Improving broiler performance and gut health using essential oils, organic acids and direct-fed microbials

---

by

Pieter Dewald Steyl

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae Agriculturae

MSc (Agric) Animal Science: Animal nutrition

In the Faculty of Natural and Agricultural Sciences

Department of Animal and Wildlife Sciences

University of Pretoria

June 2019

Supervisor: Dr. C. Jansen van Rensburg

Declaration

I, Pieter Dewald Steyl hereby declare that this thesis, submitted for the MSc (Agric) Animal Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other University.

_____

Dewald Steyl

Pretoria

25 June 2019

Acknowledgements

I want to start by thanking my heavenly Father for making this thesis possible and for giving me strength to finish my work with endurance. His hand and guidance were present throughout the whole process and may all glory be given to Him. Romans 11:36 “For from Him and through Him and to Him are all things. To Him be the glory forever! Amen.”

I want to thank Dr Christine Jansen van Rensburg for all her advice and support during the trial. Her guidance and patience were of great help during the process of finishing my MSc.

I want to thank Cargill for the opportunity to use their products in the trial and for the financial support that enabled me to conduct the study.

A special thanks to Kelly Brannan for helping with the setup of each trial and for giving me guidance during the weighing and sample collection days.

I want to thank Roelf Coertze for assisting with the statistical analysis of the data and interpretation of the results.

I want to thank my wife, Bianca, for all her moral support and help with each trial. You have been a great motivator which enabled me to finish the work with endurance. Your love and patience were of great value in the process of writing this thesis.

To my parents, Dawid and Annette, thank you for love and support during my MSc trial. A special thanks to my father for the financial support during my studies. You have enabled me to pursue my passion while completing my degree.

I thank Armann Reynecke for his assistance during the trial, and his willingness to assist me for countless hours with sample collection and care of the birds.

Last but not least, I want to thank Philagro for their sponsorship of a bursary during my MSc degree.

Abstract

The reduction of antibiotic growth promoter (AGP) use in broiler feed resulted in necrotic enteritis (NE) emerging as a common broiler disease worldwide. In this study, two trials with feed additives and combinations thereof were tested against an AGP (Zinc Bacitracin). In the first trial, broilers were raised in a non-challenged environment and in the second trial, broilers were predisposed to conditions which led to the development of NE. The key risk factor for the development of NE is an intestinal environment that favours the growth of the organism *Clostridium perfringens*. Day-old, male Ross 308 broilers were randomly distributed in an environmentally controlled broiler house, with 12 replicate pens per treatment and 23 birds per pen at the start of each trial. Treatment diets were fed from day 0 for a 35 day grow-out cycle. Apart from a negative control (no feed additives) and a positive control (AGP), three additional treatments were included for each trial. In the first trial, the feed of the first treatment was supplemented with a mixture of essential oil (EO) compounds (Biacid Nucleus, Provimi). In the second treatment a direct fed microbial (*Bacillus subtilis*; DFM) was added to the feed, while a prebiotic (Mannoseoligosacharide, Provimi) was added to the third treatment. When given in combination with AGP, the alternative feed additives resulted in longer duodenal villi (1990.79 µm vs 1876.35 µm) and ileal villi (790.6 µm vs 713.96 µm) than when given alone. However, the alternative feed additives under non-challenging conditions showed no direct increase in performance when given alone or in combination with AGP. In the second trial, the first treatment consisted of a direct fed microbial (*Bacillus subtilis*; DFM), while a blend of essential oil compounds and organic acids (Biacid, Provimi) were added to the second treatment. The feed of a third treatment was supplemented with a mixture of essential oil compounds (Biacid Nucleus, Provimi) and DFM. At 10 days of age, birds received a coccidial vaccine (Immunocox, Ceva) at 10x the prescribed dosage and on day 14 they were orally inoculated with *Clostridium perfringens*. The birds that received Biacid were significantly ($P \leq 0.05$) heavier at 28 days of age compared to birds from the negative control group (1438 g vs 1385 g). Although body weight of the broilers at 35 days of age did not differ significantly ($P > 0.05$) between treatments, feed conversion ratio (FCR; g feed intake/g body weight gain) over the rearing period was significantly ($P \leq 0.05$) lower for broilers supplemented with Biacid (1.83) compared to broilers in the negative control (1.94). Broilers that received the AGP had an FCR of 1.87, while broilers from the DFM and DFM plus Biacid Nucleus treatments had FCRs of 1.87 and 1.84, respectively. The NE scores of broilers supplemented with Biacid and Biacid Nuclease was significantly ($P \leq 0.05$) lower than the NE scores in broilers from the negative control group. It was concluded that Biacid, a combination of essential oils and organic acids, improved performance of broilers that were subjected to conditions favouring the development of NE in broilers.

Table of Content

List of Abbreviations

AEC	Animal Ethics Committee
AGP	Antibiotic growth promoter
AMR	Antimicrobial resistance
AOAC	Association of Analytical Communities
BW	Body weight
CBA	Conjugated bile acids
CFU	Coligny Forming Unit
DT104	Definite Type 104
DAFF	Department of Agriculture, Forestry and Fishery
EO	Essential oils
EU	European Union
FCR	Feed conversion ratio
FOS	Fructo-oligosaccharides
GIT	Gastrointestinal tract
GLM	Generalized linear model
Ig	Immunoglobulin
LAB	Lactic-acid producing bacteria
MOS	Mannose oligosaccharides
NE	Necrotic enteritis
NSP	Non-starch polysaccharides
SE	Standard Error
Spp	Species
SCFA	Short chain fatty acids
WHO	World health organization
VRE	Vancomycin-resistant enterococci
ZB	Zinc Bacitracin

List of Tables

List of Figures

Chapter 1

Introduction

Feed cost alone, in a broiler operation, can contribute up to 75% of the total cost of production. For the poultry industry to function as economically as possible, there needs to be a reduction in feed cost and increase in the efficiency of feed utilization (Rafiullah & Sajid, 2011). Since it is not always possible to reduce feed cost without reducing the quality of feed, the broiler industry relies on improved efficiency to offset the cost of production. The problem with low quality feed in broiler diets is that it can create a variety of problems which eventually results in poor performance and consequently lower returns (Rafiullah & Sajid, 2011). Low quality feeds often contain nutrients indigestible to the animal, resulting in a lot of feed being wasted to the environment. In some instances these feeds can cause sub-clinical infections due to inadequate processing or contamination of feed ingredients (Rafiullah & Sajid, 2011). To increase the efficiency of broiler production, producers need to improve housing conditions, focus on selection for better growth and make use of feed formulation software that matches nutrient requirements of the birds with the nutrient content of feedstuff (M'Sadeq *et al.*, 2015).

The health status of the bird is influenced by its gut health, which consists of three important components, namely, the immune system, the gut microbial population and the integrity of the gut morphology. Because the health of the gastrointestinal tract (GIT) has a direct influence on the digestion of nutrients and immune response, any disturbance of this process can result in enteric disease (M'Sadeq *et al.*, 2015). A weaker immune system makes the bird more vulnerable for *Escherichia coli, Salmonella* spp*.* and *Clostridium perfringens* infections*.* These pathogenic microbes populate the GIT of the host where they will start to compete for nutrients. This will eventually lead to reduced growth rates and performance and potential enteric diseases (Gunal *et al.*, 2006). The most common enteric disease imposing a major economic burden on the poultry industry of today is necrotic enteritis (NE). It has been estimated that NE costs the global poultry industry close to 6 billion dollars annually (Youngsub *et al.*, 2018). NE is caused by the bacterium *C. perfringens* which can be found in the intestine of healthy chickens and the surrounding environment. This bacteria only causes enteric destruction when it grows unchecked, producing toxins (Ao *et al.*, 2012). It is believed that these toxins in the intestinal tract can lead to changes in the intestinal morphology such as shorter villi heights and crypt depths. This will consequently lead to poor digestion and absorption of nutrients, ultimately reducing bird performance (Timbermont *et al.*, 2011; Ao & Choct, 2013). There are a few important predisposing factors for NE, including stress, nutrition, pathogens and the most common factor, coccidiosis. Coccidiosis stimulates mucus production that causes proliferation of mucolytic bacteria such as *C. perfringens* (Lensing *et al.*, 2010; Timbermont *et al.*, 2011).

For decades, broiler productivity was enhanced successfully by in feed inclusion of antibiotic growth promoters (AGP) (Huyghebaert *et al.*, 2011). These antibiotic feed additives have been included in poultry diets not only to promote growth, but to protect the health and maximise the genetic potential of broilers through the modulation of gut microbiota and the host's immune system. AGP works directly on the gut microflora that reduces animal efficiency by competing with the host for nutrients, consequently reducing the absorptive surface areas of the intestine and by causing diseases such as NE. Inclusion of antibiotics in the feed will stabilise the intestinal microbial flora thereby preventing proliferation of the intestinal pathogens (Bozkurt *et al.*,

2008). The livestock industry is becoming more publicly orientated in this day and age, therefore the poultry industry has to shift their attention towards addressing public concern for environmental and food safety. Recently, there has become an increasing concern about the use of antibiotics sub-therapeutically (Paiva & McElroy, 2014; Dittoe *et al.*, 2018). There is a trend that more chickens will be produced under antibiotic free conditions due to the concern of antibiotic resistant pathogens and antibiotic residues in animal products (Bozkurt *et al.*, 2008; Ao *et al.*, 2012). A subsequent ban of AGPs was instituted in the European Union (EU) in January 2006 (Khan & Iqbal, 2015; Pritchard, 2016). One of the major disadvantages of banning AGP is the higher prevalence of economically important diseases such as NE. It will not be long before political pressures force the South African poultry industry to stop using AGP in feeds. As a result, nutritionists will have to find alternative methods of increasing broiler health and performance (Kleyn, 2015).

The ban of AGPs in other countries has led to the search and development of alternative feed additives that will secure the health of the animals and improve the efficiency of poultry production. Alternative feed additives such as essential oils, prebiotics, probiotics, organic acids, and functional carbohydrates have been successfully implemented in poultry diets (M'Sadeq *et al.*, 2015; Dittoe *et al.*, 2018). The main prerequisites for these alternative feed additives are antimicrobial activities, a positive effect on feed conversion and growth of food animals. These products should have a defined mode of action and repeatable performance benefits under commercial conditions (Seal *et al.*, 2013; Pritchard, 2016). The benefits of using these alternatives are no antibiotic resistance development, improved intestinal microflora balance, improved immunity, reduced pathogenic loads and increased energy substrates for intestinal integrity. Besides using alternative feed additives in an antibiotic free environment, novel managerial and environmental measures need to be implemented to prevent diseases. These include modulation of the gut microbes, reducing pathogens in the environment through better management and vaccination to augment an immune response (M'Sadeq *et al.*, 2015). Further research is necessary to find alternatives to antibiotics in the feed, allowing removal of AGP from poultry diets to prevent antimicrobial resistance (AMR) development. This will allow the poultry industry to produce a protein source that adheres to the consumer preference.

The first aim of this study was to test three alternative feed additives on the performance and gut morphology of broilers in unchallenged conditions, with and without in-feed antibiotic growth promoters. The second aim was to test three alternative feed additives on broilers reared under conditions that induced subclinical NE, also in the presence and absence of antibiotic growth promoters, to evaluate the effect on gut health and performance of these challenged broilers.

Hypothesis:

H0: The three feed additives will not improve broiler performance and health under non-challenged conditions.

HA: The three feed additives will improve broiler performance and health under non-challenged conditions.

H0: The three feed additives will not improve broiler performance and health under conditions that will predispose them to NE.

HA: The three feed additives will improve broiler performance and health under conditions that will predispose them to NE.

