85.0	1.830 ± 0.063
Control + CP61	M	369.0 ± 23.8	1571.2 ± 130.5	2615.7 ± 168.0	1.879 ± 0.133
Control + CP61	F	347.7 ± 25.8	1375.5 ± 64.1	2349.0 ± 98.1	1.898 ± 0.055
Cocci control	M	331.9 ± 28.1	1596.4 ± 48.5	2543.2 ± 66.2	1.753 ± 0.018
Cocci control	F	335.6 ± 28.3	1363.6 ± 70.9	2317.9 ± 144.4	1.836 ± 0.069
Cocci + CP61	M	356.4 ± 26.6	1573.2 ± 138.0	2634.8 ± 197.4	1.788 ± 0.046
Cocci + CP61	F	314.5 ± 27.1	1392.1 ± 115.0	2273.3 ± 81.1	1.855 ± 0.125
Mean		350.1	1530.7	2493	1.834

Table 9. Jejunal digesta viscosity and excreta moisture in birds experimentally infected with *Eimeria* and *C. perfringens* (CP)

	Excreta moisture day 21 %			Viscosity on day 21 mPa.s		
AGP control	72.6	±	2.0	6.7	±	2.0
CP61	73.2	±	2.7	10.0	±	4.9
Cocci control	73.4	±	2.6	7.0	±	1.4
Cocci + CP61	73.3	±	2.5	7.0	±	2.3
Mean	73.13			7.46		

Table 10. Lesion score and occurrence of *C. perfringens* in birds experimentally infected with *Eimeria* and *C. perfringens*

	Lesion score[1.] Jejunum			Lesion score[1.] Ileum			Occurrence of *C. perfringens* Jejunum		
AGP control	0.39	±	0.76	0.69	±	0.70	0.33	±	0.31
CP61	0.51	±	0.55	0.75	±	0.49	0.47	±	0.41
Cocci control	0.48	±	0.58	0.90	±	0.46	0.32	±	0.35
Cocci + CP61	0.55	±	0.58	1.19	±	0.57	0.55	±	0.46
Mean	0.49			0.89			0.42		

1. Lesion scoring: 0 = no lesions, no inflammation, 1 = slight redness of the intestine, 2 = small lesions, red intestine, 3 = visible gross lesions.
* The occurrence of CP was determined using a scale 0-3 with 0 being no growth to 3 being massive growth of CP.

Control *C. perfringens* infected

Figure 6. Effect of experimental challenge with *C. perfringens* on morphological development of jejunum at 22 or 28 days of age

3.4 Discussion

The description of the pathogenesis of a subclinical NE infection varies widely. In general only a small number of birds (less than 1%) will show the classical signs of the disease. It has been recognised that even minor damage to the intestine as a result of the proliferation of *C. perfringens* can reduce weight gain by more than 200 g (van der Sluis 2000a) and reduce feed efficiency at 35 days by up to 5% (Kaldhusdal & Løvland 2000). Often the subclinical form of the disease is not recognised and remains untreated. Under the present Australian conditions, the use of AGPs such as zinc-bacitracin and/or avilamycin is a practical tool in controlling the proliferation of *C. perfringens* and protecting producers from potential losses from the disease. When looking for a potential alternative to AGPs, it has to be recognised that these products must be able to control the subclinical form of the disease and not necessarily its clinical form.

The lack of reliable experimental models hampers the search for such alternatives. Although there are a large number of possible models reported in the scientific literature, it is questionable how repeatable these models are outside the environment in which they have been tested. Most models rely on a dual infection with *Eimeria* followed by an oral challenge with a large dosage of *C. perfringens*. Furthermore, diets with a high proportion of fishmeal or a protein content above the actual requirements of the chicks appears to be beneficial for the experimental reproduction of NE. Most NE models are in essence based on the model developed by Truscott and Al-Sheikhly (1977). Under this model, birds are raised in brooders and are fed a high protein turkey starter diet between 0–14 days of age. On day 15, feed is withdrawn for 20 hours and over the next three days the birds are given 10 g of a slurry of feed and thioglycollate broth containing approximately 10^7 CFU of *C. perfringens* Type A. According to the original report by Truscott and Al-Sheikhly (1977), this model would result in NE related mortalities of 8–19%. Versions of this model include the dual challenge with *Eimeria* species (*E. acervulina, E. maxima, E. brunetti* or *E. necatrix)* (Baba et al. 1992; Branton et al. 1997; Shane et al. 1985) or the use of a highly artificial diet containing a 1:1 mixture of turkey starter and fishmeal (Brennan 2000; Cowen et al. 1987; Prescott 1979). Some studies included a direct oral challenge with *C. perfringens* (George et al. 1982; Hofacre et al. 2003) or the use of floor pens with old litter (Wicker et al. 1977). Under experimental conditions, there is no clear indication which of the above models is the most successful in reproducing the disease. Mortality and morbidity rates vary widely, between 0% (Baba et al. 1992) and over 60% (Hofacre et al. 2003).

In order to successfully test any alternatives to AGPs the first step is to find a reliable and reproducible model for a subclinical clostridial infection. In the current study, a number of experiments have been conducted to investigate the effects of a *C. perfringens* strain and *Eimeria* challenge on the development of the disease using some of the models mentioned above. None of the chosen procedures resulted in death or visible gross lesions in the intestine of birds, although the intestine of birds infected with *Eimeria* alone or in combination with *C. perfringens* had increased mucosal secretion and redness compared to that of the control birds. There is no clear trend whether the dual infection with *Eimeria* and *C. perfringens* is more successful in reproducing the disease compared to an infection with *C. perfringens* alone. Both treatment groups showed reduced growth performance compared to the control. Results would also suggest that birds were severely affected by the challenge with *Eimeria* alone. Based on the four experiments, it is difficult to attribute the loss in growth performance to the challenge with *Eimeria* or the combined challenge with *Eimeria* and *C. perfringens*.

The inclusion of a high proportion of wheat in the diet and the occurrence of coccidiosis are recognised as important precursors to the development of NE. Mucosal damage to the small intestine combined with high digesta viscosity caused by the presence of soluble NSP in wheat will lead to increased bacterial activity in the small intestine (Choct et al. 1996), creating an environment conducive for the growth of *C. perfringens.* It is of interest that coccidial infections have a marked impact on digesta viscosity. Waldenstedt et al. (2000) have found that coccidial infections reduce digesta viscosity in the small intestine and therefore reduce the risk of bacterial overgrowth in the jejunum and ileum. The results from the four experiments described here clearly show a large variability in the response to an experimental challenge with *Eimeria* or *C. perfringens* or both. The most reliable indicator of a bacterial overgrowth as a result of the challenge with *C. perfringens* is the reduction in feed efficiency in challenged birds. In all four experiments when birds were challenged with *C. perfringens* alone FCR was reduced between 3–5%.

4. The use of feed supplements to control an experimentally introduced clostridial infection

Four experiments were conducted to examine the efficacy of alternative feed additives in controlling the proliferation of *C. perfringens* using the previously established challenge model (Section 3). All additives for these experiments were obtained from commercial sources. The following is a short list highlighting the main characteristics of the additives used.

Enzymes: The main activity in all enzymes tested was xylanase with a known affinity for soluble arabinoxylans. However, no further details were provided on the exact origin or the activities of the products tested.

Organic acids: All the acid products contained a mixture of lactic, fumaric, acetic and benzoic acids. Exact ratios and differences between the products tested are not available.

Prebiotics: The origin of prebiotics used in these studies is unkown. According to our own analyses as well as some information from the supplier, prebiotics are based on manno- or gluco-oligosaccharides.

Probiotics: All probiotics tested contained *Bacillus subtilis*. In the earlier experiments the probiotics were supplied as a broth extract, whereas in the later experiments the probiotics were supplied as a cell extract. At the time of testing, the probiotics were still only an experimental product.

Four climate-controlled rooms located at UNE were thoroughly cleaned and disinfected with Divason Q-cide to ensure a completely *Eimeria* and *C. perfringens*-free environment. Cobb broiler chickens were purchased from a local hatchery (Baiada hatchery, Kootingal) between March and November 2002. Chickens were raised from hatch to the start of the experiment in small multi-tier brooders located in climate-controlled rooms at UNE and were fed a commercial broiler starter diet (Ridley AgriProducts, Tamworth NSW). Access to birds was restricted to nominated personnel to ensure complete biosecurity.

4.1 Effects of enzymes and organic acids in controlling the proliferation of *C. perfringens*

4.1.1 Experiment 7 – experimental design

Forty-eight male and 48 female Cobb broiler chicks were used in this experiment. The same design as in experiment five was used to establish the effect of an *Eimeria*l infection prior to the infection with *C. perfringens* on the severity of the NE infection (Section 3.3.2). However, the sex of the birds was

included as an additional factor to establish differences in susceptibility to the experimental introduction of NE infections in male versus female birds.

On day five, all birds were weighed and feed was changed to the experimental NE diet. Birds were relocated to ‘single-bird’ metabolisable energy cages fitted with special inserts for keeping very young chicks (two birds per cage) and were allocated to two controls and four experimental treatments (eight replicates each of male and female/treatment). Each treatment group was located in a separate room in order to minimise the likelihood of cross-contamination between the four treatments.

1. AGP control.
2. *C. perfringens* (R61) control = Model A.
3. Model A + organic acid A 4kg/t.
4. Model A + organic acid B 5 kg/t.
5. Model A + enzyme A 1 kg/t.
6. Model A + enzyme B 1kg/t.

The same experimental challenge procedure was used as described in Section 3.3.2.

4.1.2 Results

The growth performance of birds receiving each of the six diets is presented in Table 11. Uninfected birds receiving AGPs had a significantly higher bodyweight at the end of the experiment compared to birds challenged with *C. perfringens*. Supplementation with organic acids or enzymes had no significant effect on bodyweight or FCR. However, there was a tendency for birds fed enzyme A to be better able to overcome the negative impact of the *C. perfringens* challenge compared to the other treatments. There were no differences in excreta moisture, ileal viscosity or the relative weight of the bursa of Fabricius because of the *C. perfringens* infection (Table 12). Interestingly, the addition of enzyme B resulted in reduced growth performance and a significant increase in the lesion score in the jejunum. It was concluded that changes in the intestinal environment as a result of the addition of enzyme B favoured the proliferation of *C. perfringens* and other potential pathogens.

Table 11. Growth performance data of birds experimentally infected with *Eimeria* and *C. perfringens* fed two types of enzyme or organic acid

	Weight after CP infection g/bird			Weight at end of experiment day 32 g/bird			FCR		
AGP control	485.1	±	42.3	1687.4	±	135.6 [a]	1.670	±	0.327
Model A	476.8	±	34.4	1440.6	±	294.9 [b]	1.838	±	0.079
Organic acid A	469.5	±	37.9	1560.3	±	168.4 [ab]	1.714	±	0.138
Organic acid B	475.1	±	51.8	1518.5	±	190.2 [ab]	1.797	±	0.204
Enzyme A	462.8	±	49.3	1586.8	±	242.0 [ab]	1.705	±	0.156
Enzyme B	473.8	±	61.5	1433.8	±	296.5 [b]	1.837	±	0.278
Mean	473.4			1487.7					
Source of variance	Probability of greater *F* value in analysis of variance								
Diet	NS			*			NS		

*P<0.05, **P<0.01, ***P<0.001.

a, b Values with different superscripts differ significantly (P<0.05).

Table 12. Secondary parameters as indicators of a subclinical necrotic enteritis infection of birds fed two types of enzymes or organic acids

	Excreta moisture			Viscosity day 20			Relative bursa weight			Lesion score[1.]					
	%			Ileum			g/g BW			Jejunum			Ileum		
AGP control	69.6	±	4.5	10.2	±	5.9	0.18	±	0.03	0.75	±	0.50[b]	1.25	±	0.50
Model A	73.1	±	2.6	11.1	±	1.0	0.16	±	0.03	0.00	±	0.00[b]	1.25	±	0.50
Organic acid A	67.5	±	11.6	9.2	±	7.2	0.17	±	0.06	0.75	±	0.96[b]	0.63	±	0.48
Organic acid B	68.1	±	6.3	8.5	±	1.6	0.17	±	0.06	0.75	±	0.96[b]	1.00	±	0.71
Enzyme A	69.4	±	6.2	6.8	±	1.3	0.18	±	0.06	0.50	±	0.41[b]	1.38	±	0.48
Enzyme B	67.4	±	8.2	4.7	±	0.9	0.15	±	0.05	1.88	±	0.25[a]	0.88	±	0.25
Mean	69.46			8.34			0.170			0.77			1.06		
Source of variance	Probability of greater *F* value in analysis of variance														
Diet	NS			NS			NS			*			NS		

*P<0.05, **P<0.01, ***P<0.001.

1. Lesion scoring: 0 = no lesions, no inflammation, 1 = slight redness of the intestine, 2 = small lesions, red intestine, 3 = visible gross lesions.

a, b Values with different superscripts differ significantly (P<0.05).

4.2 Effect of a prebiotic in controlling a subclinical necrotic enteritis infection

Most diets used for the experimental introduction of NE contain 5–10% fishmeal. Although the mode of action by which high levels of fishmeal influence the proliferation of *C. perfringens* is still unclear, it has been established that challenge models without the inclusion of fishmeal either fail to reproduce the disease or result in a mild, subclinical form of the disease. On the other hand, it has been shown that excessive levels of fishmeal, e.g., more than 35% in the diet, given for a prolonged period will lead to a clinical disease model with mortalities in excess of 50% (Hofacre et al. 2003). These models are only useful to test the efficacy of antibiotics specific to *C. perfringens*. The purpose of the project described in this report was to test the efficacy of alternative feed supplements to control the proliferation of *C. perfringens*.

The challenge model described in Section 3 is clearly effective in introducing a mild, subclinical infection associated with an increased proliferation of *C. perfringens*. In order to demonstrate a more profound effect of a feed additive on bird health and subsequently growth performance, a more severe disease challenge is necessary.

The objective of the experiment described here was to determine the possibility of producing a more severe disease challenge of broiler chickens using high levels of fishmeal in the diet. Furthermore, the effects of an experimental prebiotic (fruit extract) with or without fishmeal were investigated. The experimental protocol used in this study is based on the model described by Brennan (2001).

4.2.1 Experiment 8 – experimental design

One hundred and forty four (144) male broiler chicks were obtained from a local hatchery (Baiada hatchery, Kootingal, NSW). Birds were raised in small brooders (10 birds/cage) located in two climate-controlled rooms at UNE. All birds were given a commercially available turkey crumble with a coccidiostat. In order to prevent the occurrence of ascites at a later stage of the experiment, light was restricted to 22 hours only (2 am–midnight). On day six, birds were transferred to metabolisable energy cages located in four climate-controlled rooms, weighed in pairs and given the experimental diets.

Two different dietary regimes prior to the challenge with *C. perfringens* were used to introduce subclinical necrotic enteritis or clostridial enteritis.

Treatment groups:

1. Control (including coccidiostat, Zn-bacitracin).
2. *C. perfringens* challenge (Model A).
3. *C. perfringens* challenge fishmeal (Model B).
4. Model A + prebiotic (1.5 kg/t).
5. Model B + prebiotic (1.5 kg/t).
6. Modle B + Zn-bacitracin.

Birds in treatment groups three, five and six (Model B) received a diet comprising 50% fishmeal and 50% wheat-based experimental diet (UNE NE diet) from day six to day 10. The fishmeal and UNE NE diet were thoroughly mixed and cold pelleted. These diets included the respective supplement at the recommended inclusion rate, e.g. 1.5 kg/t of prebiotic for the pelleted diet. On day 10 feed residue was weighed and discarded and the feed was changed to the UNE NE diet plus supplements as per the treatment plan and fed to the birds to the end of the experiment.