Chapter 2

Literature review

2.1 Introduction

The South African poultry industry forms a large segment of the agricultural sector and contributes more than 16% of its share to the gross domestic product (Bolton, 2015). An increase in demands for higher quality protein, improvement in management, better housing conditions and genetic selection have transformed the broiler industry from backyard activities over 10 decades into a highly integrated industry where success requires economic investments (Cooper, 2007; Bolton, 2015). With this development came a lot of challenges. The main challenge the industry is currently facing is the United States' African Growth and Opportunity Act. This agreement puts a lot of pressure on South African broiler producers which cannot compete with cheap imports. According to the South African Poultry Association, 457 347 tons of broiler meat was imported during the year 2015. This was already 24% higher than total imports during 2014. In June 2016 alone, 54 048 tons of broiler products were imported into South Africa. Frozen broiler imports came from EU (46.9%), from Brazil (42.8%) and from the US (5.5%). Although South African poultry producers are efficient compared with international producers, the rising feed costs and persistent droughts faced these past years create an environment where growers are becoming less competitive in global markets (Bolton, 2015). The South African poultry industry is also facing a global trend driven by consumers to move away from in feed antibiotics. The aim of this review is to provide an overview of the consequences when removing AGP from broiler diets and to evaluate the alternative feed additives that can effectively replace AGP.

2.2 Etiology of necrotic enteritis

Necrotic enteritis is a notorious enteric disease in the poultry industry and cost the industry up to six billion US dollars annually (Youngsub *et al.*, 2018). In the sub-acute form, the disease can result in dehydration, diarrhea and lower feed intake. The subclinical form of the disease is becoming more prominent in the industry and this has been identified by reports showing an increase in intestinal mucosal damage even though there is no increase in mortality (Antonissen *et al.*, 2014). Birds that suffer from the disease sub-clinically have a lower performance due to decreased digestion and absorption of nutrients (Lorenzoni, 2010; Shojadoost *et al.*, 2012). A decrease in performance and increased mortality will significantly affect the profitability of broiler production and therefore it is important to investigate the cause of the disease.

Necrotic enteritis is caused by toxins produced by *C. perfringens*, which are spore forming, anaerobic, gram-positive bacteria occurring ubiquitously in the environment and the GIT of animals and humans (Antonissen *et al.*, 2014; Caly *et al.*, 2015). The main risk for development of the disease is an intestinal environment that favours the growth of *C. perfringens.* A small number of *C. perfringens* can be found in the GIT of poultry under normal conditions (Cooper & Songer, 2009; M'Sadeq *et al.*, 2015). The amount of *C. perfringens*, however, is different between healthy birds and NE-affected birds. The population can be less than 10^2 to 10^4 colony-forming units (CFU) per gram intestinal contents in healthy birds compared to more than 10^7 CFU/g intestinal contents in NE infected birds. The presence of *C. perfringens* alone does not always lead to the development of NE. In most cases, there needs to be predisposing factors such as the diet, animal

husbandry and immune suppression (Cooper & Songer, 2009; Antonissen *et al.*, 2014). The nature of the diet is a common non-bacterial factor that can lead to the establishment of NE. Research have shown that feed such as barley, rye or wheat, containing high levels of non-starch polysaccharides (NSP), predispose chickens to NE (Yegani & Korver, 2008; Shojadoost *et al.*, 2012). Soluble NSPs form high molecular weight aggregates in the digesta, consequently increasing the viscosity of digesta, which reduces the diffusion of enzymes towards the substrates. The decrease in digestion will slow the passage rate of digesta through the intestine, allowing pathogens to proliferate due to the excess nutrients available. This might explain why there is a correlation between the amount of clostridia and intestinal viscosity (Black, 2000; Shojadoost *et al.*, 2012). Certain feed ingredients can also lead to the development of NE by oversupplying nutrients for the microbial population. A study have found that diets containing fishmeal in higher levels than normally allowed, can lead to the overgrowth of *C. perfringens* (Williams, 2005). The high levels of fishmeal increase the concentration of certain amino acids in the GIT. Glycine, for example, is one of the predisposing amino acids responsible for the production of alpha toxin (Shojadoost *et al.*, 2012) by *C. perfringens*, which increases intestinal damage, leading to the development of NE. (Yegani & Korver, 2008; Shojadoost *et al.*, 2012). Although the diet is an important factor that contributes to the development of NE, it has been found that coccidiosis is one of the main predisposing diseases that leads to the development of NE in poultry (McDevitt *et al.*, 2007; Cooper & Songer, 2009; Lee *et al.*, 2011; Timbermont *et al.*, 2011). Coccidiosis is a parasitic disease caused by *Eimeria* spp. with some species (*E. maxima*) causing more severe diseases than others. The mechanism on how coccidiosis predisposes the bird to NE have been studied thoroughly and it was found that eimeria parasites colonise the small intestine and kill epithelial cells through their life cycle. Damage to the epithelial layer causes leakage of plasma into the GIT, which provides a protein-rich nutrient substrate for *C. perfringens*, allowing the bacteria to proliferate and produce toxins (Williams, 2005; Cooper, 2007; Timbermont *et al.*, 2011; Shojadoost *et al.*, 2012). Wet litter combined with high animal densities are the key factors for the accelerated replication of coccidia, and therefore animal husbandry is important to prevent coccidial outbreaks (Cooper & Songer, 2009; Lorenzoni, 2010). The traditional prevention of NE is based on the constant inclusion of low doses of antibiotics and the adequate control of coccidia. Since the ban of AGP it has become increasingly important to prevent coccidial infections and other factors that will stimulate the proliferation of *C. perfringens* (Al-Sheikhly & Al-Saieg, 1979; Lorenzoni, 2010).

C. perfringens causes damage to the intestinal epithelium through the toxins it produces. A total of seventeen exotoxins and enterotoxin-encoding genes (Lee *et al.*, 2011) have been identified and the bacterial types (A, B, C, D or E) are classified according to the toxins (Cooper & Songer, 2009) they produce (alpha, beta, epsilon and iota). In the past it was believed that the α-toxin is the major toxin involved in NE. Although this toxin is produced by all five types of bacteria, there is evidence arguing strongly against the role of α-toxin in NE outbreaks (Nauerby *et al.*, 2003). There has been an increase in reports that α-toxin lacks a role in NE development and one such study showed that when using an α-toxin-negative-mutant of *C. perfringens* , the isogenic mutant toxin was still able to cause NE lesions (Van Immerseel *et al.*, 2009). The NetB toxin has been identified by genomic sequencing of a gene that had been sampled from an NE isolate. There is also reports that most of the NE outbreak strains carry the NetB gene, stressing the importance of this toxin in the development of NE, since *C. perfringins* isolates from other diseases lacks the NetB gene (Van Immerseel *et al.*, 2009). During the initial stages of NE, there is an increase in the release of proteolytic enzymes which

disrupts the lining of the GIT (Van Immerseel *et al.*, 2009). This will reduce the absorptive surface of the small intestine due to shorter and fewer functional villi present. The consequence of this is that there will be a malabsorption of nutrients which have a negative impact on production parameters such as body weight gain and feed conversion.

2.3 Antibiotic growth promoters

The South African poultry industry has an advantage over Europe and most of the USA broiler producers by still being allowed to use AGP in feed. AGP are cost effective at very low doses, yielding a higher return on investment, giving the broiler industry a competitive edge in meat production. The term AGP is used to describe any medicine that destroys or inhibits bacteria and is administered at a low dose. This is called sub-therapeutic use because instead of treating a specific disease, the antibiotics are used to improve the growth and health of the animal it is administered to (Barton, 2000; Van Immerseel *et al.*, 2009).

The exact mechanism of AGP have not been completely elucidated. Knudsen (2001) proposed five mechanisms in his studies by which AGP could achieve its goal in improving weight gain. The first mechanism proposed is the improved efficiency of nutrient uptake from the gut in AGP treated animals. Besides preventing pathogens from competing for nutrients, sub-therapeutic use of antibiotics can change the architecture of the gut wall that results in a thinner epithelial layer (Dittoe *et al.*, 2018; Salim *et al.*, 2018). This thinning of the gut wall allows the host to absorb and utilise more nutrients (Cox, 2016). The reason for this thinner epithelial layer can be explained by a reduction in the muscular thickness of jejunum and ileum when birds are treated with antibiotics (Barton, 2000; Gunal *et al.*, 2006; Van Immerseel *et al.*, 2009). Secondly, the sub-therapeutic use of antibiotics reduces the bacterial load within the GIT of the bird, allowing for more nutrients to be utilised by the host. This means that more of the available nutrients will be used for growth rather than energy demanding processes such as an immune response or disease resistance (Bozkurt *et al.*, 2008; Van Immerseel *et al.*, 2009; Lorenzoni, 2010). Thirdly, AGP reduces harmful gut microbes that may cause subclinical infections and consequently reduce the host's production performance. AGP decreases the pathogenic load in the intestine, and consequently the toxins produced during pathogenic infections.

During a pathogenic infection, lymphocytes accumulate to kill pathogenic bacteria, causing inflammation that leads to muscular thickening in the intestine (Gunal *et al.*, 2006; Erik & Knudsen, 2007). During a severe inflammatory response, 41% of the observed growth depression in broilers can be explained by immune responses and associated inflammation (Bozkurt *et al.*, 2008; Lorenzoni, 2010). A decrease in the pathogenic bacterial load will prevent the excess production of toxins responsible for causing lesions on the intestinal mucosa. Less epithelial damage can increase the energy available for production and growth since the healing process involves the use of resources to repair damaged cells (Gunal *et al.*, 2006; Lorenzoni, 2010). Fourthly, the production of growth-suppressing toxins or metabolites is reduced in AGP treated animals, for example, *C. perfringens* type- A producing Beta-toxins that cause intestinal epithelial necrosis (Das *et al.*, 2008). The fifth proposed mechanism of AGP is the reduction in microbial deconjugation of conjugated bile acids (CBA). The majority of lipids are digested and absorbed in the small intestine and CBA consists of a hydrophobic steroid core that is conjugated with glycine or taurine. CBA is thus amphipathic and their function is to emulsify and solubilise lipids for fat digestion (Lin, 2014). Anaerobic bacteria such as lactobacilli can often colonise the

small intestine in large numbers. These bacteria have bile salt hydrolase enzymatic activity that deconjugates bile salts and consequently decrease bile salt absorption. It can thus be concluded that bile salt hydrolase activity physiologically impacts the host by disturbing the CBA-mediated fat metabolism and endocrine function (Lin, 2014; Cox, 2016). Early studies have found that the inclusion of AGP in the diet demonstrated enhanced bioavailability of α-tocopheryl acetate in broilers, which can be attributed to reduced concentrations of unconjugated bile salts. AGP supplementation also led to improved weigh gain and fat digestion, as well as decreased populations of *Lactobacillus salivarius* (Lin, 2014; Cox, 2016).

Although the South African poultry industry is still using AGP sub-therapeutically, the trends experienced in other parts of the world will dictate the industry's future in South Africa (Kleyn, 2015). Concerns regarding the use of AGPs began soon after they were implemented during the 1950's. During 1969, the Swann committee was established by the British government with the aim to reduce the use of AGP and consequently reduce the risk of resistance development to drugs used in human medicine (Barton, 2000; Cogliani *et al.*, 2011). The response came after scientists discovered transferable oxytetracycline resistance from food animals to *Salmonella enterica*, the most common organism to cause food-borne diseases (Cogliani *et al.*, 2011; Hur *et al.*, 2012) The Swann committee recommended that antibiotics used in animal feed should have no therapeutic application in human medicine which can potentially impair the efficacy of therapeutic antibiotics (Aarestrup, 1999). As a result penicillin and tetracycline used as growth promoters were terminated in many European countries during 1972-1974 (Cogliani *et al.*, 2011). In 1986, Sweden became the first country to ban the use of antibiotics in animal feeds without a veterinary prescription after consumers lost confidence in meat safety (Barton, 2000; Casewell *et al.*, 2003; Cogliani *et al.*, 2011; Maron *et al.*, 2013; Dittoe *et al.*, 2018). The use of antibiotics sub-therapeutically came under a lot of public scrutiny following the ban of AGP in Sweden (Gunal *et al.*, 2006). This was the main driving force behind the removal of avoparcin and virginiamycin in Denmark between 1995 and 1998. The amount of AGP used sub-therapeutically the following 6 years decreased by up to 35%, while there was an increase in therapeutic use of antibiotics. The increase in therapeutic use of antibiotics forced the government to place a monetary cap on veterinarians' profits from antibiotic sales. (Casewell *et al.*, 2003; Maron *et al.*, 2013). The European Union soon followed with a ban of major AGP tylosin, spiramycin, bacitracin, virginiamycin, carbadox and olaquindox between 1997 and 1999 as a precautionary principle (Cogliani *et al.*, 2011). This move forced the rest of the industry to ban AGP use in the EU on the 1st January 2006 (Bozkurt *et al.*, 2008; Maron *et al.*, 2013; Pritchard, 2016).

Resistance to antibiotics when using AGP in animal feed is the main reason for banning AGP in most parts of the world (Salim *et al.*, 2018). The sub-therapeutic use of antibiotics may exert genetic pressure on certain bacterial communities, favouring those that are able to resist antibiotic challenges (Erik & Knudsen, 2007; Lorenzoni, 2010). This created a concern among the consumers that antibiotics used by humans and animals results in the development of AMR among food-borne bacteria that could interfere with the efficacy of health therapies (Papatsiros *et al.*, 2013; Seal *et al.*, 2013). The first concern is that antibiotic resistant genes can transfer from animal enteric flora to human pathogens. An example of this is antibiotic resistance was reported in salmonella soon after AGP were fed in animal diets. Since salmonella is recognised as a food borne pathogen, concerns were raised soon after they discovered antibiotic resistance in these bacteria during 1984, especially the emergence of Definite Type 104 (DT104) (Aarestrup, 1999; Barton, 2000; Hur *et al.*, 2012). *Salmonella typhimurium* DT 104 is a multidrug resistant strain that became increasingly widespread

throughout the world during the 1990's. During this period, DT 104 acquired decreased susceptibility to quinolones as a result of a chromosomal mutation in the *gyr A* gene, which encodes the target sites for quinolones (Furuya & Lowy, 2006).The second concern is that therapeutic antibiotics used to treat human diseases, will not affect the antibiotic-resistant bacteria and thus cannot prevent the diseases in humans, because of the long-term exposure these microbes had to the antibiotics that have been administered to food producing animals (Barton, 2000; Seal *et al.*, 2013). The continued use of antibiotics results in the accumulation of antibiotics in the GIT of animals, which leads to the accumulation of antibiotic resistant genes in the animal. According to Schjorring & Krogfelt (2011), the indigenous flora can become a reservoir of antibiotic resistant genes. The main disadvantage of this is that these microbes can transfer the resistant genes further to pathogenic bacteria through a process known as horizontal gene transfer, limiting the treatment possibilities. During the process of horizontal gene transfer, genetic material contained in the DNA of microbes is transferred between bacteria of the same species or different species. Bacteria can acquire these resistant genes through different mechanisms (Figure 2.1). The first mechanism is referred to as conjugation, where bacteria fuse and exchange plasmids and chromosome fragments. The other two are transfection, where viruses infect bacteria and in the process pass genes from one organism to the next, and transformation, where bacteria undergo lyses, releasing genes in the environment which can be picked up by other bacteria, leading to the development of resistance (Bbosa *et al.*, 2014).

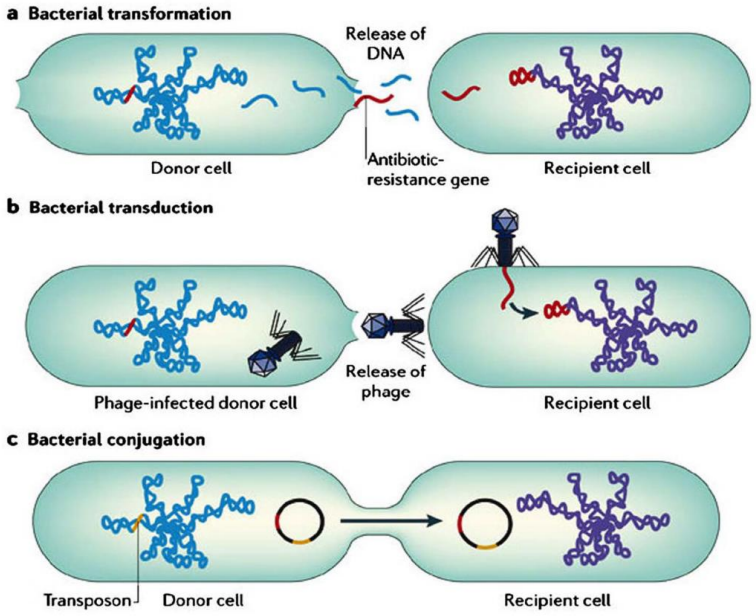


Figure 2.1 Antimicrobial-resistant bacteria in the community setting (Bbosa *et al.*, 2014)

When humans consume products from these animals containing antibiotic resistant microorganisms, the gene pool of antimicrobial-resistant genes are transferred to them, leading to a built up of antibiotic-resistant microorganisms in humans (Schjorring & Krogfelt, 2011). Consequently, the antibiotics used to treat human diseases are becoming less bellicose as time persists. When AMR are transferred to bacterial pathogens occurring in humans, the resistance to drugs will increase suffering among patients and raise death rates due to treatment failures (Cogliani *et al.*, 2011).

In the past, most of the concerns regarding AMR in animals were directed towards pathogenic bacteria such as salmonella and *Escherichia coli*, with fewer interest in commensal organisms like enterococci or campylobacter, which is considered to be less pathogenic in animals (Barton, 2000). Investigations done in multiple countries, however, have confirmed that there is a close association between the use of AGP and resistance to medically important antibiotics, especially those used to treat enterococci (Wegener, 2003). There are three proposed mechanisms on how these bacteria develop resistance against antibiotics to defend themselves. The first mechanism is where resistant bacteria retain the same sensitive target as antibiotic sensitive bacteria, but prevent antibiotics from reaching it by modifying the bacteria. An example is the β lactamase enzyme activity of some resistant bacteria which cleaves the four membered β lactam ring of the antibiotic, rendering it inactive (Hawkey, 1998). Most of these β lactamases are widespread among many bacterial species and act against penicillin and cephalosporin. The second mechanism of resistance development is where bacteria pump out antibiotics faster than it can enter the cell or preventing it from entering the cell at all. The third proposed mechanism is where bacteria produce an alternative target site such as an enzyme, which is resistant to antibiotic inhibition. The bacteria will continue to produce the sensitive site as well, but the antibiotics will not target it when alternative sites are present (Hawkey, 1998).

The aim of banning AGP in the poultry industry is to limit the use of antibiotics that increases the occurrence of resistant bacteria in the birds and consequently restore the microbial flora of these animals to a beneficial microbial population (Cogliani *et al.*, 2011). Denmark successfully banned the use of antibiotics, resulting in a reduction from 210 tons antibiotics in 1994 to 94 tons in 2000. Before Denmark started to ban the use of avoparcin in livestock, more than 80% of the broilers had been identified with vancomycin-resistant enterococci (VRE), while 20% of swine had VRE. In 2001 this number decreased to 3% in both poultry and swine (Singer *et al.*, 2003; Jacobsen & Jensen, 2004). Although some studies observed a diminution of the bacterial resistant pool in both humans and animals following the ban of AGP, other studies revealed that these agents had important prophylactic activity, and their withdrawal from the industry resulted in deterioration of animal welfare (Casewell *et al.*, 2003). There were reports from Danish farmers that the incidence of enteric infections increased during the time after the ban in 2000. This was supported by other findings where Danpo in Denmark diagnosed 25 flocks with NE compared to 1-2 flocks when AGP was used (Casewell *et al.*, 2003; Jacobsen & Jensen, 2004).

In France, the incidence of NE rose from 4% to almost 13% over a four-year period following the ban of AGP. Despite the efforts to improve disease control through better management, increasing evidence suggests that once antibiotics are removed from the diet, there is an increase in diseases and infections, which consequently increase the use of antibiotics therapeutically (Yegani & Korver, 2008; Cooper & Songer, 2009). The use of therapeutic antibiotics continues in the livestock industry despite being banned for prophylactic use, while multidrug resistant bacteria continues to spread among food animals. In Dutch farms there is still a high prevalence of resistant *E. coli* and salmonella in broilers while fluoroquinolene resistant *Campylobacter* spp. are becoming a major problem in poultry. From this experience it is clear that banning AGP without replacing it with alternative feed additives, will result in increased use of antimicrobials therapeutically (Cogliani *et al.*, 2011; Timbermont *et al.*, 2011). It is therefore important, not only to improve management and disease control, but to find alternative feed additives with the same prophylactic properties as AGP, with a positive effect on feed conversion and growth (Seal *et al.*, 2013).

Alternative feed additives

2.4 Essential oils (Phytobiotics)

Plant products have been used from ancient times to treat humans and animals. The Interest in using plant extracts in livestock feed has increased in the past decade, mainly due to concerns about the development of AMR when using antibiotics (Wallace *et al.*, 2010). Plant extracts are less toxic and residue free, making them the ideal alternative to AGP when used as a feed additive in animal production (Yang *et al.*, 2009). The ban of antibiotics in animal feed has led to the rapid expansion of organic farming (Lorenzoni, 2010) and this is driving the search for alternative feed additives that are safe, while promoting the animal's production performance and improving the quality of the food derived from those animals (Papatsiros *et al.*, 2013). Plant extracts are not only considered to be antibacterial, but have additional properties that are beneficial to the animal. Menthol for example can stimulate feed intake while increasing endogenous secretions such as bile (Wallace *et al.*, 2010; Papatsiros *et al.*, 2013). Plant products, also known as phytobiotics, can be classified into four subgroups: 1) herbs, 2) botanicals, 3) essential oils and 4) oleoresins (Yang *et al.*, 2009). The main plant extract focused on in this study was essential oils (EO) and how they can effectively replace AGP in animal feeds.

Essential oils are volatile, aromatic compounds with different structures and properties. Cinnamaldehyde for example is an aliphatic aldehyde while carvacrol and thymol are phenolic compounds (Bento *et al.*, 2013). EO act mainly along the digestive tract of the animal and can be used to stimulate feed intake by improving the animal's appetite. EO can also alter the bacterial population to benefit the wellbeing of the animal and stimulate intestinal endogenous enzymes to aid in digestion. A study by Jang *et al.* (2007) investigated the effects of EO on digestive enzymes and found that feeding EO extracted from herbs improved the secretion of digestive enzymes from the pancreas in broilers. EO can increase the concentration of amylase and other endogenous enzymes such as trypsin that can alter food substrates, for example reducing the digesta viscosity (Williams & Losa, 2001). The benefit of this is that birds will have a healthy gut due to faster passage rates, and the percentage of sticky droppings is also reduced, especially in wheat and barley diets. Feed efficiency was found to increase by 5% from 1 to 40 days in a broiler trial using wheat and barley, when supplemented with EO (Williams & Losa, 2001). In addition to digestive enzymes, EO also increases the secretion of leucine aminopeptidase and alkaline phosphatase. These enzymes are released by the microvilli membranes in the GIT and play an important role in the degradation and absorption of nutrients (Jang *et al.*, 2007).