Birds in treatment groups one, two and four (control and Model A) received the UNE NE diet plus supplements as per the treatment plan from day six to the end of the experiment. On days 11, 12 and 13, the feed for treatment groups two to six was withheld for 3 hours prior to an oral infection with 1 ml *C. perfringens* culture (approx. 10^9 CFU/ml). Broth culture was prepared using the same procedure as described in Section 3.3.2.

On day 20 and day 27 four birds from each treatment group were killed by cervical dislocation. The contents of the jejunum were collected in sterile McCartney tubes for enumeration of *C. perfringens* on *C. perfringens* agar (Oxoid Heidelberg). The intestine (jejunum and ileum) was collected, examined and scored for possible damage due to clostridial enteritis. The pH of ileal content was determined using a miniature glass electrode (EcoScan 5/6 pH meter, Eutech Instruments Pte. Ltd., Singapore). Post mortems were carried out on all dead birds to score NE lesions.

4.2.2 Results

The results obtained for growth performance are shown in Table 13 and the corresponding results on the secondary parameters as indicators of a clostridial infection are presented in Table 14. Model A resulted in significant growth depression in birds. Model B where 50% of fishmeal was included in the diet prior to the challenge had no impact on mortality or the severity of the infection. Moreover, the inclusion of fishmeal resulted in similar growth performance to the unchallenged control group with AGPs, despite the fact that birds fed fishmeal had a significantly increased lesion score in the jejunum and had a tendency for higher lesion scores in the ileum. Addition of a prebiotic to the experimental diet resulted in similar growth performance to birds fed diets without additives (treatment 2). It also appears that the inclusion of the prebiotic decreased the number of *C. perfringens* in the small intestine.

In conclusion, both Models were not totally successful in reproducing a subclinical NE under experimental conditions.

Table 13. Growth performance data of birds experimentally infected with *Eimeria* and *C. perfringens* using a challenge model with fishmeal

	Weight (g/bird) after CP infection	Weight (g/bird) at end of experiment day 32	FCR
Control	421.0 ± 42.5^{a}	1439.5 ± 200.9^{a}	1.874 ± 0.175^{b}
Model A	374.8 ± 61.2^{b}	1033.6 ± 296.0^{c}	2.444 ± 0.311^{a}
Model B	427.1 ± 25.3^{a}	1460.3 ± 244.2^{a}	1.975 ± 0.424^{b}
Model A + prebiotic	371.8 ± 62.8^{b}	1143.5 ± 303.1^{bc}	2.359 ± 0.543^{a}
Model B+ prebiotic	419.7 ± 35.4^{a}	1370.3 ± 182.8^{a}	1.963 ± 0.242^{b}
Model B + AGP	422.9 ± 52.0^{a}	1350.7 ± 292.2^{ab}	2.019 ± 0.209^{b}
Mean	406.7	1308.7	2.091
Source of variance	Probability of greater *F* value in analysis of variance		
Diet	***	***	***

*P<0.05, **P<0.01, ***P<0.001.

a, b, c Values with different superscripts differ significantly (P<0.05).

Table 14. Secondary parameters as indicators of a subclinical necrotic enteritis infection of birds fed high levels of fishmeal

	Relative bursa weight g/g BW			pH			Lesion score[1.] Jejunum			Lesion score[1.] Ileum			Occurence of CP positive	Occurence of CP total no.
Control	0.18	±	0.05	6.58	±	0.09	0.38	±	0.84[c]	0.75	±	0.52[b]	0	7
Model A	0.16	±	0.07	6.78	±	0.82	1.32	±	0.88[a]	0.93	±	1.03[a]	2	7
Model B	0.19	±	0.06	6.39	±	0.62	1.31	±	0.70[a]	0.78	±	0.66[ab]	0	7
Model A + prebiotic	0.15	±	0.06	6.76	±	0.74	0.75	±	0.86[bc]	1.02	±	0.72[ab]	1	7
FM + CP + prebiotic	0.18	±	0.05	6.55	±	0.36	0.86	±	0.70[abc]	1.03	±	0.86[a]	1	7
FM + CP + AGP	0.16	±	0.06	6.37	±	0.58	1.00	±	0.42[ab]	0.36	±	0.57[a]	0	7
Mean	0.17			6.75			0.90			0.80			4	42
Source of variance	Probability of greater *F* value in analysis of variance													
Diet	NS			NS			***			**				

*P<0.05, **P<0.01, ***P<0.001.

1. Lesion scoring: 0 = no lesions, no inflammation, 1 = slight redness of the intestine, 2 = small lesions, red intestine, 3 = visible gross lesions.

a, b, c Values with different superscripts differ significantly (*P*<0.05).

4.3 Effect of prebiotics and probiotics in controlling a subclinical necrotic enteritis infection

Previous experiments have shown that Model A only causes a mild clostridial infection with a clear NE lesion present in the intestinal of birds. The infection is associated with a small increase in intestinal lesions and a numerical decrease in growth performance of infected birds. Furthermore, the introduction of Model B, i.e., including a diet with 50% fishmeal as part of the model, had no effect on the proliferation of *C. perfringens* or the occurrence of intestinal lesions (Section 4.2). The key to controlling the severity of an experimental NE infection is the *C. perfringens* strain used for the challenge (Hofacre 2004; personal communication). The strain used in all previous experiments was isolated from a bird which had an NE lesion score 4, however, it appears that this strain is less suitable for the introduction of a severe experimental NE challenge. In this experiment, the difference between the currently used *C. perfringens* strain and a strain isolated in Victoria from a field case of NE (Dr PC Scott kindly made it available for research purpose at UNE) was determined.

In contradiction to our expectation, the addition of an experimental gluco-oligosaccharide had a detrimental effect on bird health and performance. The lack of protection against the experimental NE challenge might be due to a non-specific stimulation of all bacterial species including *C. perfringens* (Maczulak et al. 1993). It has also been reported that some oligosaccharides such as glucan-based oligosaccharides can support the growth of pathogenic bacteria (Huyghebaert 2003).

The aims of this experiment were to test a new *C. perfringens* isolate for the experimental introduction of NE, and to test the efficacy of two prebiotics and two probiotics as AGP alternatives.

4.3.1 Experiment 9 – experimental design

A total of 136 male broiler chickens wereobtaind from a local hatchery (Baiada hatchery in Kootingal, NSW). Model A was used in this experiment (see Section 4.2.1). Birds were allocated to seven experimental treatments replicated 10 times (except for treatment 3 that was replicated 8 times).

Treatment groups:

1. AGP control.
2. Model A.
3. Modified Model A (with field strain – NE-1).
4. Model A+ prebiotic A 2 kg/t.
5. Model A + prebiotic B 2 kg/t.
6. Model A + probiotic A 3 kg/t.
7. Model A + probiotic B 3 kg/t.

On day 20 and day 27 four birds from each treatment group were killed by cervical dislocation. The contents of the jejunum were collected in sterile McCartney tubes for enumeration of *C. perfringens* on *C. perfringens* agar (Oxoid Heidelberg). Each intestine (jejunum and ileum) was collected, examined and scored for possible damage due to clostridial enteritis. The pH of ileal content was determined using a miniature glass electrode (EcoScan 5/6 pH meter, Eutech Instruments Pte. Ltd., Singapore).

4.3.2 Results

Throughout the experiment, no significant differences in live weight or FCR between challenged and unchallenged birds were found (Table 15). There was no difference between the two strains of *C. perfringens* on any parameters determined in this experiment. In fact, birds challenged with *C. perfringens* showed a tendency for better growth performance than birds fed the AGP control diet. Excreta moisture and pH in the small intestine were not affected by inoculation with *C. perfringens* or by the addition of feed supplements (Table 16).

Table 15. Growth performance data of birds experimentally infected with *Eimeria* and *C. perfringens* fed two types of prebiotic or probiotic

	Weight after CP infection g/bird			Weight at end of experiment day 34 g/bird			FCR		
AGP control	513.9	±	29.1	1605.0	±	88.7	1.921	±	0.074
Model A	518.5	±	68.6	1738.0	±	38.0	1.761	±	0.080
Modified Model A (NE-1)	515.7	±	37.0	1604.4	±	227.6	1.928	±	0.247
Model A + Prebiotic A	508.9	±	68.9	1606.2	±	221.5	1.846	±	0.237
Model A + Prebiotic B	517.9	±	54.0	1676.4	±	137.1	1.860	±	0.199
Model A + Probiotic A	536.2	±	55.7	1745.5	±	131.8	1.772	±	0.093
Model A + Probiotic B	513.4	±	88.1	1667.8	±	165.5	1.789	±	0.185
Mean	518.3			1661.7			1.840		

Table 16. Secondary parameters as indicators of a subclinical NE infection of birds fed two types of prebiotic or probiotic

	Excreta moisture %			pH Proventriculus			pH Duodenum			pH Jejunum			pH Ileum		
AGP control	63.0	±	4.3	3.64	±	0.45	5.81	±	0.17	6.06	±	0.13	63.0	±	4.3
Model A	62.0	±	6.2	3.56	±	0.89	5.68	±	0.22	5.93	±	0.24	62.0	±	6.2
Modified Model A (NE-1)	59.1	±	12.4	3.70	±	0.88	5.77	±	0.26	6.10	±	0.11	59.1	±	12.4
Model A + Prebiotic A	65.3	±	4.7	3.77	±	0.69	5.75	±	0.26	6.08	±	0.27	65.3	±	4.7
Model A + Prebiotic B	62.9	±	5.4	3.87	±	0.55	5.78	±	0.42	5.95	±	0.19	62.9	±	5.4
Model A + Probiotic A	64.7	±	4.7	3.79	±	0.35	5.62	±	0.24	5.92	±	0.21	64.7	±	4.7
Model A + Probiotic B	63.2	±	7.2	3.27	±	0.79	5.80	±	0.22	5.88	±	0.13	63.2	±	7.2
Mean	63.1			3.66			5.74			6.00			6.66		

4.4 Effect of graded levels of a probiotic on growth performance and gross intestinal lesions

Probiotics are defined as exogenous microbial cultures which maintain or 'improve' the indigenous microflora (Fuller 1992). These micro-organisms act in a manner of competitive exclusion, hence depriving pathogens such as *C. perfringens* of a potential niche for proliferation. In addition, by-products from fermentation will lower the intestinal pH and create an environment less favourable for potential pathogens. The precise mode of action of probiotics, however, is not known. In addition to the direct impact on the indigenous microflora, probiotics also have an impact on the structure of the intestinal mucosa, the function of the epithelium and the immune system (Simon 2003).

A number of review papers in the literature discuss the benefits of probiotics as alternatives to AGPs (Fioramonti et al. 2003; Ghadban 2002; Holzapfel et al. 1998; Simon 2003; van Eys 2003). However, little is published on the efficacy of probiotics on the prevention of clostridial infections.

The objective of this experiment was to establish how effective a new probiotic product was in minimising losses in growth performance due to a subclinical NE infection.

4.4.1 Experiment 10 – experimental design

Two experiments were conducted simultaneously using 500 male broiler chickens. Both experiments were conducted over a six-week period at the University of New England from October 24 to December 5 2002. Chickens were raised from hatch to six days of age in one large floor pen. The birds were given commercial turkey crumbles with a coccidiostat (monensin).

In both experiments, birds were allocated to the following six dietary treatments:

1. AGP control + challenge.
2. Model A.
3. Model A + probiotic 0.6 g/kg.
4. Model A + probiotic 1.2 g/kg.
5. Model A + probiotic 1.8 g/kg.
6. Model A + probiotic 3.0 g/kg.

Experiment 10/1 – Cage Trial

On day six, 134 birds were transferred into 68 metabolisable energy cages located in four climate-controlled rooms, weighed in pairs and given the experimental diets. Birds were allocated to six treatment groups (12 replicates for the control (treatment 1) and 11 replicates for all other treatments). On day 11, feed was withdrawn for 20 hours prior to the oral infection with *C. perfringens* (R61). The infection procedure and preparation of broth has been previously described (Section 3.3.2). On day 20 and day 27, six birds from each treatment group were killed by cervical dislocation. Ileal contents (10 cm from the Meckel's diverticulum) were collected in sterile McCartney tubes and immediately diluted with 10 ml of thioglycollate broth before processing for enumeration of *C. perfringens*. Each intestinal segment (jejunum and ileum) was examined and scored for possible damage due to clostridial enteritis.

Experiment 10/2 – Floor Pen Trial

On day 6 all remaining healthy birds were split into six groups (55–60 birds per pen) and allocated to the same six experimental treatments as birds in experiment 10/1. On day 11 feed was withdrawn for 20 hours prior to the oral infection with *C. perfringens* (R61). On day 12, birds in treatment group 2–6 were fed a slurry of experimental diet and broth culture containing approximately 10^8–10^9 CFU of

C. perfringens (ratio of feed to *C. perfringens* inoculum 1:1.5). On day 13 and day 14, feed was withheld for 3 hours prior the inoculation with *C. perfringens*.

On day day 23, five to 10 birds from each treatment group were killed by cervical dislocation. Ileal contents (10 cm from the Meckel's diverticulum) were collected in sterile McCartney tubes and immediately diluted with 10 ml of thioglycollate broth before processing for enumeration of *Clostridium perfringens*. Each intestine (jejunum and ileum) was examined and scored for possible damage due to clostridial enteritis.

4.4.2 Results

Cage trial

Growth rates of birds fed the control diet were similar to that of birds challenged with *C. perfringens* (Figure 7 and Table 17). Neither the inclusion of the probiotic nor its dosage level had any effect on the measured performance parameters. Birds challenged with *C. perfringens* had a tendency for more frequent occurrence and higher numbers of the pathogen in the small intestine (Table 18). Furthermore, challenged birds had a significantly higher ($P<0.05$) lesion score in the jejunum.