Antimicrobial activity and immune stimulation are two of the most beneficial multifunctional properties derived from EO (Jang *et al.*, 2007; Wallace *et al.*, 2010). Research done on the antimicrobial activity of EO has mainly focused on *in vitro* studies, while more studies are being done with live poultry flocks to prove that EO can effectively replace AGP in the feed. One such study showed that the blend of EO could be used to control *C. perfringens*, *Salmonella typhimurium* and *E. coli* through its antimicrobial activity (Griggs & Jacobs, 2005). Although there is a synergistic effect when a blend of oils is given, the mechanism of action differs

between these components in the way they exert their antimicrobial activities. Thymol and carvacrol for example, bind with the hydrogens on the cell membrane which affects membrane permeability by releasing K^+ ions and ATP. Cinnamaldehyde and eugenol on the other hand, bind with proteins on the cell membrane and inhibit the activity of cell enzymes, consequently leading to the destruction of the cell (Nazzaro *et al.*, 2013). All these components have more than one mechanism, but in principle these compounds disturb the integrity and function of bacterial cell membranes. The antibacterial activity of EO is thus a function of both the chemical structure of the feed additive and the target sites on the bacterial cell wall. (Bento *et al.*, 2013). There are many more ways in which EO can act along the cell structure of microbes to result in a loss of microbial viability (Figure 2.2).

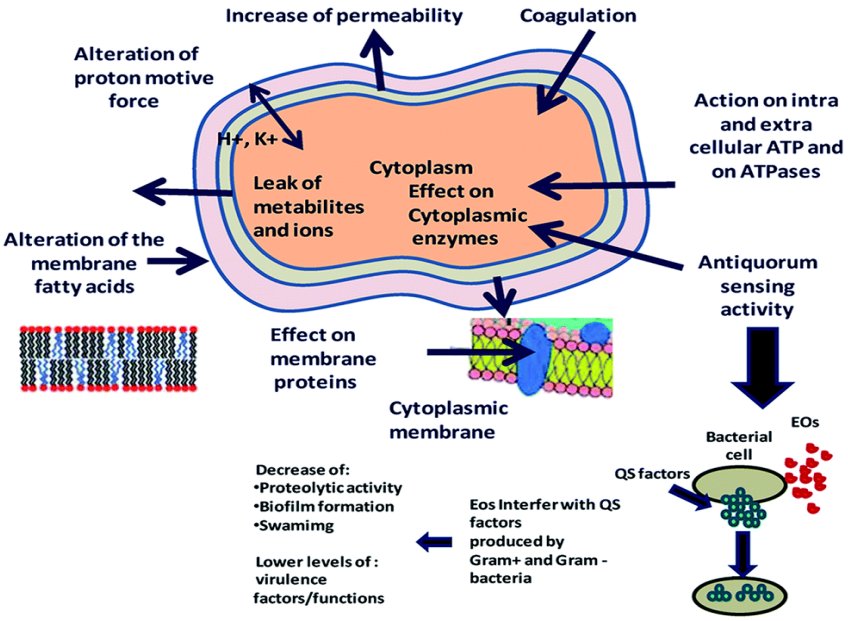


Figure 2.2 Mechanism of action and target sites of essential oils on microbial cells (Nazzaro *et al.*, 2013)

Although EO provide an alternative to antibiotics, some classes of EO are not effective enough against bacterial species. For example, the antimicrobial activity for ginger and pepper is rather weak compared to garlic and cinnamaldehyde (Huyghebaert *et al.*, 2011). Like antibiotics, EO can modify the gut microflora and reduce microbial load by preventing bacteria from proliferating. In addition to being antimicrobial, EO have some anticoccidial properties (Yang *et al.*, 2009) by reducing the intestinal populations of *E. coli* (Jang *et al.*, 2007). EO can increase the turnover of the gut lining and prevent a coccidial attack by maintaining a more healthy population of gut cells (Ferket, 2004). Although this mode of action can increase the animal's maintenance requirement, due to the higher proportion of enterocyte turnover, it prevents coccidial infections which is one of the main pre-requisites for developing NE (Al-Sheikhly & Al-Saieg, 1979).

The polysaccharide components of EO are the most important immunoactive components when it comes to establishing a healthy immune system. A review by Yang *et al.* (2009) found that the complex mixture of bioactive components can enhance both the cellular and humoral immune responses of chickens. An EO blend that consisted out of thymol and cinnemaldehyde have shown to increase the proportion of butyrate in the caecum, which is an important energy source for the epithelial cells in the gut that helps to support intestinal

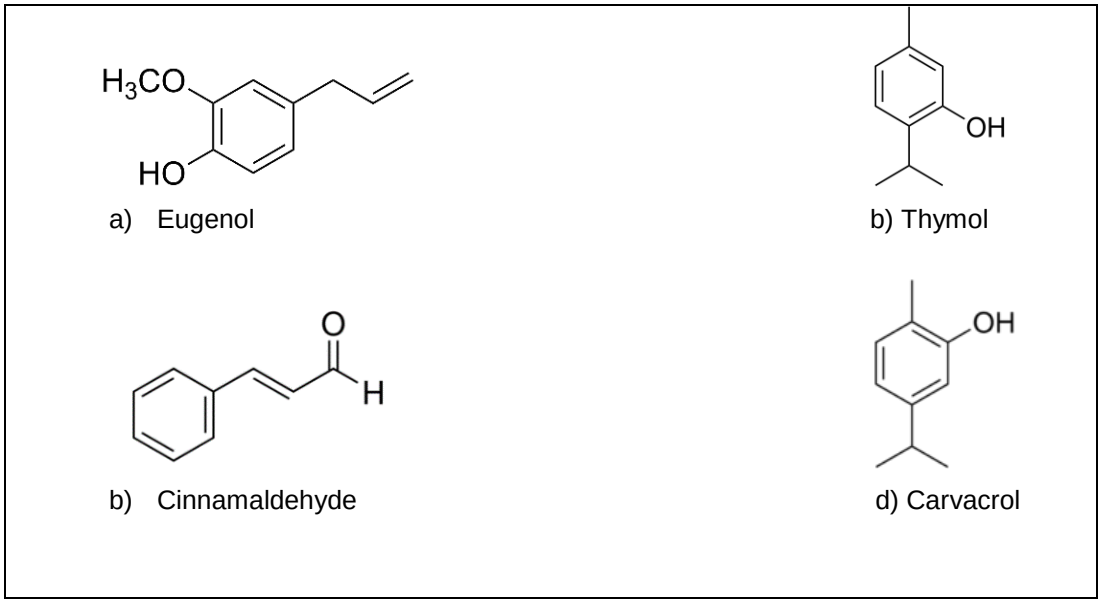
immunity. This blend of EO have also shown to improve the immuno-competence of broiler chicks by increasing the immunoglobulin A secretion in the caecum and ileum of chicks (Bento *et al.*, 2013).

Numerous studies have proven that EO have selective antibacterial properties and can be used to effectively replace AGP in animal diets (Griggs & Jacobs, 2005; Bento *et al.*, 2013). Although some studies have found contradicting results when using EO, it has the potential to achieve an environmental friendly broiler production system (Alçiçek *et al.*, 2004). The four major EO used in the study was thymol, carvacrol, cinnamaldehyde and eugenol. Table 2.1 shows a summary of the modes of action of each of these EO.

Table 2.1 Different essential oils (EO) with their mode of action against pathogens

Essential oil	Mode of action on pathogens	Source
Thymol	Disrupts the cell membrane and citrate metabolic pathway	(Trombetta *et al.*, 2005; Di Pasqua *et al.*, 2007)
Carvacrol	Inhibits the ATPase activity in cells and causes leakage of cell ions, leading to cell destruction	(Gill & Holley, 2006a; Gill & Holley, 2006b; Di Pasqua *et al.*, 2007)
Cinnamaldehyde	Inhibits the ATPase activity leading to membrane disruption	(Gill & Holley, 2004; Gill & Holley, 2006a; Gill & Holley, 2006b)
Eugenol	Blocks the efflux pump and inhibits cell activity by inhibiting the ATPase activity. Reduces the virulence Factors at certain concentrations in the cell.	(Gill & Holley, 2006a; Gill & Holley, 2006b; Di Pasqua *et al.*, 2007; Hemaiswarya & Doble, 2009; Qiu *et al.*, 2010; Bolla *et al.*, 2011)

The structure of EO's are illustrated in Figure 2.3.



a) Eugenol

b) Thymol

b) Cinnamaldehyde

d) Carvacrol

Figure 2.3 The structure of eugenol (a), thymol (b), cinnamaldehyde (c) and carvacrol (d) (Marchese *et al.*, 2016)

2.5 Organic acids

Organic acids are carbon containing compounds that have been used for more than a decade in animal feed. Organic acids was initially used to inhibit fungal and microbial growth in feed, but it has been discovered that the salts from organic acids have additional benefits as well (M'Sadeq *et al.*, 2015). In this study, organic acids with a blend of EO were used. There are different forms of organic acids (Table 2.2), and those associated with specific anti-microbial activity are short chain acids (C1-C7) and can be either classified as monocarboxylic acids (formic, acetic, propionic and butyric acid) or carboxylic which contains the hydroxyl group (lactic, malic, tartaric and citric acid) attached to the α bond of the general R-COOH structure (Dibner & Buttin, 2002; Khan & Iqbal, 2015). Organic acids have different chemical and physical properties and can either be used as a blend of acids or individual acids (Wang *et al.*, 2009). Many organic acids are available as sodium, potassium or calcium salts, used in either the drinking water or feed. The advantage of using these acids as salts is that they are easier to handle, especially during the process of feed production. These salts are also odourless, causing less irritation to people handling the products. (Huyghebaert *et al.*, 2011).

Acidification with different organic acids can reduce the pathogenic toxicity by preventing pathogens from colonising the gut, thus inhibiting damage to epithelial cells (Dittoe *et al.*, 2018). When organic acids dissociate, they are able to diffuse through the lipophilic membrane of bacteria and fungi. Once they penetrate the bacterial cytoplasm, the non-ionized acids decompose into H^+ ions and A^- ions, which disrupts the enzymatic reactions and transport system of the bacteria (M'Sadeq *et al.*, 2015).This disruption occurs when the bacteria starts to compensate for the reduced pH inside the cell by using the H^+-ATPase pump to return the intracellular levels to a normal pH. Bacteria normally have a pH around 7 (Lorenzoni, 2010) inside the cytoplasm, which ensures a stable structure of macromolecules inside the cell. This whole pH regulation process requires energy, which will reduce the energy available for bacteria to proliferate, thus preventing bacteria from growing (Lambert & Stratford, 1999). Organic acids can also exert their antimicrobial activity by disrupting the stability of the cytoplasmic membrane, which causes the electron transport chain to uncouple and disrupt normal ATP metabolism (Lorenzoni, 2010).

Table 2.2 Characteristics of organic acids (Broom, 2015)

Organic acid	Formula	Form	p*K*a	Water solubility
Formic	HCOOH	Liquid	3.75	
Acetic	CH_3COOH	Liquid	4.76	
Propionic	C_2H_5COOH	Liquid	4.87	
Butyric	C_3H_7COOH	Liquid	4.83	
Lactic	C_2H_5OCOOH	Liquid	3.83	
Sorbic	C_5H_7COOH	Solid	4.76	
Benzoic	C_6H_5COOH	Solid	4.2	

The p*K*a value of organic acids is defined as the pH at which half of the acid will dissociate (Lambert & Stratford, 1999) and is an important aspect of the antimicrobial activity. Most organic acids have a p*K*a value between 3 and 5 (Dibner & Buttin, 2002; Khan & Iqbal, 2015). The proportion of undissociated acid increases as the pH declines. When the pH of the environment through which the organic acid passes is higher than the p*K*a value of the acid, it will start to dissociate (Lambert & Stratford, 1999). That is why organic acids dissociate once they enter the pH neutral zone of bacterial cells where they then exert their antimicrobial activity (Broom, 2015; Salim *et al.*, 2018). Thus, within the GIT of the animal, organic acids will have a greater antimicrobial effect in the lower pH regions of the gut than in higher pH regions (Broom, 2015). Once the organic acid is ingested, it is first exposed to the microbial load, and is particularly effective against *E. coli* and other intolerant species of the foregut. The lower pH of the upper gut favours the antimicrobial activity and absorption of organic acids into the epithelium. This antimicrobial activity in the crop is important as it is the major site of colonisation of *E. coli* and salmonella (Dibner & Buttin, 2002). It is however of high importance that organic acids reach the small intestine of the animal to prevent the colonisation of opportunistic bacteria which competes with the host for nutrients.

A review by Khan *et al.* (2015) highlighted contradicting results regarding the efficacy of organic acids. Certain organic acids are only absorbed in the upper tract of the animal and never ends up in the small intestine, which may lead to reduced efficacy. A study done on the salmonella load in chickens reported that the concentration of salmonella in the upper digestive tract was significantly reduced compared to the lower tract after the feed was acidified with a combination of sodium chloride and citric acid (Mohyla *et al.*, 2007). Recently, researchers have focused more on transporting the organic acids further down the GIT by microencapsulating the organic acids. This should prevent absorption at the upper tract and ensure a more efficient release of organic acids in the small intestine, which is essential for their antimicrobial activity throughout the GIT (Van Immerseel *et al.*, 2006; Huyghebaert *et al.*, 2011). There are a few other variables that can lead to decreased efficacy of the organic acids, including: the p*K*a value of the acid, the chemical formula, molecular weight, the population of microorganism and the buffering capacity of the feed. One way to overcome these limitations is to feed a blend of organic acids instead of individual acids. A blend of organic acids represents a larger range of p*K*a values, which ensures a broad spectrum activity against pathogens while it combines the good qualities of different acids (Huyghebaert *et al.*, 2011; Khan & Iqbal, 2015). When using this blend of organic acids, the additive effects between acids are utilised, for example medium chain fatty acids with a chain length between 8 and 12 carbon atoms are heavier to absorb at first, and facilitate the absorption of short chain fatty acids (Huyghebaert *et al.*, 2011).

The positive effects of organic acids in broilers have been well documented. In a review by M'Sadeq *et al.* (2015), several studies have shown that the inclusion of organic acids in broiler diets enhanced growth and feed utilization, while reducing the proliferation of pathogenic bacteria in the intestine. According to Gunal *et al.* (2006), the acidification of diets with various weak acids such as formic, fumaric, lactic and sorbic acids are able to decrease colonisation of pathogens and the production of toxic metabolites. Similarly, Khan *et al.* (2015) suggested that organic acid mixtures of fumaric acid, calcium format, calcium propionate, potassium sorbate, calcium butyrate and calcium lactate were more effective against intestinal *E. coli* and salmonella compared to AGP. Hamed & Hassan (2013) reported similar results in their study when they found a significant reduction in bacterial counts in the caeca when two groups of birds received a blend of acetic acid and an organic acid

mixture (3 ml/L water). The organic acid mixture consisting of acetic acid, phosphoric acid, lactic acid, fumaric acid and tartaric acid was administered through the drinking water to Japanese quails 7 days after an infection (Hamed & Hassan, 2013). Similar results on salmonella was reported when feeding broilers 0.3% caproic acid (Griggs & Jacob, 2005). In a different salmonella challenged study, layer chick diets were supplemented with a mixture of propionic acid and formic acid. These chicks received a *S. pullorum* challenge at three days of age and was fed the acid supplemented diets from three days onwards. Over the 21-day challenge period, the combination between propionic and formic acid significantly reduced the *S. pullorum* recovery in the crop and caeca (Broom, 2015). There are, however, some contradicting results when it comes to lowering the clostridium counts during treatments. Paul *et al.* (2007) found that ammonium formate and calcium propionate (3 mg/kg) reduced coliform counts compared to control treatments, but the clostridium counts remained unaffected. The same study showed that ammonium formate lowered the *E. coli* counts in the gut, but clostridium counts remained the same.

It should be considered that the bactericidal effects depend mainly on the susceptibility of the strain. Research discovered that certain bacterial species can tolerate acidic environments better than others species and it may be related to the ability of the strain to regulate their intracellular pH. Lactobacilli for example are much more resistant to a drop in pH compared to bacillus. This is mainly because the minimum inhibitory concentration of acetic acid is 250 times lower for *Bacillus subtilus* than for lactobacillus (Lorenzoni, 2010). There are, however, studies that show beneficial effects of organic acids during NE outbreaks. One such study showed that 0.45% potassium diformate was able to prevent *C. perfringens* infections and consequently NE (Mikkelsen *et al.*, 2009). Apart from reduced pathogen loads in animals treated with organic acids, an increase in performance was also recorded, probably due to a lower pathogenic load competing with the host for nutrients. Fumaric acid improved feed efficiency by 4% in broilers while body weight gain increased 0.5-1% without an effect on feed use. The effects of buffered propionic acid with and without bacitracin was tested and the results showed a significant increase in dressing percentage for female broilers, while there was a decrease in abdominal fat in the male broilers (Dibner & Buttin, 2002).

Some studies also reported enhanced mineral absorption when adding organic acids to broiler feed (Gunal *et al.*, 2006; Fattah *et al.*, 2008). Furthermore, Khan & Iqbal (2015) have reported that organic acids were able to raise gastric proteolysis in broilers, improving the digestibility of protein and amino acids. The lower pH in the gut after supplementing organic acids increased the pepsin activity which activated the release of hormones, gastrin and cholecystokinin. The increase in pancreatic secretion was the main reason for improved protein digestibility, due to high concentrations of trypsinogen, chymotrypsinogen A, chymotrypsinogen B, procarboxy A and procarboxy B (Khan & Iqbal, 2015). Butyric acid is an important short chain fatty acid when it comes to gut health. Not only is it an important energy source, which stimulates proliferation of epithelial cells in the GIT, it also has anti-inflammatory properties (Papatsiros *et al.*, 2013). Butyric acid is able to strengthen the mucosal barrier of the GIT by producing antimicrobial peptides in the mucus while it is stimulating the expression of tight junction proteins to decrease intestinal permeability (Huyghebaert *et al.*, 2011; Papatsiros *et al.*, 2013). As an energy source, butyrate helps to maintain intestinal villi structure, consequently supporting absorption of nutrients that can contribute to improved performance. Butyric acid was also reported to have anti-coccidial effects, as birds fed butyrate were able to better withstand coccidial challenges (Timbermont *et al.*, 2010).

2.6 Probiotics (Direct-fed microbials)

The definition of the word probiotic has evolved over the past few decades. It was originally used to describe substances produced by a protozoa to stimulate the growth of another and later the word was used to describe animal feed supplements which had beneficial effects on the host by affecting the gut flora (Fuller, 1989). According to the currently adopted definition by the World Health Organisation, probiotics are mono or mixed cultures of live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (Alloui *et al.*, 2013; Popova, 2017). Probiotics can thus be classified as live microorganisms that can be administered to animals through either the water or the feed in order to populate the intestine of the host. Once in the GIT, these beneficial microorganisms will start to alter their environment, which is especially important in young animals in which the intestinal bacteria have not been established (Dittoe *et al.*, 2018). Adding probiotics to the diet of young chicks populate the intestine with beneficial bacteria, thus preventing the colonisation of the gut by opportunistic pathogens (Lorenzoni, 2010).

The major microbial groups in monogastric animals are *Bacteroides*, *Clostridium*, *Bifidobacterium*, *Eubacterium*, *Lactobacillus*, *Enterobacteriaceae*, *Streptococcus*, *Fusobacterium*, *Peptostreptococcus* and *Propionibacterium* spp. (Gaggia *et al.*, 2010). Just a few days after hatching, the GIT of the chick is already colonised with microflora consisting of more than 300 strains, which make up a total count of 10^{14} colony forming units (CFU) per gram of digesta. The caeca contain the highest count of bacteria which mainly consists out of lactobacilli, *E. coli* and other gram-positive aerobes, with lower counts of bacteriodes and bifidobacteria. As the chicken matures, the bacterial population changes as lactobacilli, bacteriodes and bifidobacteria numbers starts to increase. Gram-positive anaerobes decrease while *E. coli* tends to remain at the same concentration (Harimurti & Hadisaputro, 2015). This microbial population of the GIT reflects the co-evolution of microorganisms with its host and the diet consumed by the host. Any changes in this microbial population can have beneficial or detrimental effects on the health, growth and maturation of the host (Lu *et al.*, 2003). A balanced microbial population constitutes an effective barrier against pathogens, while it produces metabolites such as vitamins and short chain fatty acids (SCFA) and stimulate the immune system in a non-inflammatory manner (Gaggia *et al.*, 2010). When in the wild, the chick attains its gut flora from the mother by direct and indirect routes (Figure 2.4). During modern rearing of the chick, access to the mother is restricted when eggs are incubated in the hatchery. The chick is no longer exposed to the normal environment of the hen where it would have acquired the full complement of characteristic microflora (Fuller, 1989). This emphasises the need for probiotics early in the life of the chick.

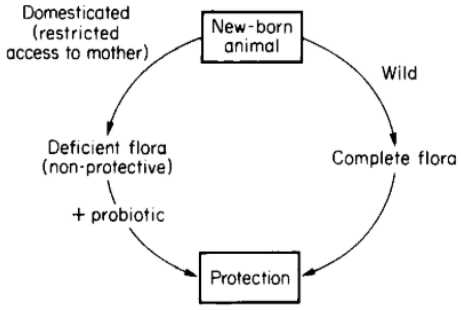


Figure 2.4 Different pathways of attaining the intestinal microorganisms in an animal (Fuller, 1989)

Poultry are mainly vulnerable to pathogens such as *E. coli, Salmonella* spp., *C. perfringens* and *Campylobacter sputorum* which populates the GIT of the host to compete for nutrients, consequently reducing the digestion of fat and fat-soluble vitamins (Engberg *et al.*, 2000; Harimurti & Hadisaputro, 2015). Any form of stress can influence the beneficial microbial population, especially lactobacilli, which tend to decrease under these situations, allowing pathogenic bacteria to proliferate. As soon as overgrowth of pathogenic bacteria begins, it predisposes the host to diseases such as diarrhea which reduce production performance (Kabir, 2009). These pathogens, however, face a multifaceted defense system, composed of lowered gastric pH, rapid transit time through the GIT, resident bacteria, epithelium and immune systems. The intestinal epithelium together with the mucus provide the first sensory line of defense against luminal pathogens. This mucus layer is formed through the interaction of mucosal secretions such as glycoproteins, trefoil peptides and surfactant phospholipids (Gunal *et al.*, 2006; Gaggia *et al.*, 2010). The resident bacteria in the gut have a dual function by stimulating the mucosal mechanisms of defense and maintaining homeostasis of the immune system and are thus able to positively affect the integrity of the gut wall (Lu *et al.*, 2003; Gaggia *et al.*, 2010). Reduced epithelial cell proliferation and mucosal atrophy increase the permeability of the intestine. The loss in gut integrity allows pathogens to enter, inducing a switch from physiological to pathological inflammation. Pathogens produce toxins and other substances such as mucinases which all interfere with the metabolism of the epithelial layer (Gunal *et al.*, 2006; Gaggia *et al.*, 2010). Research from the past two decades has found evidence of cross-talk between pathogens and the epithelial layer in GIT, which can result in the rearrangement of epithelial cells upon colonisation by pathogens (Patterson & Burkholder, 2003). Hooper *et al.* (2001) found that there was cross-talk between *Bacteroides thetaiotaomicron* and the epithelial cells, which resulted in the secretion of glycans that was eventually utilised by the bacteria. It is believed that other intestinal bacteria, including probiotics, can act in a similar manner to enhance the proliferation of these bacteria in the gut wall (Hooper *et al.*, 2001). Toxins produced by pathogens caused damage to the gut wall. Shorter and thinner villi were associated with toxins (Awad *et al.*, 2009), which consequently reduced the surface area for absorption in the intestine and lead to decreased growth and performance during pathogen invasion. Changes in the gut microbiota was also noticed. Increasing evidence indicates that with an increase in pathogen colonisation, beneficial bacteria such as bifidobacteria and lactobacilli decrease (Kabir, 2009; Gaggia *et al.*, 2010). This decrease in beneficial bacteria also occurs under physiological and psychological stressors. During stressful conditions, the general trend observed is a decrease in lactobacilli and an increase in coliforms. Hormonal changes during stress affect the production of mucus, which may reduce the components of the gut flora associated with it. During conditions in which the microbial balance is affected, probiotics are of potential value (Fuller, 1989).