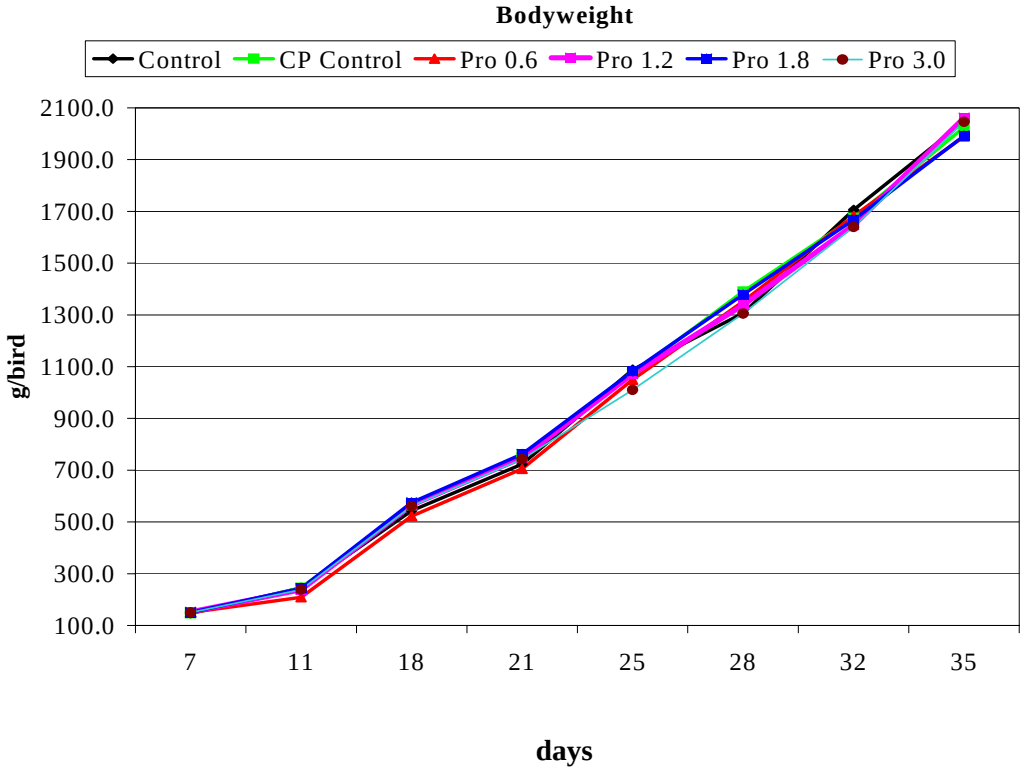


Figure 7. Development of bodyweight of birds experimentally infected with *Eimeria* and *C. perfringens* and fed a probiotic at four different dosages raised in cages

Table 17. Growth performance data of birds experimentally infected with *Eimeria* and *C. perfringens* and fed a probiotic at four different dosages raised in cages

	Weight after CP infection			Weight at end of experiment day 32			Feed intake			FCR		
	g/bird			g/bird			g/bird					
AGP control	543	±	60	1706	±	153	2539	±	272	1.638	±	0.139
Model A	564	±	55	16756	±	86	2545	±	164	1.662	±	0.045
Model A + prob 0.6	523	±	104	1683	±	122	2710	±	213	1.768	±	0.126
Model A + prob 1.2	565	±	71	1647	±	150	2581	±	230	1.735	±	0.168
Model A + prob 1.8	575	±	65	1668	±	117	2598	±	105	1.719	±	0.144
Model A + prob 3.0	561	±	40	1640	±	212	2432	±	237	1.641	±	0.074
Mean	555.3			1674.9			2568.1			1.689		

Table 18. Occurrence of lesions and *C. perfringens* as indicators of a subclinical NE infection of birds fed a probiotic at four different dosages raised in cages

	Lesions[1.]	Occurrence of *C perfringens*		
	Jejunum	10^4 CFU/g	Infected birds	Total birds tested
AGP control	0.17 ± 0.33[b]	3.1	1	9
Model A	0.80 ± 0.59[a]	32.6	5	9
Model A + prob 0.6	0.60 ± 0.66[ab]	14.7	3	9
Model A + prob 1.2	0.67 ± 0.54[ab]	3.4	2	9
Model A + prob 1.8	0.77 ± 0.56[a]	21.2	3	9
Model A + prob 3.0	0.95 ± 0.83[a]	16.6	2	9
Mean	0.65	17.3	16	54
Source of variance	Probability of greater *F* value in analysis of variance			
Diet	*		NS	

*P<0.05, **P<0.01, ***P<0.001. a, b Values with different superscripts differ significantly (P<0.05).
1. Lesion scoring: 0 = no lesions, no inflammation, 1 = slight redness of the intestine, 2 = small lesions, red intestine, 3 = visible gross lesions.

Floor Pen trial

Growth rates of birds raised in floor pens were in general higher compared to those of birds raised in cages regardless of dietary treatments (Figure 8, Table 19). In contrast to the cage trial it was found that bodyweight of birds raised in floor pens was significantly affected by the experimental challenge with *C. perfringens*. Birds given a low level of the probiotic and those given no supplements had significantly lower bodyweight at the end of the experiment compared to birds fed the control diet or diets containing increased levels of the probiotic. As expected birds fed these diets also had increased occurrence of *C. perfringens* and higher numbers of pathogens in the small intestine (Table 20).

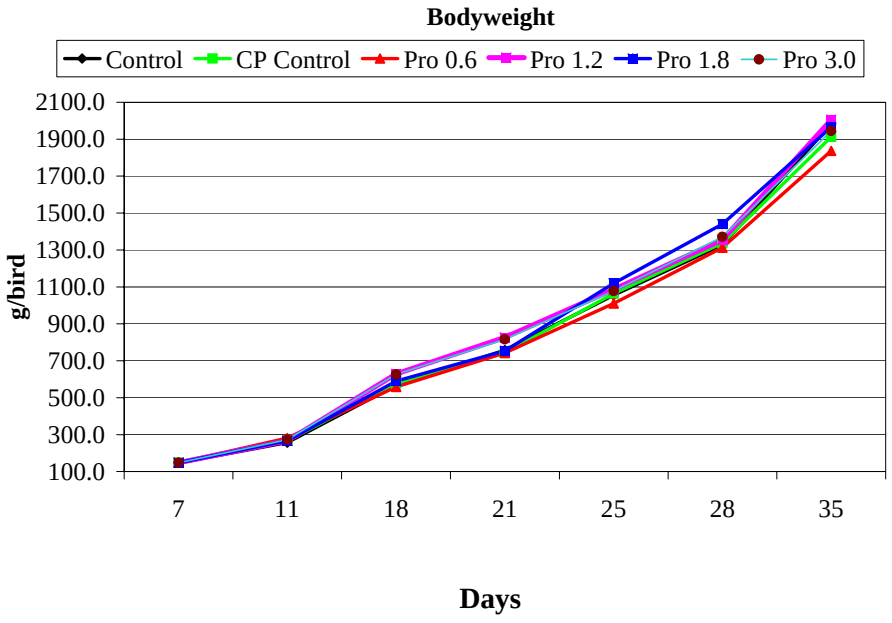


Figure 8. Development of bodyweight of birds experimentally infected with *Eimeria* and *C. perfringens* and fed a probiotic at four different dosages raised in floor pens

Table 19. Growth performance data of birds experimentally infected with *Eimeria* and *C. perfringens* and fed a probiotic at four different dosages raised in floor pens

	Weight after CP infection g/bird	Weight at end of experiment g/bird	Feed intake g/bird	FCR
AGP control	567.6 ± 18.9	1983 ± 75.2[a]	3159	1.652
Model A	576.7 ± 6.9	1912 ± 52.2[ab]	3177	1.718
Model A + prob 0.6	558.5 ± 27.1	1837 ± 38.6[b]	3349	1.754
Model A + prob 1.2	629.0 ± 65.8	2005 ± 37.7[a]	3383	1.683
Model A + prob 1.8	589.7 ± 14.3	1966 ± 24.5[a]	3202	1.661
Model A + prob 3.0	626.9 ± 73.2	1945 ± 95.4[a]	2950	1.747
Mean	591.4	1941	3052	1.703
Source of variance	Probability of greater *F* value in analysis of variance			
Diet	NS	**	NS	NS

*P<0.05, **P<0.01, ***P<0.001.

a, b Values with different superscripts differ significantly (P<0.05).

Table 20. Occurrence of lesions and *C. perfringens* as indicators of a subclinical necrotic enteritis infection of birds fed a probiotic at four different dosages raised in floor pens

	Lesions[1]	Occurrence of *C perfringens*		
	Jejunum	10^4CFU/g	infected birds	total birds tested
AGP control	0.30 ± 0.46	0.0	0	3
Model A	0.63 ± 0.72	8.5	2	3
Model A + prob 0.6	0.53 ± 0.61	1.2	1	3
Model A + prob 1.2	0.83 ± 0.56	15.0	1	3
Model A + prob 1.8	0.70 ± 0.65	0.0	0	3
Model A + prob 3.0	0.30 ± 0.37	0.0	0	3
Mean	0.55	4.70	4	18

1. Lesion scoring: 0 = no lesions, no inflammation, 1 = slight redness of the intestine, 2 = small lesions, red intestine, 3 = visible gross lesions.

4.5 Discussion

Challenging broiler chicks with a large number of *C. perfringens* is no guarantee for the development of typical field-type NE (Truscott & Al-Sheikhly 1977). The keys for reproduction of the clinical form of the disease are sufficient numbers of *C. perfringens* and the production of sufficient toxin by multiplication of the infused bacterium. In the four experiments described in this section, birds did not die from, or develop a clinical form of, necrotic enteritis. However, birds challenged with *C. perfringens* without any supplement (*C. perfringens* control) had lower bodyweight and increased FCR at day 32 compared to birds fed the control diet with AGPs. Indicators of a possible infection such as lesion scores in the small intestine and the occurrence of *C. perfringens* in the small intestine confirmed the negative impact on bird health of the oral challenge with *C. perfringens*.

Lesion scores measured at day 27 were the lowest in the control group in all four experiments. This is most likely due to the subclinical effects of the *C. perfringens* challenge. Other parameters measured such as the relative weight of the bursa, or the pH in the intestine remained unaffected among diets. The fact that *C. perfringens* was only found in some of the challenged birds and birds did not show any obvious symptoms of the disease would indicate that the *C. perfringens* challenge used in all experiments led to a disturbance of the normal microflora in the intestine or caused the so-called dysbacteriosis. In contrast to clinical enteritis, birds affected by dysbacteriosis show no obvious symptoms and have a normal mortality rate, except their performance is lower than that of healthy birds with a balanced microflora. These differences would suggest that in the current experiments birds were successfully infected with subclinical clostridial enteritis.

It has to be noted that in all four experiments mortality due to ascites was extremely high (>15%). In addition some birds had an enlarged crop and proventriculus. The causes of these symptoms remain unclear. Tissue analysis excluded infectious proventriculitis as the main cause and it can only be speculated that the dilatation of the crop and proventriculus was the result of increased levels of biogenic amines in the diets due to the high inclusion rate of fishmeal. Biogenic amines, such as histamine, cadaverine, putrescine, spermine or spermidine are formed by microbial decarboxylation of amino acids. It has been reported that the inclusion of putrescine in diets can result in increased growth (Smith 1990), but on the other hand, it is also known that increased levels of biogenic amines in feedstuffs will lead to reduced feed efficiency (Brugh & Wilson 1986) and can result in ulcers or lesions in the proventriculus (Barnes et al. 2001).

All feed ingredients used in these experiments were sourced from a local supplier. Neither the raw ingredients nor the prepared diets were analysed for the presence of biogenic amines or other potential toxins. Fishmeal available in Australia is not of high quality (den Brinker et al. 2003). Therefore it cannot be excluded that elevated levels of biogenic amines in the experimental diets led to the dilatation of the crop and proventriculus. Birds in all dietary treatments were affected at a similar rate. Although it cannot be excluded that the enlarged proventriculus had some effect on the overall bird performance, the actual comparisons between experimental treatments are still relevant.

There are a large number of commercial feed additives used to improve broiler growth performance. However, to date only AGPs are effective in controlling a subclinical clostridial infection. In the four experiments discussed in this section, no significant differences between the unsupplemented control group (*C. perfringens* control) and diets containing alternatives to AGPs were found. However, birds that received an organic acid or an enzyme preparation (enzyme A) had numerically improved bodyweight and reduced FCR compared to the unsupplemented control. Interestingly, the addition of a second enzyme (enzyme B) had no impact on growth performance or the challenge by *C. perfringens*. The benefits of using feed enzymes to reduce digesta viscosity and subsequently improve growth performance are well established. By altering nutrient digestion and digesta passage rates in the small intestine, the addition of enzymes can also have a direct influence on the proliferation of unwanted bacteria (Choct & Sinlae 2000). The exact mode of action of a feed enzyme depends very much on the type of enzyme and the available substrates (Choct et al. 2004). In particular, the depolymerisation of soluble or insoluble NSP can have an impact on the overall nutrient digestibility and the composition of the microflora. A significant increase in the lesion score when enzyme B was added to the diet would indicate greater microbial activity in the upper intestine that had a negative impact on growth performance of broilers.

Organic acids can be successfully used in broiler diets to improve feed efficiency or bodyweight gain (Patten & Waldroup 1988; Skinner et al. 1991). The mechanisms by which organic acids improve growth performance and animal health are related to the change in gastrointestinal pH and the ability to inhibit the growth of unwanted microbes (Dibner & Buttin 2002). Although both organic acid preparations tested in this study are based on the same three basic acids, the differences in the ratio of the individual acids altered the effect on broiler growth performance. The lack of consistency is most likely related to factors such as the ability of the acid mixture to disassociate or uncontrolled variables such as buffering capacity or the differences in general composition of the microflora.

Another way to control the growth of potential pathogens such as *C. perfringens* is to manipulate the beneficial microflora in the intestine of broilers. Essentially, this means to either provide substrates required by these types of bacteria (prebiotics) or alternatively to include beneficial micro-organisms in the feed (probiotics). The inclusion of prebiotics or probiotics as feed supplements to control the proliferation of *C. perfringens* had little or even negative effects on the growth performance of broilers. Unfortunately, the exact composition of the prebiotic products used in this study was unknown. Prebiotics such as fructo-, xylo- or gluco-oligosaccharides can be utilised as substrates by bifidobacteria or lactobacilli (Gibson & Roberfroid 1995) whereas manno-oligosaccharides have been reported to bind to pathogens using type-1 fimbriae as well as modulate the immune system (Spring 2003). The responses to the addition of prebiotics and probiotics to broiler diets are variable (Simon 2002). First, the exact mode of action of these additives is still relatively unknown and secondly, probiotics can stimulate the growth of both beneficial but also potentially harmful bacteria (Maczulak et al. 1993).

The results of these four experiments indicate that some of the tested products can effectively maintain performance of birds challenged with *C. perfringens*. The presence of biogenic amines or a viral infection and the fact that the chosen challenge model resulted only in a subclinical infection make it very difficult to clearly establish the absolute benefit of these products.

5. Evaluation of feed supplements for their ability to control a subclinical necrotic enteritis infection using a modified model

5.1 Effect of a probiotic in controlling a subclinical necrotic enteritis infection

Results from the previous experiment investigating the effects of a newly developed probiotic (*B. subtilis* spores) on the control of *C. perfringens* proliferation have been inconclusive. The objectives of the current experiment were (1) to introduce a new model with a number of extra elements incorporated; and (2) to test the efficacy of a new formulation of the above-mentioned probiotic on the control of clostridial enteritis.

5.1.1 Experiment 11 – experimental design

Three hundred male broiler chicks were purchased from a local hatchery (Baiada hatchery, Kootingal) and placed in groups of 50 birds in six large floor pens (1.5 m x 1.5 m) fitted with a hover brooder and automated drinker lines. Each pen was allocated to one of the following six treatments:

1. Control (including coccidiostat, Zn-bacitracin at 50 ppm)
2. *C. perfringens* challenge (strain R-61).
3. *C. perfringens* challenge (strain NE-1).
4. *C. perfringens* challenge (NE-1) + probiotic 5 g/t.
5. *C. perfringens* challenge (NE-1) + probiotic 50 g/t.
6. *C. perfringens* challenge (NE-1) + probiotic 500 g/t.

Birds were fed experimental diets from day one (Table 21). On day eight, feed residue was recorded; feed was discarded and was changed to a high protein diet based on 40% fishmeal (Table 21). Both diets included the respective supplement at the recommended inclusion rate.

On day 14, feed was withdrawn for 20 hours prior to an oral infection with *C. perfringens.* Birds in treatment groups 2–6 were infected with 2 ml of *C. perfringens* broth culture containing approximately 10^8–10^9 CFU/ml. Birds in group one were given sterile thioglycollate solution. This procedure was repeated twice on days 16 and 17; however, on those days feed was withheld for only 3–4 hours prior to the inoculation with *C. perfringens*.