Microorganisms that are predominately used as probiotics include *Bacillus, Bifidobacterium, Enterococcus, E. coli, Lactobacillus, Lactococcus, Streptococcus* spp. and undefined mixed cultures. Lactobacilli and bifidobacteria are mostly used in human probiotics where bacillus, enterococcus and saccharomyces yeasts are mostly used in livestock (Simon *et al.*, 2001; Dittoe *et al.*, 2018). There has been an increase in research on feeding lactobacilli to broiler chickens. Commercial probiotics can either be single or multi-strains of beneficial bacteria. *In vivo* studies on probiotics are generally time consuming and expensive, therefore producers use *in vitro* tests as a selection criteria to reduce the number of strains and find the most effective microorganism (Taheri *et al.*, 2009). The selection of a probiotic strain should, however, consider

certain attributes such as being of host origin, must be non-pathogenic to the animal, acid and bile resistant, adhere to the gut epithelial tissue, and be persistent in the GIT for a short period of time. Probiotics should also produce antimicrobial substances for them to effectively replace AGP (Popova, 2017). One of the concerns when using undefined cultures of probiotic is the theoretical lack of consistency of the final product. This can result in different performances of these probiotics under field conditions (Lorenzoni, 2010).

Probiotics can influence the intestinal microbiota in a diverse way. One such mechanism of probiotics that has been studied extensively is the ability of these beneficial bacteria to exclude pathogens from attaching to the gut through a process known as competitive exclusion (Dittoe *et al.*, 2018; Salim *et al.*, 2018). During this process, beneficial bacteria will block the cellular receptors on the surface of epithelial cells and therefore mechanically avoid the entrance of pathogenic bacteria (Lorenzoni, 2010). Competition for adhesion sites are crucial for the survival of bacteria since they need to avoid peristalsis movement during the digestion of feed (Alloui *et al.*, 2013; Harimurti & Hadisaputro, 2015). Exclusion of pathogens is especially important in newly hatched chicks as they do not receive maternal antibodies like young mammals (Simon *et al.*, 2001). The competitive exclusion approach instantly provides the newly hatched chick with an adult intestinal microbiome (Patterson & Burkholder, 2003), allowing the competitive excluding bacteria to establish in the GIT where they become competitive or antagonistic to pathogenic bacteria (Gaggia *et al.*, 2010). This approach was first described by Nurmi and Rantala (1973) which discovered that feeding faecal organisms from adult chickens to day old chicks prevented colonisation of the gut by *Salmonella infantis*. Competitive exclusion is thus a very effective measure to protect newly hatched chicks against salmonella and other enteric pathogens (Kabir, 2009). It has been shown experimentally that the treatment of chicks with probiotics were able to protect the chicks against pathogens such as *C. jejuni, Listeria monocytogenes, E. coli, Yersinia enterocolitica* and *C. perfringens* (Nisbet, 2002; Schneitz, 2005). Gaggia *et al.* (2010), reported that lactobacilli probiotics significantly reduced the *Salmonella enteritidis* recovery in challenged neonatal broilers.

Other methods besides competitive exclusion, in which probiotics inhibits pathogens, have been studied. Beneficial bacteria, for instance, can reduce intestinal pH by producing certain metabolites and thereby preventing pathogens from proliferating in the GIT (Lorenzoni, 2010; Alloui *et al.*, 2013). The lactic acid produced by beneficial microbes potentially increases the permeability in the outer membrane of gram-positive pathogens which facilitates the diffusion of antimicrobial substrates produced by the host's epithelium and other bacteria (Alakomi *et al.*, 2000). The SCFA produced by probiotics are also able to diffuse through the membrane of pathogens into the more alkaline environment where they dissociate to acidify the cytoplasm of the cell (Broom, 2015; M'Sadeq *et al.*, 2015) Pathogens are also sensitive to butyrate, as it is known to decrease salmonella by specific suppression of salmonella pathogenicity island I genes. There seems to be a correlation between the amount of lactic-acid producing bacteria (LAB) and butyrate produced. LAB includes species of *Lactobacillus, Lactococcus, Streptococcus, Pediococcus* and *Leuconostoc* genera (Alloui *et al.*, 2013). LAB, however, do not produce the butyrate, instead they increase butyric concentration indirectly by stimulating the proliferation of butyrate producing bacteria through a mechanism known as cross feeding (Harimurti & Hadisaputro, 2015). Butyrate also has anti-coccidial effects as birds fed butyrate are able to better withstand coccidial challenges (Timbermont *et al.*, 2010).

Another possible method by which probiotics may reduce the viability of pathogens is through the synthesis of substances that can destroy pathogenic bacteria. These substances include H_2O_2 and

bacteriocins, which are molecules with bactericidal properties on genetically related organisms (Gillor *et al.*, 2008; Lorenzoni, 2010). Bacteriocins can be divided into two main groups: those produced by gram-positive bacteria and those by gram-negative bacteria. One limitation of bacteriocins produced by gram-positive bacteria is that they seldom inhibit commonly encountered pathogenic bacteria such as enterobacter and salmonella. However, bacteriocins that are produced by gram-negative bacteria are able to inhibit the growth of these pathogenic bacteria (Gillor *et al.*, 2008).

Another important mode of action of probiotics is its ability to stimulate the immune system to control pathogenic infections. Immunity that results from the exposure of the gut to a variety of antigens such as dietary protein and pathogenic bacteria is important in the defense of young animals against possible enteric infections (Jin *et al.*, 1997). The microflora in the GIT has a significant impact on the body's immune system. When administrating probiotics to the animal, the gut microbiota is manipulated, which influences the development of the immune system. Bacteria initially colonising the GIT can influence the establishment of future microbial populations by influencing the epithelial cell gene expression. As the GIT is part of the immune system it needs constant stimulation from the commensal bacteria for maturation (Lorenzoni, 2010). The number of intraepithelial lymphocytes, plasma cells and Peyer's patches are lower in animals raised under germ-free environments compared to animals raised under conventional systems. Probiotics are able to interact with the Peyer's patches and intestinal epithelial cell, thereby activating mucosal immunity by stimulating these plasma cells, IgA secretion and migration of intestinal T-cells (Lorenzoni, 2010; Harimurti & Hadisaputro, 2015). Jin *et al.* (1997) reported in their review that birds treated with *L. reuteri* exhibited longer villi and deeper crypts, which is generally a response associated with enhanced T-cell function and increased production on anti-salmonella IgM antibodies. When probiotics are administered to the animal, they interact with the enterocytes, goblet cells and M cells from the Peyer's patches. These interactions increase the number of IgA-producing cells accompanied by the production of both IgM and IgA, which is important for the immunity of the mucosa since it forms the barrier against pathogenic microorganisms (Otutumi *et al.*, 2012).

2.7 Prebiotics

Similar to probiotics, the aim of administering prebiotics is to modify the intestinal bacterial community. The key difference is that prebiotics are non-digestible but fermentable carbohydrates that serves as a nutrient source to the pre-existent intestinal bacterial populations (Lorenzoni, 2010; Dittoe *et al.*, 2018). Prebiotics can therefore stimulate the growth of bacteria normally present in the GIT and already adapted to the environment (Yang *et al.*, 2009). Due to the presence of ß-links between the sugar molecules, prebiotics are only susceptible to bacterial fermentation and cannot be digested by the host. The non-digestibility ensure that prebiotics can end up in the colon where they serve as an nutrient source for bacteria (Papatsiros *et al.*, 2013). Another important feature that prebiotics have is that they are well fermented by beneficial bacteria but poorly fermented by pathogenic bacteria (Crittenden & Playne, 2008). For a feed additive to be classified as a prebiotic, it must not be hydrolysed or absorbed in the small intestine. It must also be a selective substrate for beneficial bacteria in the large intestine and stimulate these bacteria to proliferate and become metabolically active to alter the intestinal microbiome towards a healthier composition (Dahiya *et al.*, 2006; Gaggia *et al.*, 2010).

The most common prebiotics are carbohydrates and oligosaccharides with different molecular structures. Dietary carbohydrates such as fibre can be classified as prebiotics but non-digestible oligosaccharides have more promising properties. Non-digestible oligosaccharides include: mannanoligosaccharides (MOS), galacto-oligosaccharides, fructo-oligosaccharides (FOS), soybean-oligosaccharides, isomalto-oligosaccharides, xylo-oligosaccharides, lactulose, inulin (Patterson & Burkholder, 2003; Ricke, 2018). These oligosaccharides are promising alternatives to antibiotics since they promote the symbiotic relationship between the host and the microflora. The two most commonly used prebiotic oligosaccharides are MOS and FOS. Although both of these oligosaccharides are beneficial to the enteric health of the host, they have different modes of action (Ferket, 2004).

MOS is derived from the outer cell wall of selected *Saccharomyces cerevisiae* and their components include protein, glucans and phosphate radicals as well as mannose (Griggs & Jacob, 2005; Yang *et al.*, 2009). Although MOS have been used in the same manner as the rest of the prebiotics mentioned above, they do not selectively enrich the beneficial bacterial populations like FOS. MOS binds to the mannose-specific lectin of gram-negative pathogens where they express type 1 receptors (Gaggia *et al.*, 2010). Bacteria use these receptors found on the cells of the host as possible binding sites. Once pathogenic bacteria bind to these receptors, they can cause disease to the host. MOS thus functions as a competitive binding site for pathogens such as *E. coli* and salmonella as the bacteria bind to it and are carried out of the intestine rather than binding to the receptors on the gut cells (Patterson & Burkholder, 2003; Gaggia *et al.*, 2010; Huyghebaert *et al.*, 2011; Papatsiros *et al.*, 2013). Griggs and Jacob (2005) noted that supplementing 2.5% mannose in broilers' drinking water reduced *Salmonella typhimurium* colonisation in the intestine of these birds. These results were confirmed in another study where 3-day old broilers were challenged with two strains of salmonella. MOS supplementation reduced the number of bacteria in the caeca and the caeca were less likely to be colonised in the treated birds than in the control birds at day 10 (Spring *et al.*, 2000). During the initial research with MOS, it was found that it had the ability to attach to pathogenic bacteria as mentioned above, but subsequent research has found that MOS also improves the host's immune system and intestinal morphology (Bozkurt *et al.*, 2008). The immune modulatory effect of MOS is based on two properties: Firstly, mannan and glucan components have antigenicity characteristics and, secondly, MOS prevents the pathogens from attaching to the intestine and allows them to be presented to immune cells as attenuated antigens (Yang *et al.*, 2009). Another unique character of MOS is its ability to enhance the protective antibody response to enhance disease resistance while at the same time suppresses the acute phase response such as inflammation which can be detrimental to production (Ferket *et al.*, 2002).