On days 11, 12 and 13, treatment groups two and four were orally infected with 1 ml of *C. perfringens* culture (approximately 10^9 CFU/ml). Two days prior to the inoculation of the birds (day nine) a master culture of *C. perfringens* broth was prepared using 0.1 ml of bacterial stock culture (stored at -40°C). The stock culture was incubated for 16 hours at 37°C and stored in a fridge (approximately 4°C) for the duration of the experiment. On days 10, 11 and 12, respectively, 5 ml of *C. perfringens* broth from the master culture was used to prepare 500 ml of fresh thioglycollate broth. Each batch was grown at 37°C for 20 hours and contained approximately 10^8 CFU of *C. perfringens.*

Table 21. Composition (%) of the basal diet and the high protein diet

Main components	Ingredients	Basal diet	High protein diet
Cereal component:	Wheat *	47.0	44.15
	Oats *	10.0	10.0
	Rice pollard	2.65	
	Wheat offal/Millrun		4.0
Oil and fat:	Tallow	3.5	
Protein components:	SBM 48	19	
	Meat and Bone meal	8	
	Peas	7.5	
	Blood	0.5	
	Fishmeal		40.0
Minerals	Lime	0.5	0.5
	Sodium bicarbonate	0.34	0.34
	Salt	0.1	0.1
	Methionine	0.33	0.33
	Lysine	0.22	0.22
	Premix#	0.2	0.2
	Threonine	0.1	0.1
	Choline Chloride	0.06	0.06

* Hammer-milled.

#The active ingredients contained in each kg premix were as follows: retinol 3.03 mg, cholecalciferol 0.09 mg, all-rac-α-tocopherol acetate 20 mg, menadione 6.3 mg, riboflavin 8 mg, pyridoxine hydrochloride 5 mg, biotin 0.01mg, niacin 30 mg, thiamine 1.5 mg, D-calcium pantothenate 15 mg, folic acid 2 mg, ethoxyquin 125 mg, Mn 75 mg, Fe 20 mg, Cu 5 mg, I 1 mg, Co 0.3 mg, Se 0.5 mg, Mo 0.16 mg cyanocobalamin 0.15 mg.

The challenge inoculum was prepared as follows: two days prior to the challenge, 10 ml of Oxoid's (Heidelberg, Vic) thioglycollate medium (USP) was prepared to company specification and infected with a small portion of *C. perfringens* stock culture stored at -40°C. The culture was incubated at 39°C overnight. The following day 10 ml of cooked meat medium (Oxoid) were inoculated with 0.5 ml of thioglycollate broth culture and incubated at 39°C for 12 hours. The actual broth culture for inoculation was prepared using thioglycollate broth medium USP containing 0.5% starch (wheat starch) and 1.0% casitione (MMSC). The broth was inoculated with 10 ml of cooked meat medium culture and incubated at 39°C for overnight prior to the oral challenge of the birds.

On days 18, 21 and 29 (three, six and 14 days after the challenge), five birds from each treatment group were killed by cervical dislocation. A small subsection of the ileum including content (10 cm from the Meckel's diverticulum) were collected in sterile McCartney tubes and immediately diluted with 10 ml of thioglycollate broth before processing for enumeration of *C. perfringens* using commercially available agar specific for the growth of *C. perfringens* (OPSP agar, CM543, Oxoid, Heidelberg).

5.1.2 Results

The effects of dietary treatment and *C. perfringens* on growth performance are presented in Table 22. The experimental challenge with *C. perfringens* had no influence on the growth performance or mortality of broilers. Supplementation with low or high levels of an experimental probiotic (*B. subtilis*) resulted in significantly reduced weight gain at day 42 and increased mortality (Table 23). No differences in the relative weight of immune organs (spleen or bursa) were found. High mortality and relatively poor performance in the control group made it very difficult to evaluate the impact of the experimental challenge and the effects of the probiotic under these conditions.

Table 22. Growth performance data of birds experimentally infected with *Eimeria* and *C. perfringens*

	Weight after CP infection g/bird			Weight at end of experiment day 42 g/bird			FCR
AGP control	464.0	±	70.4[ab]	2200.4	±	234.8[a]	2.004
CP control R61	486.8	±	63.4[ab]	2245.2	±	195.4[a]	1.716
CP CSIRO NE-1	492.0	±	43.5[ab]	2208.2	±	280.3[a]	1.727
CP + prob 5	490.0	±	75.9[ab]	2022.8	±	245.2[b]	1.774
CP + prob 50	499.5	±	72.6[a]	2304.7	±	147.3[a]	1.819
CP + prob 500	457.4	±	64.5[b]	2037.7	±	243.0[b]	1.746
Mean	479.1			2158.3			
Source of variance	Probability of greater *F* value in analysis of variance						
Diet	*			***			N/A

*P<0.05, **P<0.01, ***P<0.001.

a, b Values with different superscripts differ significantly (P<0.05).

Table 23. Occurrence of lesions and *C. perfringens* as indicators of a subclinical necrotic enteritis infection of birds fed a probiotic

	Relative weight spleen day 21		Relative weight bursa day 21		Occurrence of CP			Mortality
	%BW		% BW		CFU/g av 10^4	infected	total	%
AGP control	0.115	± 0.032	0.194	± 0.026	0.6	1	15	14.0
CP control R61	0.146	± 0.062	0.207	± 0.018	9.2	10	15	10.0
CP CSIRO NE-1	0.110	± 0.026	0.208	± 0.065	1.3	7	15	8.0
CP + prob 5	0.111	± 0.019	0.207	± 0.047	2.5	8	15	22.0
CP + prob 50	0.111	± 0.038	0.279	± 0.094	136.0	8	15	14.0
CP + prob 500	0.090	± 0.033	0.227	± 0.038	212.2	9	15	18.0
Mean	0.113		0.223		60.3	43	90	14.3

5.2 Effect of four feed additives on a subclinical necrotic enteritis infection

Repeatable and reliable reproduction of a subclinical NE infection has been extremely challenging. Previous sections in this report (Sections 3 and 4) have described a number of different models, all of which result in a mild clostridial enteritis. In order to effectively test potential alternatives for the control of clostridial infection it is necessary to develop a model causing a more severe infection resulting in mortality rates of approximately 5–8% in the unsupplemented control group. After a concerted effort to gather more information on the factors predisposing the occurrence of NE, a number of meetings with industry experts (Jim Aspinal, Jules D'Assonville, Margaret Curtis, Tom Grimes, Rod Jenner, Peter Scott) and other researchers (Bot Hughes, Magne Kaldhusdal) took place between 2002-2003. From our discussions, it became apparent that diet and *C. perfringens* were only two of the many factors that had to be manipulated in order to develop a repeatable model for reproducing NE under experimental conditions. The factors included gut damage, environmental stress, and manipulation of the challenge organism. An ideal model would cause a mild (subclinical) NE infection with a mortality rate under 10%. The model developed by Brennan et al. (2003) was to become a basis for our model (the UNE NE Model).

Increased proliferation of unwanted bacteria such as *C. perfringens* can lead to degradation of digestive enzymes and bile salts and will lead to excessive colonisation of the absorptive surface (Smits & Annison 1996). There is considerable evidence that the addition of enzymes to wheat-based diets will improve the rate of digestion and absorption and subsequently will have an impact on the rate of proliferation of the intestinal microflora (Huyghebaert 2003). Similarly, the use of probiotics has been proven beneficial in controlling the intestinal microflora (Cavazzoni et al. 1998). The actual mode of action of these probiotics is still poorly understood, however, it is believed that probiotics can stimulate the growth of 'positive' bacteria such as *Lactobacillus* by the formation of specific substrates (lactate) required by *Lactobacillus* as well as simply establishing a microflora which builds resistance to the colonisation of pathogens (Huyghebaert 2003).

Organic acids have a limited effect on the actual colonisation of bacteria in the intestine. The primary effect of organic acids is antimicrobial through pH depression in the gut, in particular within the organisms themselves (Dibner & Buttin 2002). Further, organic acids also have a direct effect on the growth performance of broilers by reducing endogenous nitrogen losses.

The objectives of the experiment were to test the ability of four potential alternative feed additives to control the occurrence of a subclinical NE infection under the UNE NE Model, which had been evaluated in a number of ancillary experiments.

5.2.1 Experiment 12 – experimental design

Three hundred (300) male broiler chicks were purchased from a local hatchery (Baiada hatchery, Kootingal) and placed in groups of 50 birds in six large floor pens (1.5 m x 1.5 m) fitted with a hover brooder and automated drinker lines. One of the following six treatments was allocated to each pen:

1. Control (including coccidiostat, Zn-bacitracin at 50 ppm).
2. UNE NE Model.
3. UNE NE Model + enzyme.
4. UNE NE Model + organic acid.
5. UNE NE Model + organic acid + enzyme.
6. UNE NE Model + probiotic.

Birds were fed the experimental diets from day one (see Table 21) and feed additives were included according to the manufacturer's recommendation; enzyme 500 ppm, organic acids 2500 ppm, organic acids + enzyme 2500 ppm, probiotics 500 ppm.

The experiment was conducted during the winter months in Australia (July–August). A severe drop in temperature during the first week and a lack of adequate heating resulted in increased mortality of up to 30–40% per pen. On day eight, all birds were weighed and physically examined to determine the overall health status of each chick. At this point in time, sick and small birds were culled and eliminated from the experiment. A total of 30 chicks per pen were retained.

A high protein diet based on 40% fishmeal (see Table 21) was fed from day eight to day 14. Birds in groups two to six were orally infected with *Eimeria* on day nine. On day 14, feed was withdrawn for 20 hours prior to an oral infection with *C. perfringens* (isolate NE-1). Preparation of the broth culture and the challenge procedure have been previously described (Section 5.1).

On days 18 and 21, six birds from each pen were randomly selected and killed by cervical dislocation. Measurement of digesta viscosity, enumeration of *C. perfringens* and assessment of jejunal lesions were performed as described in Section 3.3.2.

5.2.2 Results

The modified challenge model resulted in significant differences in growth performance of unchallenged birds fed the AGP-containing diet and challenged birds without feed supplements (Table 24). The immediate impact of the oral challenge on daily weight gain is shown in Figure 9. Enumeration of *C. perfringens* in the small intestine and necropsies of birds that died during the experiment confirmed the occurrence of a mild NE infection (Table 26). In the uninoculated AGP control group no *C. perfringens* was found in the upper small intestine. In contrast, a minimum of 8×10^7 *C. perfringens* were present in the intestine of challenged birds. Five birds were diagnosed with NE and lesions in the jejunum were scored in excess of 3. Birds fed enzyme or the AGP had significantly better growth performance compared to birds without additives or with organic acids or probiotics. Addition of a probiotic or a mixture of organic acids and enzymes resulted in similar growth performance compared to the unsupplemented control diet.

Table 24. Growth performance data of birds experimentally infected with *Eimeria* and *C. perfringens* and fed four different alternatives to AGP

	Weight after *C. perfringens* infection			Weight at end of experiment day 42			FCR
	g/bird			g/bird			
AGP control	481.4	±	50.1^{ab}	2297.8	±	183.8^{ab}	1.726
CP control	384.8	±	52.2^{d}	1977.4	±	368.4^{c}	2.028
Enzyme	504.8	±	71.2^{ab}	2373.4	±	264.6^{a}	1.778
Organic acid	460.0	±	64.6^{bc}	2095.9	±	223.9^{bc}	1.931
Organic acid + enzyme	454.4	±	52.9^{bc}	2037.5	±	333.3^{c}	1.913
Probiotic	427.8	±	63.7^{c}	2047.3	±	299.5^{c}	1.972
Mean	453.3			2141.0			1.891
Source of variance	Probability of greater *F* value in analysis of variance						
Diet	***			***			N/A

*P<0.05, **P<0.01, ***P<0.001.

a, b, c, d Values with different superscripts differ significantly (P<0.05).

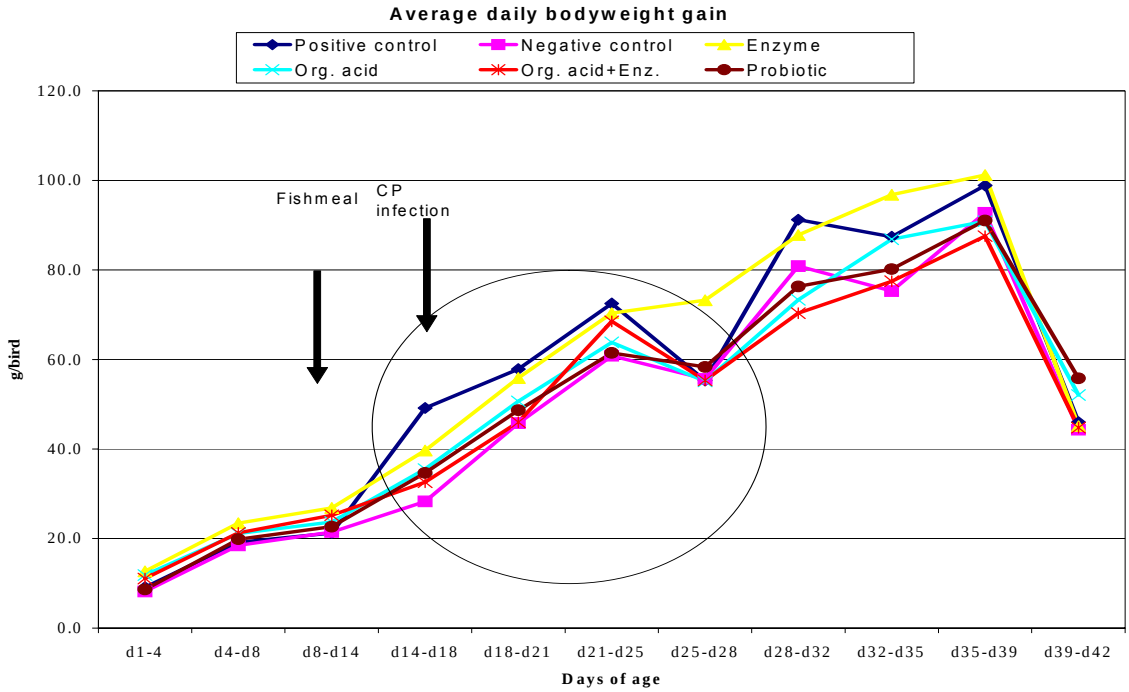


Figure 9. Average daily weight gain of broilers experimentally infected with *Eimeria* and *C. perfringens* fed four different alternatives to AGP

Table 25. Viscosity and occurrence of lesions in birds experimentally infected with *Eimeria* and *C. perfringens* and fed four different alternatives to AGP

	Viscosity			Lesions1. Jejunum					
	mPa.s			day 18			day 21		
AGP control	10.6	±	1.1bc	0.04	±	0.1b	0.00	±	0.0b
CP control	13.6	±	1.9ab	1.71	±	1.2a	0.88	±	0.9a
Enzyme	3.1	±	0.6c	0.79	±	0.6ab	1.46	±	0.8ab
Organic acid	19.5	±	9.1ab	0.96	±	1.4ab	1.13	±	0.8a
Organic acid + enzyme	12.8	±	5.3ab	1.13	±	0.5a	1.13	±	0.9a
Probiotic	10.6	±	0.8bc	1.33	±	0.3a	0.92	±	0.8ab
Mean	11.7			0.99			0.92		
Source of variance	Probability of greater F value in analysis of variance								
Diet	***			***			***		

*P<0.05, **P<0.01, ***P<0.001.

1. Lesion scoring: 0 = no lesions, no inflammation, 1 = slight redness of the intestine, 2 = small lesions, red intestine, 3 = visible gross lesions.

a, b, c Values with different superscripts differ significantly (P<0.05).

Table 26. Occurrence of *C. perfringens* and mortality of birds experimentally infected with *Eimeria* and *C. perfringens* and fed four different alternatives to AGP

	Occurrence of *C. perfringens* 10^6				Mortality (no. birds)	
	day 18		day 21		NE	Total
AGP control	0.0	0.0[c]	0.0	0.0[b]	0	3
CP control	203.0	63.1[a]	122.7	72.3[ab]	3	3
Enzyme	86.0	39.5[b]	110.3	97.3[ab]	1	3
Organic acid	85.4	38.3[b]	65.0	54.8[ab]	0	1
Organic acid + enzyme	185.6	30.1[a]	187.0	57.0[a]	1	5
Probiotic	186.8	53.2[a]	64.0	72.4[ab]	1	4
Mean	124.5		91.5			
Source of variance	Probability of greater *F* value in analysis of variance					
Diet	***		NS			

*P<0.05, **P<0.01, ***P<0.001.

a, b, c Values with different superscripts differ significantly (P<0.05).

5.3 Discussion

The modification made to the originally proposed challenge model had a significant impact on the severity of the disease. For the first time, birds orally challenged with *C. perfringens* died as a result of massive intestinal lesions with the classical symptoms of an NE infection. Modifications to the existing model included dietary changes, introduction of environmental stressors, and inoculaton of a mild infection with *Eimeria*. It has been suggested that a frequent change of diet and the inclusion of a protein rich diet immediately before the *C. perfringens* challenge will destabilise the microflora in the intestine (Brennan 2001). It is known that damage to the intestine promotes establishment and rapid proliferation of *C. perfringens* (Al-Sheikhly & Truscott 1977). A potential problem was that a challenge with *Eimeria* spp. could make it difficult to distinguish between a coccidial infection and the occurrence of a subclinical NE infection. However, in the field the outbreaks of NE usually coincide with a failure of coccidial control or in management practice. An experienced person can also distinguish between lesions caused by coccidial infections and that occurred as a result of NE.

This experiment (Experiment 12) used an isolate originated from a field case of NE in Victoria, which had not been passaged. The actual isolates used in previous challenge models, however, were isolated from a dead bird after it was artificially infected with *C. perfringens*. Thus, some of the isolates were passaged at least once before being used in experiments. Although there is no evidence in the scientific literature that passaging reducing the virulence of *C. perfringens*, it cannot be excluded that the repeated re-culturing of the bacterium had a negative influence on its ability to colonise the intestine. Veterinary advice given to the authors by Dr. T. Grimes (personal communication) suggested that the use of a 'fresh' isolate of *C. perfringens* from a field case of the disease would increase the chances of success of the model. Other factors introduced in the current model include imposition of environmental stressors, such as withdrawl of feed or temporary deprivision of water, and manipulation of the organism in addition to the original protocol used in our previour experiments.

Under experimental conditions, it is not known which of the above was the predisposing factor in reproducing a subclinical NE infection. Regardless, the described model can be successfully used to test the efficacy of alternative feed supplements to control clostridial related diseases. Studies described in previous sections indicated that the use of a xylanase product in birds challenged with NE (enzyme A, Section 4.1) could lead to a similar growth performance to unchallenged birds. The reduction in intestinal viscosity and the subsequent improvement in nutrient digestion and absorption in the presence of the enzyme seemed to give adequate protection against the artificial challenge with *C. perfringens*. These findings are in agreement with findngs of Elwinger and Teglöf (1991). Reduced digesta viscosity and a subsequently enhanced passage rate and more rapid and extensive digestion of all nutrients reduced the available substrates for *C. perfringens*, and hence reduced the ability of *C. perfringens* to proliferate.

Only the use of a specific xylanase in the wheat-based experimental diets was successful in maintaining the growth performance and reducing the numbers of *C. perfringens* in challenged birds. Also, the use of a specific organic acid product had a significant impact on the actual numbers of *C. perfringens* in the small intestine. This clearly shows that adding an organic acid to the diets can modulate the microflora and could subsequently improve performance. Results from this study were not consistent with previously published data, which showed a significant negative effect of organic acids on growth performance and FCR (Dibner & Buttin 2002).

The key for intestinal health is clearly the ability to maintain a healthy and stable microflora. Progress in the knowledge of the complex ecosystem in the intestine of broilers could make it possible to develop more specific probiotics. Functional benefits of probiotics such as *B. subtilis* include the production of bacteriocins or direct modulation of the microflora (Simon 2003). However, in this study, despite the apparent changes in the microflora no benefit in the growth performance of birds was measured.

These results clearly show that the proposed and tested changes to the NE model developed at UNE have a significant impact on the severity of the disease. The results also confirm that no additive could offer a complete protection against NE although feed enzymes appeared to overcome performance losses caused by a subclinical NE infection.

6. Effect of dietary additives and early feeding on broiler chickens challenged with *C. perfringens*

This study investigated the effects of early nutrition and dietary additives on bird performance, immune competence and gut morphology in broilers under NE challenge as part of a PhD study conducted at the University of New England (Ao, 2004) using the UNE NE Model.

6.1 Experiment 13 – experimental design

A 6 × 2 factorial design was used in this trial. Six dietary treatments consisted of a negative control (control), positive control (Zn-bacitracin, designated as “antibiotic”), enzyme, enzyme + probiotic (designated as “probiotic”), enzyme + prebiotic (Allzyme PT + mannan oligosaccharide, designated as “prebiotic”), enzyme + prebiotic + organic acidifier (designated as “acidifier”). The birds were fed under two holding times; immediate access to both feed and water after hatch (fed) and 48 hours delayed access to both feed and water post hatch (held). Feed additives were supplemented at a rate of 0.5 kg/t (enzyme), 2.0 kg/t (mannan oligosaccharide), 3.0 kg/t (organic acidifier), and 0.25 kg/t (probiotic). Zn-bacitracin was supplemented at the recommended level (50 ppm).

The birds were fed cold-pelleted starter diets through the first three weeks and then cold-pelleted finisher diets. Both the starter and finisher diets were wheat-soyabean meal (SBM) based (the UNE NE diet). The birds were fed a high-protein diet containing 40% fishmeal (with the full dose of supplements) from day eight to day 14 prior to the inoculation with *C. perfringens*.

Six hundred (600) male Cobb broiler chicks were obtained from a local hatchery (Baiada hatchery, Kootingal, NSW) within two hours of hatch. The birds were reared in floor pens in groups of 50 birds per pen. All the birds were wing-tagged and weighed individually upon arrival to the experimental facility. Half of the birds were given access to feed and water immediately after they were weighed, while the other half of the birds were held in chicken boxes in a warm room for 48 hours to mimic the conditions in an incubator prior to giving access to feed and water.

The experimental protocol is as set out in Sections 5.1 and 5.2. The *C. perfringens* inoculant was prepared using the isolate NE-1. Necropsies of all dead birds from day 14 onwards were conducted to determine the cause of death. Mortality associated with lesions of confluent necrosis or sloughing of the epithelial lining of the intestinal tract was considered to be caused by NE (Hembolt & Bryant 1971). For birds that died as a result of NE, intestinal swab samples from the infected area were taken and plated onto *C. perfringens* agar (Oxoid, UK). Total mortality and mortality due to necrotic enteritis were recorded daily.

On days 17 and 20, four birds from each treatment group were randomly selected, weighed, and killed by cervical dislocation. The numbers of *C. perfringens* (CFU/g digesta) in the ileum were measured by plating onto *C. perfringens* agar plate. The small intestine from each killed bird was incised longitudinally and examined for evidence of gross necrotic enteritis lesions (0 = none, 1 = mild, 2 = moderate, 3 = marked/severe).

6.2 Results and discussion

Results are shown in Figures 10 to 12 and Tables 27 to 35. Fed birds had significantly higher bodyweight (BW) throughout the experiment ($P<0.001$). Birds given the prebiotic, acidifier, enzyme and probiotic all had heavier bodyweight at 14 days of age (before *C. perfringens* challenge) ($P<0.001$). The body weight of antibiotic-supplemented birds was similar to that of the control birds at 14 days of age. Furthermore, the held birds given antibiotic had lighter body weight than the control birds ($P<0.001$) at day 14. The data of feed efficiency are not compared for statistical purposes and not presented here because in each treatment group there was a single pen of 50 birds.

The held birds lost 12.3% of their initial body weight during the first 48 hours post hatch, while the fed birds gained 25.6% of the initial body weight during the same period. A higher relative growth occurred in held birds immediately after the birds were given access to nutrients from day three to day 28 ($P<0.001$) (Table 30). However, the growth rate of fed birds became higher during week five ($P<0.001$). Birds given dietary supplements had higher body growth during the first two weeks before *C. perfringens* infection ($P<0.001$). However, the birds given antibiotic exhibited higher body growth from the second week post hatch ($P<0.001$). During week three, when the outbreak of NE occurred, only the birds given the antibiotic-supplemented diet had a higher growth rate, compared to the other treatment groups ($P<0.001$). From week four onwards, birds given the enzyme had higher body growth rate ($P<0.001$).

The NE-related and non-NE related mortalities for the held birds were 14.6% and 6.9%, respectively, both were numerically higher than that of fed birds, which was 12.2% and 2.8%, respectively.

Only the antibiotic was completely preventative of NE outbreak. Holding time had no effect on NE lesion scores and *C. perfringens* counts.

The fed birds had significantly heavier relative bursa weight at 21 days of age ($P<0.05$). Acidifier and probiotic tended to enhance the bursa development when the bird was seven days of age ($P = 0.1$). All the dietary supplements, especially the prebiotic, significantly increased the spleen weight at 14 days of age ($P<0.05$).

There was interaction between early nutrition and dietary treatments on the IL-6 production ($P<0.05$). Only the fed birds given prebiotic supplementation had significantly higher IL-6 production (Figure 10). Early nutrition had no effect on IL-6 production in the birds given acidifier, enzyme, probiotic and antibiotic supplemented diets. The proliferation of the T-cells was significantly enhanced by early access to nutrients ($P<0.001$). All the dietary supplements, except antibiotic, reduced T-cell proliferation ($P<0.05$).

The additives tended to reduce ileal pH ($P<0.01$), which appeared to be largely due to acetic acid content ($P = 0.1$). Overall, birds given dietary supplements, especially the enzyme and acidifier, tended to have higher ileal total VFA contents ($P<0.1$).

Birds given acidifier and enzyme had a lower caecal pH value ($P<0.01$). Birds given the enzyme and acidifier had higher levels of caecal acetic ($P<0.05$), propionic ($P<0.01$), butyric ($P<0.01$) acids, as well as total caecal VFA contents ($P<0.01$).

Table 27. Effects of dietary supplementation and holding time on body and organ growth at seven days of age[1].

	BW (g)	Bursa/BW (%)	Spleen/BW (%)	Pancreas/BW (%)	Liver/BW (%)
Fed					
Control	114^{a}	0.17^{b}	0.071	0.40^{c}	3.41^{b}
Antibiotic	111^{a}	0.17^{b}	0.101	0.56^{ab}	4.20^{ab}
Prebiotic	117^{a}	0.13^{b}	0.085	0.56^{ab}	3.98^{ab}
Acidifier	116^{a}	0.18^{b}	0.082	0.54^{ab}	4.40^{a}
Enzyme	116^{a}	0.14^{b}	0.102	0.53^{abc}	3.84^{ab}
Probiotic	113^{a}	0.28^{a}	0.097	0.66^{a}	3.97^{ab}
Held					
Control	87^{c}	0.13^{b}	0.087	0.57^{ab}	3.74^{ab}
Antibiotic	77^{d}	0.17^{b}	0.080	0.57^{ab}	3.74^{ab}
Prebiotic	87^{c}	0.17^{b}	0.075	0.48^{bc}	4.06^{ab}
Acidifier	94^{b}	0.16^{b}	0.084	0.50^{bc}	3.90^{ab}
Enzyme	92^{bc}	0.16^{b}	0.069	0.59^{ab}	4.02^{ab}
Probiotic	92^{bc}	0.16^{b}	0.087	0.52^{abc}	4.45^{a}
Pooled SE	2	0.03	0.012	0.04	0.24
P Value					
Diet	0.001	0.137	0.857	0.177	0.145
Holding time	0.001	0.285	0.189	0.975	0.892
Diet × Holding time	0.011	0.115	0.415	0.018	0.214

1.Values are means of four replicates.

a, b, c, d Means within a column with different superscripts are significantly different ($P<0.05$).

Table 28. Effects of dietary supplementation and holding time on body and organ growth at 14 days of age[1].

	BW (g)	Bursa/BW (%)	Spleen/BW (%)	Pancreas/BW (%)	Liver/BW (%)
Fed					
Control	302^{b}	0.23	0.086^{abc}	0.54^{ab}	4.35^{b}
Antibiotic	306^{ab}	0.18	0.096^{abc}	0.60^{ab}	4.41^{b}
Prebiotic	322^{ab}	0.17	0.120^{a}	0.64^{a}	4.58^{ab}
Acidifier	317^{ab}	0.19	0.071^{bc}	0.57^{ab}	4.42^{b}
Enzyme	324^{a}	0.23	0.092^{abc}	0.47^{b}	4.84^{ab}
Probiotic	320^{ab}	0.21	0.092^{abc}	0.50^{ab}	4.69^{ab}
Held					
Control	254^{e}	0.19	0.059^{c}	0.62^{ab}	4.13^{b}
Antibiotic	234^{f}	0.17	0.108^{ab}	0.64^{a}	4.46^{b}
Prebiotic	259de	0.18	0.096^{abc}	0.49^{ab}	4.59^{ab}
Acidifier	276^{cd}	0.17	0.096^{abc}	0.60^{ab}	4.35^{b}
Enzyme	269^{cde}	0.20	0.086^{abc}	0.50^{b}	4.78^{ab}
Probiotic	280^{c}	0.19	0.084^{abc}	0.64^{a}	5.32^{a}
Pooled SE	6	0.02	0.011	0.05	0.25
P Value					
Diet	0.001	0.408	0.056	0.137	0.055
Holding time	0.001	0.212	0.488	0.303	0.719
Diet × Holding time	0.095	0.944	0.188	0.092	0.641

1.Values are means of four replicates.

a, b, c, d, e, f Means within a column with different superscripts are significantly different ($P<0.05$).

Table 29. Effects of dietary supplementation and holding time on body and organ growth at 21 days of age[1].

	BW (g)	Bursa/BW (%)	Spleen/BW (%)	Pancreas/BW (%)	Liver/BW (%)
Fed					
Control	662^{b}	0.22^{ab}	0.117^{ab}	0.42^{ab}	3.75^{c}
Antibiotic	805^{a}	0.23^{ab}	0.106^{abc}	0.38^{b}	3.81^{c}
Prebiotic	685^{b}	0.29^{a}	0.089^{abc}	0.42^{ab}	4.17^{abc}
Acidifier	707^{b}	0.22^{ab}	0.126^{a}	0.49^{a}	4.72^{a}
Enzyme	653^{b}	0.26^{ab}	0.120^{ab}	0.38^{b}	4.13^{abc}
Probiotic	692^{b}	0.22^{ab}	0.072^{c}	0.40^{ab}	3.95^{bc}
Held					
Control	583^{c}	0.19^{b}	0.097^{abc}	0.42^{ab}	3.88^{bc}
Antibiotic	655^{b}	0.24^{ab}	0.103^{abc}	0.40^{ab}	3.81^{c}
Prebiotic	537^{c}	0.19^{b}	0.097^{abc}	0.43^{ab}	4.57^{ab}
Acidifier	587^{c}	0.23^{ab}	0.079^{bc}	0.47^{ab}	4.36^{abc}
Enzyme	546^{c}	0.20^{b}	0.099^{abc}	0.43^{ab}	4.51^{ab}
Probiotic	553^{c}	0.21^{ab}	0.109^{abc}	0.47^{ab}	4.28^{abc}
Pooled SE	19	0.03	0.013	0.03	0.21
P Value					
Diet	0.001	0.721	0.625	0.090	0.004
Holding time	0.001	0.045	0.302	0.234	0.234
Diet × Holding time	0.355	0.255	0.049	0.786	0.436

1.Values are means of four replicates.

a, b, c Means within a column with different superscripts are significantly different ($P<0.05$).