Supplementation of diets with an oligosaccharide such as FOS may improve the gut microbial population, including a reduction in salmonella colonization, making it a viable option in salmonella control and AGP free programs (Alloui *et al.*, 2013). The chemical structure of FOS consists of short chain polymers of ß 1-2 linked fructose units attached to a D-glucosyl residue at the end of the chain (Ferket, 2004; Hajati & Rezaei, 2010). FOS are found in numerous plants such as onions, garlic, banana, chicory, asparagus and wheat. It influences the enteric microflora by acting as a substrate for beneficial microbes, which consequently excludes the colonisation of pathogens. FOS provides enrichment for beneficial bacteria such as lactobacilli and bifidobacteria (Ferket, 2004) and reduces the colonisation by pathogenic bacteria (Dahiya *et al.*, 2006; Gaggia *et al.*, 2010) . Bifidobacteria in particular benefit from the addition of prebiotics. A study done by Crittenden and

Playne (2008) reported that bifidobacteria can increase as much as 2 logs after prebiotics have been administered to broiler diets. Patterson *et al.* (2003) also found that the caecal concentration of lactobacilli and bifidobacteria was increased 7-fold and 24-fold, respectively, in young broilers receiving FOS enriched diets. These bifidobacteria have enzymes that are able to digest a broad variety of oligosaccharides and complex carbohydrates to use them as an energy source. In addition, bifidobacteria are able to internalise oligosaccharides and digest them internally. This prevents the release of simple sugars into the GIT which may serve as a possible nutrient for other bacteria such as pathogens (Lorenzoni, 2010). The increased numbers of bifidobacteria play an important role in the health and composition of the microbial population. Bifidobacteria have antibacterial effects and can suppress the growth of pathogens, especially against *E. coli*, by producing bacteriocins and by lowering the pH through the production of short chain fatty acids (Hajati & Rezaei, 2010).

There are studies that showed the advantage of using prebiotics in animal diets. One such study found that the addition of FOS at 0.4% inclusion lowered the number of *E. coli* while increasing the number of bifidobacteria and lactobacilli in broiler chickens. Oligosaccharide beta-glucans of yeast cell wall origin is believed to improve performance of the host through its immunomodulatory effect. It enhances phagocytosis and the proliferation of monocytes and macrophages. This interaction between macrophages and glucans plays a big role in immune stimulation (Huyghebaert *et al.*, 2011). Tian *et al.* (2016) showed that ß-glucans improved phagocytosis and inflammatory cytokine production, which lead to the improvement of the host's gut ecosystem by increasing lactobacilli and bifidobacteria. Another important function of FOS is its ability to improve the activities of enzymes such as proteases and amylases, enhancing the digestion and utilisation of nutrients (Xu *et al.*, 2003). Xu *et al.* (2003) also reported that chicks supplemented with diets containing FOS had longer ileal villi heights compared to the negative control.

2.8 Conclusion

The consequences of banning AGP's in Europe has led to the search of alternative feed additives to effectively replace antibiotics as disease preventative measures. The South African broiler industry will need to be prepared, especially since the global trend shows how the consumers are becoming more focused on environmental and food safety. It is promising to see how the latest research creates a paradigm shift in our understanding of NE, which we can use as a tool to control the incidence of NE in broilers once the South African poultry industry is forced to remove AGP completely from the feed.

Chapter 3

Trial 1: The effects of antibiotic growth promoters and alternative feed additives on performance and gut morphology of broilers in a non-challenged environment

3.1 Introduction

The first trial was conducted under non-challenging conditions in a broiler facility on the Hillcrest Experimental Farm, University of Pretoria. All procedures used in this trial were approved by the Animal Ethics Committee (AEC) of the University of Pretoria (EC008-16).

3.2 Materials and methods

3.2.1 Housing and care

Housing and care of the birds were done in such a way as to represent as far as possible commercial broiler production conditions. Prior to placing the day-old chicks, the broiler house was washed, disinfected, and pre-heated to the comfort zone of the chicks of 36ºC ambient temperature and at least 34ºC litter (floor) temperature. Pine shavings was spread on the floor of the pens to absorb waste and to assist with insulation from the floor. A total of 2208 day-old male chicks were obtained from a commercial hatchery. Only male Ross birds from the same breeder flock were used for these trials to minimise variation between birds. On arrival, all birds were selected randomly and weighed as a group of 23 to determine the average body weight per pen. Weighed groups were then placed in floor pens of the environmentally controlled broiler house. Automatic heaters provided the optimum temperature to keep the birds in their desired comfort zone. Ventilation was controlled automatically to ensure optimum oxygen supply and removal of ammonia and carbon dioxide. A lighting program consisting of 23 hours of light and 1 hour of dark was provided during the first week of life to the chicks to stimulate normal daily feed and water intake. Thereafter, the length of daylight was reduced to 16 hours of light according to the Ross' Broiler Management Guide (Broiler management manual Ross-308, 2014). Birds were monitored on a daily basis by the principal investigators and supervisor as well as students and staff on the farm to ensure optimum growing conditions and bird comfort throughout the 35-day trial period. Any variation from the individual's normal behaviour or from its general appearance or appearance of the excreta was noted. Special attention was given to individual birds that were sick or in poor condition. Any bird judged to be suffering pain, distress, or appearing to be unlikely to survive was humanly euthanised and subjected to necropsy to determine the cause. Culled or dead birds were weighed to correct for feed conversion ratio (FCR).

3.2.2 Pen design

Pens treatment designation followed a completely randomised block design to minimise the influence of variations in the house environment (Figure 3.1). There were 96 pens of 1.5 meters x 1.5 meters with 23 birds housed per pen to simulate typical stocking densities used in the broiler industry. Pens were divided into 8 treatment groups consisting of 12 replicate pens per treatment to provide sufficient statistical power to the study. All birds had free access to feed and water at all times, provided by a tube feeder and 5 nipple drinkers

per pen. During the first week after chick placement one extra pan feeder and one extra fountain drinker was provided per pen in order to encourage and assist chicks to eat and drink.

Block 6

7*	3
1	5
8	4
2	6

Block 3

8	4
2	6
1	5
3	7

Block 5

1	5
3	7
2	6
4	8

Block 2

2	6
4	8
3	7
5	1

Block 4

3	7
5	1
4	8
6	2

Block 1

4	8
6	2
5	1
7	3

* Numbers indicate the dietary treatment applied to the specific pen

Figure 3.1 Pen arrangements of treatments and replications for one of the two experimental houses, demonstrating the blocking of treatments

3.2.3 Dietary treatments

The birds received a nutrient dense broiler ration mixed at a small-scale feed mill (Pennville Animal Nutrient Solutions) according to nutrient specifications recommended by the breeder company (Ross Breeders). Feed consumption was monitored and the feeders refilled when needed on a daily basis to ensure *ad libitum* feed intake. A four-phase feeding program was implemented with pre-starter, starter, grower and finisher feeds. The pre-starter was fed from day 0-7 as crumbles and starter was also fed as crumbles from day 7-21. The grower was fed as pellets from day 21-28 and finisher also fed as pellets from day 28-35. All groups received identical feed with the only difference between treatments being the specific additive supplemented in the feed. The dietary feed additives were blended with the premix to be used for the relevant treatments. The negative control consisted out of the base diet without any feed additives blended with the premix. The same basal diet was mixed for all 8 treatments after which the premixes with the different additives listed below were added to the basal feed and remixed to form the 8 different dietary treatments shown in Table 3.1. The additives used during Trial 1:

1. Zinc Bacitracin 15 % m/m (ZB; antibiotic growth promoter) (Ceva Animal Health, South Africa; Reg. No. G1070)
2. Enviva Pro (a three strain Bacillus probiotic product) (Chemunique, South Africa; Reg. No. V23200)
3. Mannoseoligosaccharides KR01 (MOS; a prebiotic) (Cargill, Minnesota, USA) DAFF import permit (no: 11/1/392) for trial purposes (date of permit: 08/02/2016)
4. Biacid Nucleus (an essential oil (EO) product) (Cargill, Minnesota, USA). DAFF import permit (no: 11/1/391) for trial purposes (date of permit: 08/02/2016)

The 8 dietary treatments (Table 3.1) consisted of a negative control (basal), a positive control (ZB), EO (Biacid Nucleus), Positive control + EO (ZB + Biacid Nucleus), Probiotic (Enviva Pro), Positive control + Probiotic (ZB + Enviva Pro), Prebiotic (MOS), Positive control + Prebiotic (ZB + MOS).

Table 3.1 Eight dietary treatments containing different inclusion levels of feed additives

Treatment name	Inclusion level (g/ton)			
	ZnBac®	Biacid Nuclease®	Enviva Pro®	Mannoseoligosaccharide®
Negative Control	0	0	0	0
Zinc Bacitracin (ZB)	333	0	0	0
Biacid Nuclease	0	100	0	0
Biacid Nuclease + Zinc Bacitracin	333	100	0	0
Enviva Pro	0	0	500	0
Enviva Pro + Zinc Bacitracin	333	0	500	0
Mannoseoligosaccharide (MOS)	0	0	0	200
Mannoseoligosaccharide + Zinc Bacitracin	333	0	0	200

The rations were mixed to specifications shown in Table 3.2.

Table 3.2 Raw material inclusion (%) and calculated nutrient composition (g/kg) of the basal broiler diet for each of the four phases

Ingredients	Pre-starter	Starter	Grower	Finisher
Maize yellow	58.50	64.00	69.63	73.73
Soya oilcake (46.5%)	33.63	28.50	20.27	16.70
Sunflower oilcake (36%)	2.00	2.50	3.00	3.00
Gluten 60	1.00	0.93	3.00	3.00
Lysine (Sint 78%)	0.28	0.28	0.33	0.36
Methionine (DL 98%)	0.26	0.24	0.16	0.15
Threonine (98%)	0.06	0.05	0.03	0.04
Oil crude soya	0.67	0.50	0.97	0.80
Feed lime (50:50 Mix)	1.74	1.55	1.42	1.28
Mono-dicalcium phosphate (Ws>70%)	0.93	0.61	0.45	0.24
Salt (Fine)	0.25	0.25	0.16	0.16
Sodium bicarbonate	0.36	0.28	0.33	0.33
Phytase (Axtra Phy 1000 FTU's)	0.01	0.01	0.01	0.01
Broiler starter premix (3kg/t)	0.30	0.30	0.00	0.00
Broiler grower premix (2.5kg/t)	0.00	0.00	0.25	0.00
Broiler finisher premix (2kg/t)	0.00	0.00	0.00	0.20
Calculated nutrient values		(g/kg)		
Moisture	106.50	106.89	105.73	106.05
Metabolisable energy, MJ/kg	11.50	11.75	12.30	12.50
Crude protein	220.37	201.86	182.84	169.78
Crude fat	35.74	35.29	40.71	39.93
Crude fibre	34.43	35.36	35.44	35.36
Ash	59.36	51.90	44.23	39.18
Calcium	10.40	9.06	8.14	7.22
Total phosphorus	5.93	5.15	4.58	4.04
Total lysine	13.60	12.28	10.70	10.02

3.2.4 Proximate and mineral analyses of feed

Representative feed samples of each phase (pre-starter, starter, grower and finisher) were sampled during production of the basal diet. The feed samples (4 samples) were analysed according to the proximate analysis system for their nutritional content at Nutrilab (Department of Animal and Wildlife Science, University of Pretoria). This system determines seven fractions in food including dry matter (AOAC, 2000, Official method of analysis 942.05), ash (AOAC, 2000, Official method of analysis 942.05), crude protein, crude fat (AOAC, 2000, Official method of Analysis 920.39), crude fibre (AOAC, 2000, Official method of Analysis 962.09), calcium (AOAC, 2000, Official method of Analysis 935.13) and total phosphorus (AOAC, 2000, Official method of Analysis 965.17). The Leco FP-428 (Leco Corporation, 3000 Lakeview Avenue, St. Joseph, MI 49085-2396) was used to analyse the nitrogen content of the feed and the method used was according to the AOAC's official method of analysis (AOAC, 2000, Official method of Analysis 988.05).
Results of the analysis are presented in Table 3.3.