Table 30. Effects of dietary supplementation and holding time on relative growth rate of birds at 3, 7, 14, 21, 28 and 35 days of age[1.]

Treatment	Relative Growth (%)					
	0–2 days	3–7 days	8–14 days	15–21 days	22–28 days	29–35 days
Fed						
Control	26^{ab}	92^{c}	166^{e}	127^{b}	76^{bcd}	55^{ab}
Antibiotic	19^{c}	96^{c}	176de	166^{a}	70^{d}	52^{abcd}
Prebiotic	30^{a}	90^{c}	173de	122^{b}	76^{bcd}	53^{abc}
Acidifier	28^{a}	93^{c}	175de	125^{b}	74^{cd}	53^{abc}
Enzyme	26^{ab}	96^{c}	178^{cde}	109^{b}	85^{ab}	57^{a}
Probiotic	22^{bc}	92^{c}	183^{bcd}	126^{b}	76^{bcd}	52^{abcd}
Held						
Control	-12[2.]	115^{b}	184^{bcd}	128^{b}	84^{ab}	47^{cde}
Antibiotic		95^{c}	204^{a}	190^{a}	84^{ab}	43^{e}
Prebiotic		115^{b}	197^{ab}	119^{b}	83^{ab}	46de
Acidifier		132^{a}	194^{ab}	114^{b}	77^{bcd}	33^{f}
Enzyme		124^{b}	193^{abc}	110^{b}	88^{a}	49^{bcde}
Probiotic		127^{a}	204^{a}	107^{b}	80^{abc}	46de
Pooled SE	2	3	5	7	3	2
P Value						
Diet	0.001	0.001	0.004	0.001	0.002	0.001
Holding time	0.001	0.001	0.001	0.760	0.001	0.001
Diet × Holding time	0.001	0.001	0.743	0.043	0.244	0.009

1.Values are means of 20 replicates.

2.Value is the mean of held birds 48 hours post hatch.

a, b, c, d, e, f Means within a column with different superscripts are significantly different ($P<0.05$).

Table 31. Effects of dietary carbohydrate source and post hatch holding time on necrotic enteritis lesion scores and *C. perfringens* counts three and six days after challenge[1]

	Day 17 (3 days after challenge)		Day 20 (6 days after challenge)	
	NE Lesion scores[2.]	**CP Counts (10^5 CFU/g)**	**NE Lesion scores[2]**	**CP Counts (10^5 CFU/g)**
Fed				
Control	2.17^{a}	6.38^{a}	1.38^{a}	0.98^{a}
Antibiotic	0.00^{c}	0.00^{b}	0.13^{bc}	0.00^{b}
Prebiotic	1.88^{a}	1.20^{ab}	0.75^{abc}	0.68^{ab}
Acidifier	1.88^{a}	1.44^{ab}	0.75^{abc}	0.33^{ab}
Enzyme	1.38^{abc}	1.63^{ab}	1.25^{a}	0.64^{ab}
Probiotic	1.63^{ab}	1.50^{ab}	0.88^{abc}	0.64^{ab}
Held				
Control	2.00^{a}	1.24^{ab}	0.75^{abc}	0.03^{b}
Antibiotic	0.25^{bc}	0.00^{b}	0.00^{c}	0.00^{b}
Prebiotic	1.63^{ab}	2.58^{ab}	1.75^{a}	1.10^{a}
Acidifier	2.38^{a}	2.56^{ab}	1.00^{ab}	0.30^{ab}
Enzyme	1.50^{ab}	4.86^{ab}	1.00^{ab}	0.83^{ab}
Probiotic	2.63^{a}	6.54^{a}	1.00^{ab}	0.94^{a}
Pooled SE	0.53	1.84	0.30	0.31
P Value				
Diet	0.001	0.278	0.005	0.085
Holding time	0.373	0.387	0.719	0.937
Diet × Holding time	0.782	0.156	0.156	0.338

1.Values are means of four replicates for each treatment group.

2.Lesion scoring: 0 = no lesions, no inflammation, 1 = slight redness of the intestine, 2 = small lesions, red intestine, 3 = visible gross lesions.

a, b, c Means within a column with different superscripts are significantly different ($P<0.05$).

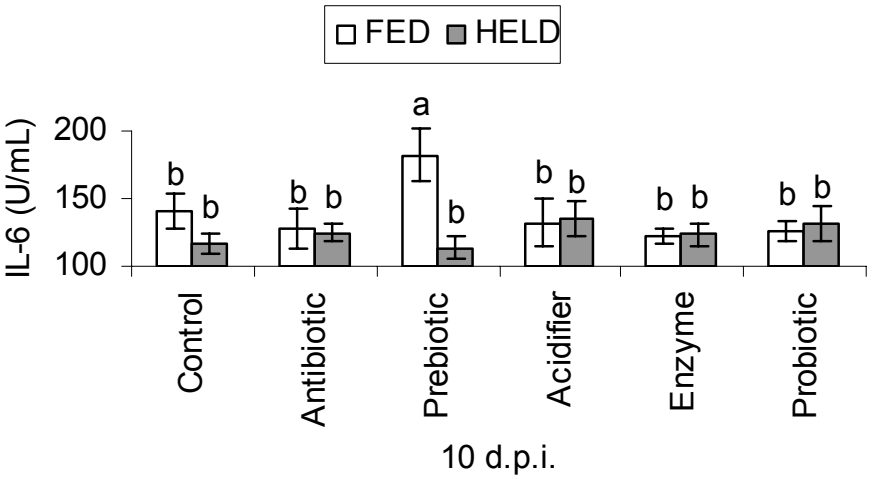


Figure 10. Chicken IL-6 production 10 days post infection (d.p.i.)

(Mean values, n = 6; error bars indicate SE; bars with different superscripts are significantly different).

Table 32. Effects of post hatch holding time and dietary supplements on T-cell proliferation of broilers 10 days post infection (d.p.i.) when stimulated with different levels of Con A, expressed by counts per minute (cpm)[1]

	T-cell proliferation (cpm)			Stimulation index
	50µg/ml Con A	10µg/ml Con A	0µg/ml Con A	Stimulated cpm/ Unstimulated cpm
Fed				
Control	3391^{a}	10666^{ab}	1227^{c}	7.8^{ab}
Antibiotic	2695^{ab}	14038^{a}	1522^{bc}	11.4^{a}
Prebiotic	2999^{ab}	4815^{bcd}	1206^{c}	5.4^{bc}
Acidifier	3363^{a}	6000^{bcd}	1785^{abc}	3.9^{bc}
Enzyme	2953^{ab}	5688^{bcd}	1993^{abc}	3.0^{c}
Probiotic	2777^{b}	7775^{bc}	1475^{bc}	5.4^{bc}
Held				
Control	1908^{ab}	3053^{cd}	1215^{c}	3.3^{bc}
Antibiotic	1096^{b}	914^{d}	772^{c}	1.5^{c}
Prebiotic	1234^{ab}	1427^{d}	1301^{c}	1.4^{c}
Acidifier	1653^{ab}	2291^{cd}	1657^{abc}	1.4^{c}
Enzyme	1577^{ab}	1904^{cd}	2830^{a}	0.9^{c}
Probiotic	2588^{ab}	3657^{cd}	2624^{ab}	1.3^{c}
Pooled SE	796	1856	393	1.5
P Value				
Diet	0.906	0.134	0.009	0.027
Holding time	0.005	0.001	0.390	0.001
Diet × Holding time	0.927	0.075	0.194	0.124

1.Values are means of six replicates.

a, b, c, d Means within a column with different superscripts are significantly different ($P<0.05$).

Table 33. Effects of dietary supplements on ileal and caecal volatile fatty acid content (as is) at day 42[1].

	Acetic Acid (uM/g)	Propionic Acid (uM/g)	Butyric Acid (uM/g)	Total VFA (uM/g)	pH
Ileum					
Control	25.5^{ab}	0.34^{a}	0.19	26.5^{ab}	7.8^{ab}
Antibiotic	21.2^{b}	0.24^{ab}	0.08	21.9^{b}	8.0^{a}
Prebiotic	30.5^{ab}	0.19^{ab}	0.07	31.2^{ab}	6.8^{c}
Acidifier	44.1^{a}	0.15^{ab}	0.44	45.1^{a}	6.6^{c}
Enzyme	45.5^{a}	0.07^{b}	0.45	46.4^{a}	7.0^{bc}
Probiotic	42.8^{ab}	0.15^{ab}	0.11	43.4^{ab}	7.1^{bc}
Pooled SE	7.7	0.07	0.22	7.7	0.3
P Value					
Diet	0.131	0.107	0.658	0.133	0.010
Caeca					
Control	19.1^{b}	2.2^{bc}	2.9^{b}	24.9^{b}	6.5^{a}
Antibiotic	20.2^{ab}	2.5^{abc}	3.9^{b}	27.6^{b}	6.3^{ab}
Prebiotic	15.3^{b}	1.0^{c}	3.9^{b}	20.9^{b}	6.7^{a}
Acidifier	21.2^{ab}	3.1^{ab}	5.5^{ab}	30.5^{ab}	6.3^{ab}
Enzyme	28.4^{a}	4.0^{a}	7.5^{a}	40.8^{a}	6.0^{b}
Probiotic	15.5^{b}	1.7^{bc}	3.2^{b}	20.9^{b}	6.5^{a}
Pooled SE	2.7	0.6	0.9	4.0	0.1
P Value					
Diet	0.023	0.012	0.008	0.014	0.012

1.Values are means of 12 replicates for dietary treatments and 16 replicates for holding time treatments.
a, b, c Means within a column with different superscripts are significantly different (P<0.05).

The fed birds given the additives tended to have a heavier relative pancreas weight at day seven (P = 0.1). While prebiotic supplementation tended to give the held bird a lighter pancreas during the first two weeks post hatch (P = 0.1). All the dietary supplements, except the antibiotic, significantly increased the relative liver weight of the birds at day 21, regardless of holding time (P<0.05).

The intestinal histology was studied before and after the *C. perfringens* challenge and the data are presented in Tables 34 and 35. At day 14, before the *C. perfringens* challenge, the fed birds and the birds given dietary supplements had thinner crypts and greater villus/crypt ratio in the jejunum (P<0.01). They also exhibited increased villus height in the jejunum (P<0.001), but not fed birds given the prebiotic or held birds given the acidifier. Early feeding and dietary supplements had a similar effect on the ileal segment, albeit less apparent. The held birds had thicker submucosa in both the jejunum and ileum (P<0.001). Early feeding had no effect on villus height of the jejunum and ileum. At day 21, only the fed birds given dietary supplements had thinner crypts and greater villus/crypt ratio in the jejunal segment (P<0.001). The held birds given the supplements had thicker crypts and lower villus/crypt ratio in the jejunum (P<0.001). In addition, the antibiotic and acidifier gave greater villus height, but only the antibiotic gave thinner crypts and higher villus/crypt ratio in the jejunum

(P<0.001). Again in the held birds, the acidifier and probiotic increased villus height, while the antibiotic reduced it in the ileum (P<0.001). The fed birds and the birds given dietary supplements had thinner crypts in the ileum (P<0.001).

Early feeding has been reported to enhance the body growth, uniformity and health status of birds (Casteel et al. 1994; Moran & Bilgili 1990; Sklan 2000). Birds with early access to nutrients had improved body weight and flock uniformity. However, those with delayed access to feed and water had higher relative growth after they were given access to nutrients. In this study, the fed birds put on 25.6% of the initial body weight during the first 48 hours post hatch, while the held birds lost 12.3% of the initial body weight during the same period, but the held birds demonstrated superior growth relative to body weight from day three to day 14, before the *C. perfringens* infection. However, after the birds were given the *C. perfringens* challenge, the fed birds seemed to exhibit better growth than the held birds. This was not the case in a previous study when the birds were reared under hygienic conditions without NE challenge, suggesting an interaction between early feeding and disease challenge. Thus, delayed access to nutrients up to 48 hours post hatch may cause a long-term compromise in the ability of birds to resist disease challenge and to gain compensatory growth. All the additives used in the current study failed to prevent the outbreak of NE and improve the growth performance. This is of practical importance because under the commercial production system, broiler chickens face many stressors such as delayed access to feed, dirty litters and frequent change of diets. Therefore, it might be worthwhile to consider early feeding, in combination with good environmental management, to sustain an efficient poultry industry in the post AGP era.

Feed additives such as acidifiers, enzymes, probiotics and prebiotics have all been reported to reduce the colonisation of the intestine by pathogens and subsequently reduce the incidence of some diseases, such as NE, with varying levels of success (Corrier et al. 1995; Sims et al. 2004; Kaldhusdal 2000; Thomson & Hinton 1997). On the other hand, early access to nutrients post hatch has also been claimed to enhance the immune competence and health status of birds, but its effect on prevention of bacterial diseases, for example NE, is not known. In the current study, the antibiotic successfully prevented the outbreak of NE, while all the other supplements failed to prevent its occurrence. The held birds had numerically higher NE scores and *C. perfringens* counts in the intestine, especially three days after the *C. perfringens* challenge. These results indicate that under severe challenge, the current alternative additives do not prevent the outbreak of NE. However, early feeding offered a degree of protection even although the reduction of NE lesion scores and *C. perfringens* counts was not significant. Early feeding may, in combination with other managerial and dietary factors, be considered as an approach to alleviate disease risks in the post-antibiotic broiler industry.

The protective immunity of birds plays an important role in preventing infectious diseases, especially after the use of AGPs is banned. Both the development of the immune system and early nutrition have been widely studied, but little is known about the effects of early nutrition on the development of the immune system of chick. Dibner et al. (1998) suggested that early oral nutrition might influence immune competency. Therefore the first week post hatch is a critical period and the nutritional status of birds may impact the immune system (Klasing 1998). The bursa of Fabricius, a primary immune organ, plays a major role in creating antibody diversity (Uni 1998) and inhibition of bursal development was found to result in failure of normal development of the spleen, the secondary immune organ (Glick 1967). The bursa and spleen were both found to be responsive to environmental stress of birds at an early age (Wyatt et al. 1986). Dibner et al. (1998) found that chicks that had early access to feed and water showed a higher bursa weight as a percentage of body weight, earlier appearance of germinal centres, and better disease resistance than their held hatch mates. This is also supported by the data of Wyatt et al. (1986) who found that broiler chicks held in incubators for 30 hours without access to feed and water before placement showed significantly lower bursa and spleen weights. This suggests that early stress to young broiler chicks, such as holding without feed and water post hatch, alters the immune capability and growth rate later in their life.

Table 34. Effects of post hatch holding time and dietary supplements on the histological development of the small intestine at 14 days of age[1].

	Jejunum				Ileum			
	Submucosa (µm)	Villus height (µm)	Crypt depth (µm)	Villus/ Crypt ratio	Submucosa (µm)	Villus height (µm)	Crypt depth (µm)	Villus/ Crypt ratio
Fed								
Control	16.9^{bcde}	1240.2^{b}	195.6^{ab}	6.7^{b}	20.0^{cde}	555.6^{b}	164.9^{ab}	3.5^{ab}
Antibiotic	15.2^{e}	1221.4^{b}	182.6^{bc}	7.1^{ab}	22.3^{abcd}	685.6^{a}	186.8^{a}	4.0^{ab}
Prebiotic	16.5^{cde}	1069.6^{cd}	158.0^{c}	7.3^{ab}	18.5^{e}	577.8^{b}	147.3^{b}	4.1^{ab}
Acidifier	17.7^{abcd}	1366.7^{a}	187.8^{bc}	7.4^{ab}	23.0^{abc}	684.2^{a}	162.6^{ab}	4.4^{a}
Enzyme	16.2^{de}	1379.7^{a}	189.7^{bc}	7.7^{ab}	21.6^{abcde}	565.8^{b}	179.3^{ab}	3.4^{b}
Probiotic	18.7^{abcd}	1353.8^{a}	170.3^{bc}	8.4^{a}	19.3^{de}	606.8^{b}	183.1^{a}	3.5^{ab}
Held								
Control	20.2^{a}	1059.5^{d}	225.0^{a}	4.9^{c}	25.2^{a}	605.0^{b}	190.4^{a}	3.5^{ab}
Antibiotic	19.5^{ab}	1241.9^{b}	203.4^{ab}	6.4^{b}	23.7^{ab}	684.9^{a}	189.7^{a}	3.7^{ab}
Prebiotic	17.9^{abcd}	1219.2^{b}	180.5^{bc}	7.2^{ab}	23.6^{abc}	686.2^{a}	178.9^{ab}	3.9^{ab}
Acidifier	18.9^{abc}	1166.8^{bc}	178.9^{bc}	7.1^{ab}	19.3^{de}	592.7^{b}	166.8^{ab}	3.9^{ab}
Enzyme	18.4^{abcd}	1364.3^{a}	200.4^{ab}	7.2^{ab}	20.9^{bcde}	607.3^{b}	147.9^{b}	4.2^{ab}
Probiotic	18.6^{abcd}	1407.9^{a}	190.6^{bc}	7.6^{ab}	23.5^{abc}	602.2^{b}	159.1^{ab}	4.1^{ab}
Pooled SE	0.8	36.0	10.1	0.4	1.1	18.2	10.0	0.3
P Value								
Diet	0.251	0.001	0.002	0.001	0.351	0.001	0.081	0.327
Holding time	0.001	0.170	0.007	0.008	0.004	0.106	0.800	0.630
Diet × Holding time	0.106	0.001	0.486	0.514	0.001	0.001	0.008	0.104

1.Values are means of 12 replicates.

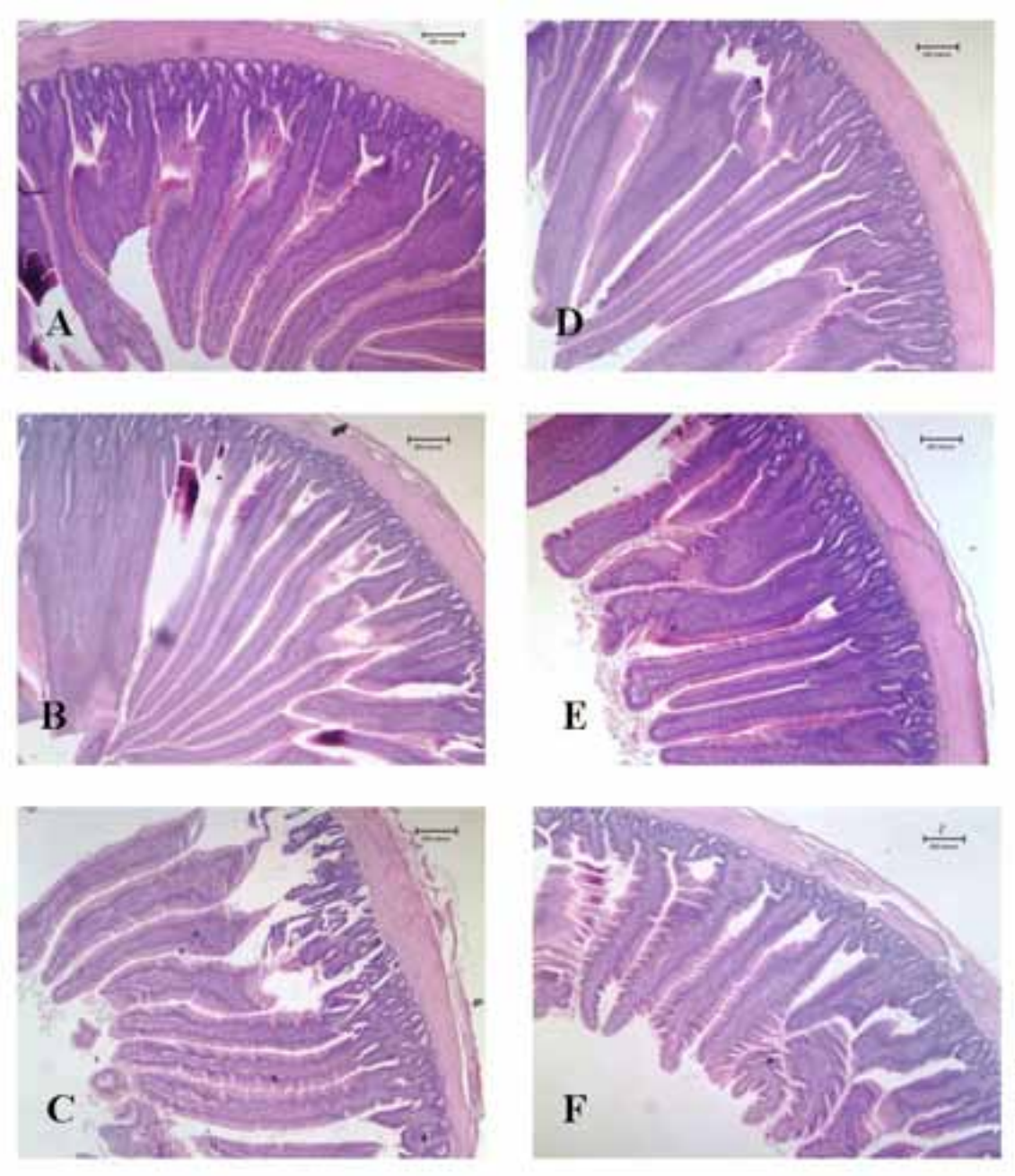
a, b, c, d, e Means within a column with different superscripts are significantly different (P<0.05).

Table 35. Effects of post hatch holding time and dietary supplements on the histological development of the small intestine at 21 days of age[1].

	Jejunum				Ileum			
	Submucosa (µm)	Villus height (µm)	Crypt depth (µm)	Villus/ Crypt ratio	Submucosa (µm)	Villus height (µm)	Crypt depth (µm)	Villus/ Crypt ratio
Fed								
Control	30.0^{a}	1417.9^{abc}	280.5^{a}	5.1^{f}	28.8^{ab}	893.2^{ab}	198.3^{a}	4.8^{d}
Antibiotic	23.2^{bcd}	1542.8^{a}	200.4^{bcde}	8.9^{b}	25.8^{bcd}	772.2^{cde}	102.0^{c}	8.0^{ab}
Prebiotic	21.2^{def}	1239.6^{cde}	174.9^{de}	7.4^{c}	24.4^{bcd}	782.4^{cde}	103.6^{c}	8.2^{ab}
Acidifier	26.6^{ab}	1418.7^{abc}	227.1^{abcd}	6.6^{cde}	28.9^{ab}	890.4^{ab}	116.7^{c}	7.6^{bc}
Enzyme	22.4^{cdef}	1225.5^{de}	200.9^{bcde}	6.2^{cdef}	32.9^{a}	734.9^{de}	105.9^{c}	7.3^{bc}
Probiotic	25.3^{bc}	1212.1^{de}	159.4^{de}	6.1^{cd}	28.1^{bc}	955.0^{a}	112.9^{c}	8.8^{ab}
Held								
Control	19.0^{ef}	1321.3^{bcd}	189.8^{cde}	7.1^{c}	26.4^{bc}	803.5^{bcd}	108.3^{c}	7.7^{abc}
Antibiotic	18.6^{f}	1435.7^{ab}	135.0^{e}	10.9^{a}	21.7^{d}	694.5^{e}	79.1^{d}	9.3^{a}
Prebiotic	19.7^{def}	1062.9^{e}	264.1^{ab}	5.3^{ef}	21.8^{d}	835.1^{bc}	115.9^{c}	7.3^{bc}
Acidifier	22.8^{bcde}	1566.2^{a}	262.1^{ab}	6.1^{cdef}	23.9^{cd}	893.3^{ab}	114.6^{c}	8.0^{ab}
Enzyme	23.3^{bcd}	1514.9^{a}	224.6^{abcd}	7.1^{c}	27.3^{bc}	807.1^{bcd}	110.9^{c}	7.9^{ab}
Probiotic	23.4^{bcd}	1384.9^{abcd}	252.5^{abc}	5.6^{def}	25.9^{bcd}	859.1^{bc}	143.2^{b}	6.2^{cd}
Pooled SE	1.3	60.0	22.3	0.4	1.4	29.3	6.3	0.5
P Value								
Diet	0.001	0.001	0.009	0.001	0.001	0.001	0.001	0.001
Holding time	0.001	0.211	0.438	0.444	0.001	0.184	0.002	0.331
Diet × Holding time	0.001	0.001	0.001	0.001	0.752	0.007	0.001	0.001

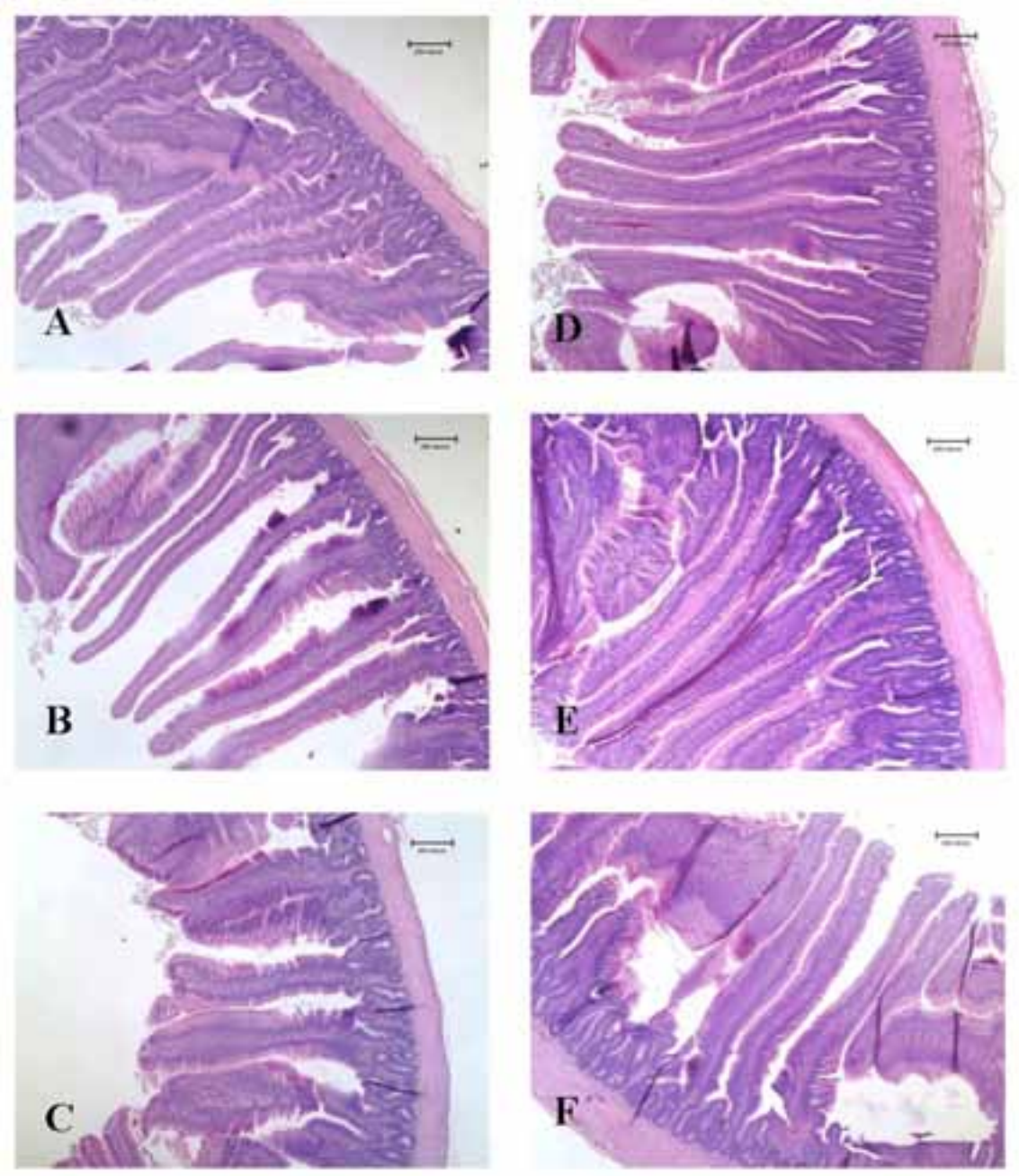
1. Values are means of 12 replicates.

[a, b, c, d, e, f] Means within a column with different superscripts are significantly different (P<0.05).



Sections were stained with hematoxylin and eosin, magnification is 50-fold, and bars are 200 μm (A: Control; B: Antibiotic; C: Prebiotic; D: Acidifier; E: Enzyme; F: Probiotic).

Figure 11. Jejunal sections from 21 day old fed chickens



Sections were stained with hematoxylin and eosin, magnification is 50-fold, and bars are 200 μm (A: Control; B: Antibiotic; C: Prebiotic; D: Acidifier; E: Enzyme; F: Probiotic).

Figure 12. Jejunal sections from 21 day old held chickens

Early access to nutrients did not affect on bursa and spleen development when birds were reared under unchallenged experimental conditions. In the current study, however, under a challenged environment, the fed birds exhibited heavier relative bursa and spleen weights. The same was also true for birds given dietary supplements. This might suggest that when birds are exposed to certain environmental stressors, early feeding and some dietary additives might assist in modulating the development of these lymphoid organs and enhance the immune competence of the bird.

The immune response to infection is controlled by a complex interplay between various cytokines (Kelso & Metcalf 1990). The multifunctional cytokine, IL-6, plays a major role in regulating immune responses, acute phase reactions and haematopoiesis (Hirano et al. 1986). This cytokine is produced

by many different cell types and acts on B-cells, T-cells, hepatocytes, haematopoietic progenitor cells and cells of the central nervous system, immediately following infection or vaccination (Gauldie et al. 1987; Hirano et al. 1986). Since T-cells are the major source of cytokines, the ability of these cells to proliferate in response to their mitogens has been used to determine the development of the immune responses of chickens (Lowenthal et al. 1994). The study suggested that susceptibility of newly hatched chicks to infection is due to a period of transient T-cell unresponsiveness to immune stimulation, a deficiency that may be amenable to cytokine therapy. In this study, only the fed birds given control and prebiotic diets had elevated IL-6 production. This indicates that there is an interaction between early nutrition and dietary supplements. Early administration of prebiotics might augment the production of IL-6.

Early feeding significantly increased T-cell proliferation. Interestingly, the T-cells of the birds given control or antibiotic had significantly enhanced ability to proliferate, while all the other additives had the opposite effects. Such an effect has not been demonstrated elsewhere. The mechanism by which this occurs is not understood. This result, together with the data on IL-6 production, suggests that early access to nutrients post hatch might enhance the immune responses to infectious challenge.

It may be concluded that to date there is no single alternative additive that could play a role similar to that played by antibiotics in terms of controlling infectious diseases and improving the immune competence of birds.

A near neutral pH condition is favourable to the growth of pathogens, including *E. coli* and *Salmonella* whilst a lower pH is more conducive to growth of beneficial bacteria, such as lactobacilli and bifidobacteria (Drasar & Barrow 1985; Hampson et al. 1985). The intestinal pH is influenced by its VFA levels. Volatile fatty acids, especially butyrate, were found to have bacteriostatic function (Hinton et al. 1990; Waechtershaeuser & Stein 2000) and act as a barrier to pathogens by inhibiting the growth and colonisation by detrimental bacteria (Mathew 2001), like *Salmonella*, both *in vitro* or *in vivo* in mice (Bohnhoff et al. 1964; Meynell 1963) and in chicks (Barnes et al. 1979).

In the caeca, the concentrations of acetate, propionate and butyrate present in the un-dissociated state progressively increase as the pH of the caecal contents decreases. The pH of caecal contents of chicks supplemented with lactose was significantly lower compared with control chicks (Tellez et al. 1993). Also, the inclusion of 0.4% and 0.8% Luprosil-NC (a product containing 53.3% propionic acid) decreased the number of coliforms and *E. coli* in the small intestine without affecting intestinal pH (Izat et al. 1990). In the current study, all the tested additives seemed to increase the total ileal VFA content, of which acetate is the main acid, and subsequently lower the ileal pH. In the caeca, however, only the acidifier and enzyme boosted the acetic, propionic and butyric acid levels and hence the total VFA contents. The caecal pH was also reduced by the acidifier and enzyme. These data indicate that these additives might elicit anti-bacterial effects by elevating intestinal VFA contents and reducing the pH of the digesta.

Hydrolysis of macromolecules in the small intestine is achieved, to a large extent, by pancreatic enzyme activities, which are correlated with body weight and intestinal weight (Sklan 2000). Undoubtedly, early access to nutrients stimulates gut development. For instance, the gastrointestinal tract increases in size and weight more rapidly in relation to body weight during early post hatch than other organs and tissues of chickens (Katanbaf et al. 1988; Lilja 1983). The preferential early growth of the small intestine occurs both in the presence and absence of feed, although in the absence of exogenous feed both the absolute and the relative growth rates are lower (Noy & Sklan 1995). The intestine is also the largest immunological organ and contains a large number of lymphocytes, most of them are T-cells located predominantly in the *Lamina propria* (Perdigon et al. 1991). In the current study, the fed birds given dietary supplements had heavier pancreas and liver relative to body weight, especially during the first week post hatch. When the birds got older, the dietary supplements gave the held birds heavier digestive organs relative to body weight. These results indicate that the dietary supplements enhanced the development of GIT and digestive organs and this preferential growth was delayed in held birds.

The interaction between the microflora and the morphology of the intestinal wall is clearly shown by alteration in the structure and morphology of the GIT of germ-free compared to conventional animals (Heneghan 1965). The villi in the small intestine of germ-free species are usually uniform in shape and slender, whereas crypts are shorter (Gordon & Bruckner-Kardoss 1961). Cook and Bird (1973) associated the presence of pathogenic micro-organisms with a change in the intestinal wall and a change in the surface area for nutrient absorption. They demonstrated a shortening of the villi and a decrease in their epithelial layers when counts of pathogenic bacteria increased. In addition, deeper crypts appear. Schneeman (1992) suggests that shorter villi relative to crypt depths result in less absorptive and more secretory cells. This change in morphology in the intestine due to pathogenic organisms is more pronounced in the upper than in the lower parts of the intestinal tract. Coliform bacteria (Truscott & Al-Sheikhly 1977) and *Clostridia* (Kaldhusdal & Hofshagen 1992) in the intestinal tract cause damage to the mucosal layer of the intestine of broiler chicks. Based on these results it is possible that morphological changes in the intestinal wall are indicative of a disturbance in the balance between non-pathogenic and pathogenic micro-organisms. In addition, data showed that chicks with immediate access to feed and water post hatch have increased villus height and surface area of the small intestine, and enhanced crypt development (Uni 1998). The length of villi within the jejunum and ileum of birds receiving an amylase-supplemented corn-soy based diet was significantly increased, with a concomitant improvement in growth rate (Ritz et al. 1995). This might suggest an increased absorptive area capable of greater absorption of available nutrients (Caspary 1992).

In the current study when the birds were 14 days old, just before the *C. perfringens* infection, all the dietary supplements increased the villus height and reduced the crypt depth in the jejunum, thus increasing the villus height/crypt depth ratio. This was particularly obvious in held birds. On day 21, i.e., seven days after the initial *C. perfringens* challenge, the fed birds given the supplements still showed reduced jejunal crypt depth and increased villus/crypt ratio, while in the held birds, the supplements increased the crypt depth and reduced the villus/crypt ratio. The morphology of the ileum followed a similar trend, albeit less pronounced. The results on the intestinal morphology suggest that under unchallenged conditions, both dietary supplements and early access to nutrients enhance the absorptive capacity and reduce the secretion in the small intestine. However, under pathogenic challenge, the gut microflora community might be disturbed for held birds given the supplements.

Addition of an enzyme, acidifier, prebiotic and probiotic to broiler diets enhanced the digestive organ development and absorptive capacity of the small intestine, and improved the growth performance of birds before the *C. perfringens* challenge. However, none of these additives gave the birds the same degree of protection against *C. perfringens* infection as the antibiotic did. Early access to feed and water post hatch improved the absorptive capacity of the small intestine, and enhanced the immune response of birds to pathogenic infection. The birds with delayed access to nutrients showed a greater compensatory growth rate before the *C. perfringens* challenge, but this effect did not persist after the infection.

In conclusion, under challenge, birds with early access to nutrients exhibit superior disease resistance and growth performance through enhanced immunity and intestinal absorption.

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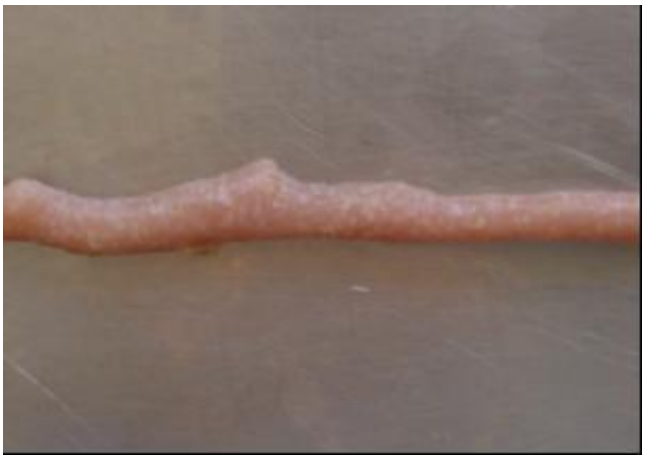
Appendix

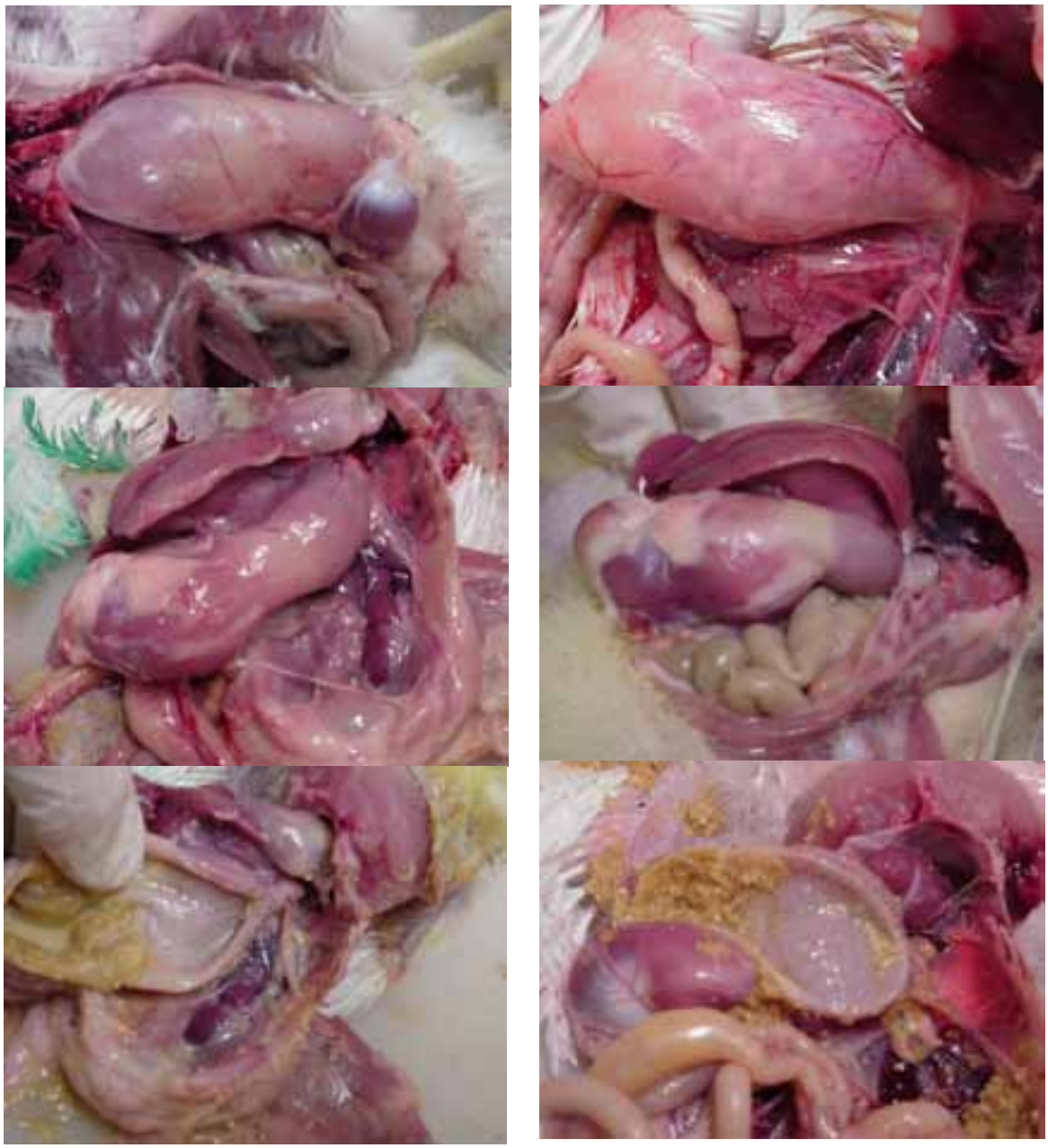
Picture 1: Single bird cages

Picture 2: Semi-commercial units

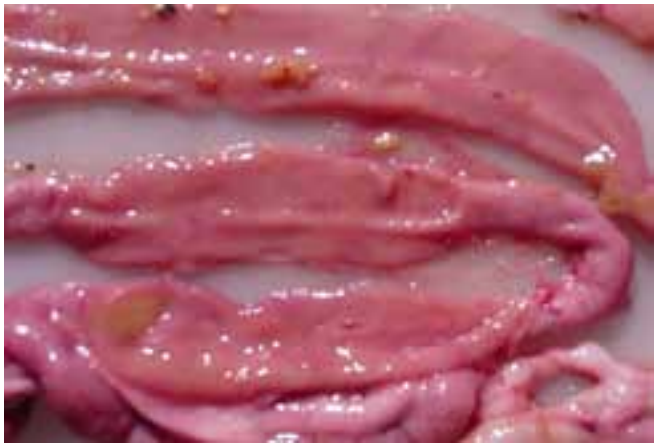
Picture 3: Small floor pens



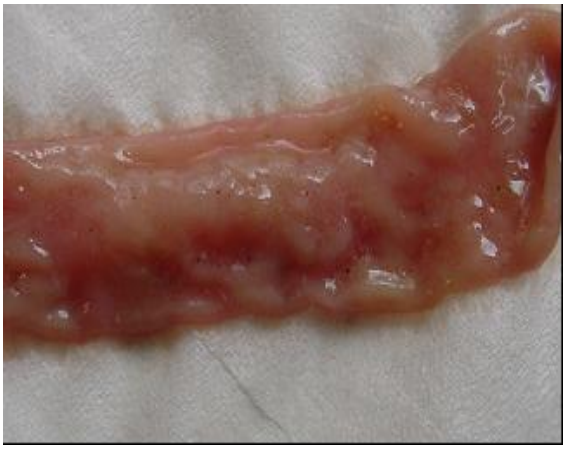
Picture 4: Occurrence of Eimeria lesions after challenge with 7000 each of sporulated oocysts of E. acervulina and E. brunetti



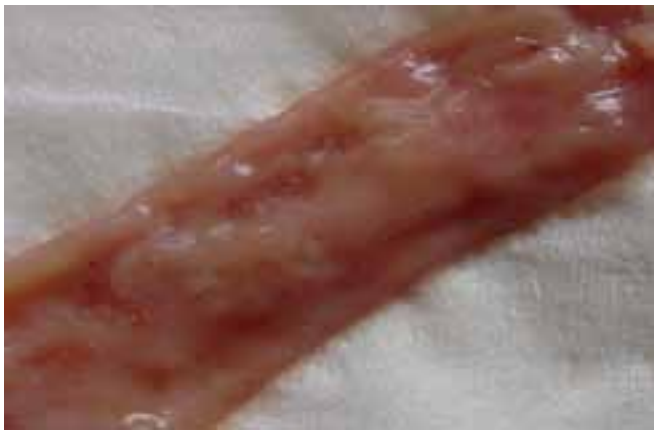
Pictures 4–9: Incidences of enlarged proventriculus (Experiments 5–8)



Picture 10: **NE lesion Score 0**

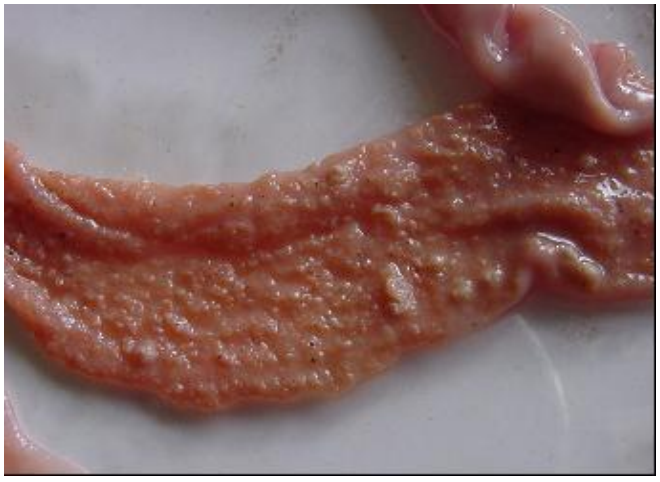


Picture 11: **NE lesion Score <1**

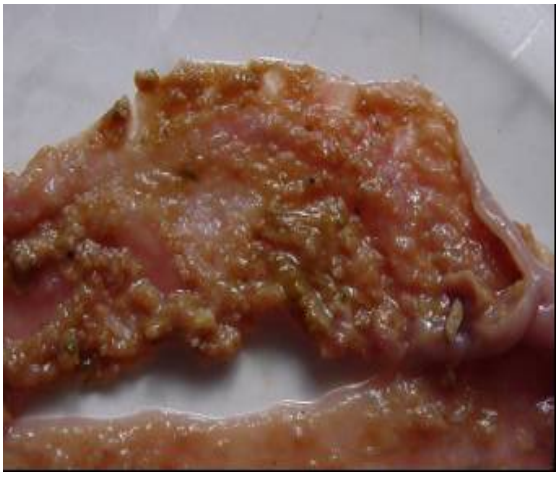


Picture 12: **NE lesion Score 1**

Picture 13: **NE lesion Score 2**



Picture 14: **NE lesion Score 3**



Picture 15: **NE lesion Score 4**

Pictures 10–15: **Necrotic enteritis lesion scores**