Table 3.3 Analysed nutrient values (on "as fed" basis) of the pre-starter, starter, grower and finisher feeds

Nutrient	Pre-starter	Starter	Grower	Finisher
Dry Matter (%)	88.7	88.4	88.0	89.6
CrudeProtein (%)	21.6	19.9	18.3	17.6
CrudeFibre (%)	3.00	3.11	3.30	3.20
CrudeFat (%)	3.68	3.60	4.50	4.29
Ash (%)	6.20	4.90	4.35	3.77
Calcium (%)	0.81	0.76	0.72	0.62
Phosphorus (%)	0.60	0.54	0.44	0.38

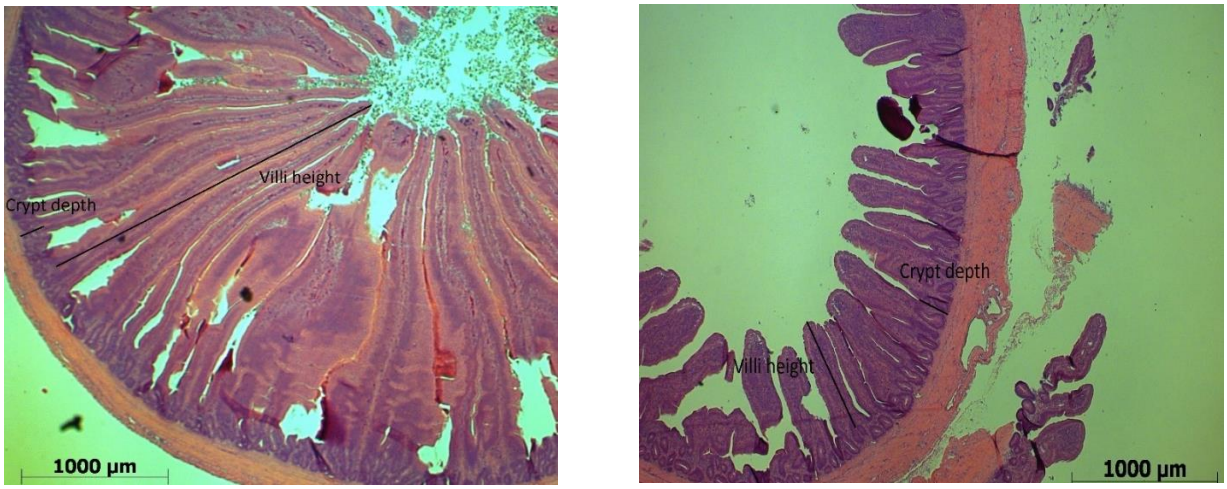
3.2.5 Performance parameters

Body weight (BW) of broiler chickens was measured on a weekly basis. The chicks were weighed before being placed (day 0) and then on day 7, 14, 21, 28 and 35. Feed intake was measured weekly on the same day that the chicks were weighed. To measure feed intake, each pen was allocated a bin of known weight. Feed was weighed into the bin at the beginning of the week and weighed again, together with any orts from the pen's feeder, at the end of the week. The total amount of consumed feed was divided by the amount of birds in each pen to determine feed intake per bird over the week period. During two daily inspections in the broiler house dead birds were collected and the weight of dead birds recorded. The weekly FCR was calculated as feed intake (g)/ body weight (g). The FCR was corrected for BW of mortalities.

3.2.6 Histomorphology

At the end of the trial (day 35), two birds per pen (24 birds per treatments) were selected, based on the average weight of the pen and sacrificed for sample collection. For histopathologic and morphometric analysis, one cm tissue samples from the duodenum, jejunum and ileum were obtained and fixed in 10% buffered formalin (100 mL of 40% formaldehyde. Four-gram phosphate, 6.5 g dibasic sodium phosphate and 900 mL of distilled water) for 48 hours. Tissues were then dehydrated by transferring it through a series of alcohols with increasing concentrations over a 24-hour period after which it was placed into xylol and

embedded in paraffin wax. A microtome was used to make cuts that were 4 µm thick. The cuts were stained with hematoxylin-eosin on microscope slides by the Department of Pathology, University of Pretoria, Onderstepoort. The values were measured using a Zeiss Axiovert 2000 with Zeiss Axiovision 4.8.2.0 software at the Microscopy Department, University of Pretoria. The morphometric indices evaluated were villi height from the tip of the villi to the crypt, crypt depth from the base of the villi to the submucosa, and the villi height to crypt depth ratio. Measurements of the villi heights and crypt depths were determined at a magnification of 5X.



a) Duodenum b) Ileum

Figure 3.2 Villi height and Crypt depth measurements of the duodenum (a) and ileum (b)

3.2.7 Statistical analyses

Statistical analysis was done with the statistical software program SAS (Statistical Analysis System, 2014). The significance between treatments was determined by analysis of variance (ANOVA) with the general linear model (GLM). Means, standard error and significance of differences between means were determined by Fischers test (Samuals, 1989) at the 95% confidence level. In all cases the level of statistical significance was $P \leq 0.05$. Repeated Measures Analysis of Variance with the GLM model (SAS, 2014) were used for repeated period measures. Means and standard error of means for the different treatments were calculated and significant differences ($P \leq 0.05$) between means were determined by Fischers test (Samuels, 1989). The linear model used is described by the following equation:

$$Y_{ij} = \mu + T_i + H_j + TH_{ij} + e_{ij}$$

Where Y_{ij} = variable studied during the period

μ = overall mean of the population

T_i = effect of the i^{th} treatment

H_j = effect of the j^{th} house

TH_{ij} = effect of the ij^{th} interaction between treatment and house

e_{ij} = error associated with each Y

3.3 Results

3.3.1 Broiler performance

Table 3.4 and 3.5 indicate the average weekly and cumulative feed intake during the 35-day trial period. During the first 7 days, the feed intake was higher ($P \leq 0.05$) on the feed containing a mixture of ZB + Enviva Pro compared to Enviva Pro alone. During the second week, there was no difference ($P > 0.05$) in feed intake between the treatments. Between 14 and 21 days, feed intake was significantly higher on the feed containing a mixture of Biacid Nuclease + ZB compared to Enviva Pro alone, with no significant difference in feed intake between the other treatments. Over the 21-day cumulative period, feed intake was higher ($P \leq 0.05$) on the feed containing the mixture of Biacid Nuclease + ZB compared to Enviva Pro, with no significant difference in feed intake between the other treatments. Over the 28-day cumulative period, the feed intake on Enviva Pro was lower ($P \leq 0.05$) compared to MOS.

Table 3.4 Mean weekly feed intake (g) of broilers over a 35-day period receiving 8 different dietary treatments

Treatments	Days of age				
	0-7	7-14	14-21	21-28	28-35
Negative control	0.16 [ab]	0.37	0.58 [ab]	1.07	1.07
Zinc Bacitracin (ZB)	0.16 [ab]	0.36	0.57 [ab]	1.06	1.12
Biacid Nuclease	0.16 [ab]	0.37	0.58 [ab]	1.05	1.09
Biacid Nuclease + Zinc Bacitracin	0.16 [ab]	0.37	0.60 [a]	1.06	1.08
Enviva Pro	0.16 [b]	0.36	0.56 [b]	1.04	1.10
Enviva Pro + Zinc Bacitracin	0.17 [a]	0.37	0.57 [ab]	1.07	1.13
Mannoseoligosaccharide (MOS)	0.17 [ab]	0.37	0.58 [ab]	1.08	1.09
Mannoseoligosaccharide + Zinc Bacitracin	0.17 [ab]	0.36	0.58 [ab]	1.07	1.13
Standard error of the mean (SE)	± 0.002	± 0.006	± 0.013	± 0.013	± 0.024

[ab]Column means with the same superscript do not differ ($P > 0.05$)

Table 3.5 Cumulative feed intake (g) of broilers over a 35-day period receiving 8 different dietary treatments

Treatments	Days of age				
	0-7	0-14	0-21	0-28	0-35
Negative control	0.16 [ab]	0.53	1.11 [ab]	2.18 [ab]	3.25
Zinc Bacitracin (ZB)	0.16 [ab]	0.52	1.10 [ab]	2.16 [ab]	3.28
Biacid Nuclease	0.16 [ab]	0.53	1.11 [ab]	2.16 [ab]	3.25
Biacid Nuclease + Zinc Bacitracin	0.16 [ab]	0.54	1.13 [a]	2.19 [ab]	3.27
Enviva Pro	0.16 [b]	0.52	1.08 [b]	2.13 [b]	3.22
Enviva Pro + Zinc Bacitracin	0.17 [a]	0.54	1.11 [ab]	2.19 [ab]	3.32
Mannoseoligosaccharide (MOS)	0.17 [ab]	0.53	1.11 [ab]	2.19 [a]	3.29
Mannoseoligosaccharide + Zinc Bacitracin	0.17 [ab]	0.52	1.11 [ab]	2.17 [ab]	3.31
Standard error of the mean (SE)	± 0.002	± 0.007	± 0.015	± 0.024	± 0.037

[ab] Column means with the same superscript do not differ ($P > 0.05$)

Table 3.6 indicates the weekly body weights of the broilers during the 35-day trial period. There was no difference ($P > 0.05$) in the weekly body weights between the dietary treatments over the duration of the trial.

Table 3.6 Mean weekly BW (g) of broilers over a 35-day period receiving 8 different dietary treatments

Treatments	Days of age					
	0	7	14	21	28	35
Negative control	35.98	180.30	458.90	892.50	1569.70	2181.00
Zinc Bacitracin (ZB)	35.65	181.00	457.90	879.70	1555.10	2160.60
Biacid Nuclease	35.62	180.70	454.40	857.00	1556.80	2190.40
Biacid Nuclease + Zinc Bacitracin	36.01	179.90	459.00	910.80	1596.10	2135.50
Enviva Pro	35.58	178.50	455.10	851.70	1550.50	2138.90
Enviva Pro + Zinc Bacitracin	35.98	181.80	456.90	874.20	1559.80	2182.40
Mannoseoligosaccharide (MOS)	36.12	181.10	459.20	876.40	1561.90	2138.80
Mannoseoligosaccharide + Zinc Bacitracin	35.47	179.70	452.40	883.00	1547.90	2145.10
Standard error of the mean (SE)	± 0.312	± 2.023	± 5.604	± 20.557	± 15.156	± 22.983

Table 3.7 indicate the weekly FCR over the 35-day trial period. During the first 7 days, birds receiving the Enviva Pro + ZB mixture had a higher ($P \leq 0.05$) FCR compared to birds on the Biacid Nuclease treatment. During days 7-14, no difference was observed ($P > 0.05$) in FCR between the dietary treatments. During 14-21 days, the FCR of birds on the Biacid Nuclease treatment was higher ($P \leq 0.05$) than all the other dietary treatments except the MOS treatment. During 21-28 days the FCR of birds on the mixture of Biacid Nuclease + ZB was lower ($P \leq 0.05$) than the FCR of birds on ZB, MOS and the mixure of MOS + ZB, with no significant difference in FCR between the rest of the treatments.

Table 3.7 Mean weekly FCR of broilers over a 35-day period receiving 8 different dietary treatments

Treatments	Days of age				
	0-7	7-14	14-21	21-28	28-35
Negative control	1.13 ^{ab}	1.35	1.34 ^{b}	1.53 ^{ab}	1.78
Zinc Bacitracin (ZB)	1.12 ^{ab}	1.31	1.36 ^{b}	1.56 ^{a}	1.88
Biacid Nuclease	1.12 ^{b}	1.36	1.40 ^{a}	1.54 ^{ab}	1.76
Biacid Nuclease + Zinc Bacitracin	1.13 ^{ab}	1.35	1.36 ^{b}	1.48 ^{b}	1.83
Enviva Pro	1.12 ^{ab}	1.34	1.36 ^{b}	1.54 ^{ab}	1.84
Enviva Pro + Zinc Bacitracin	1.15 ^{a}	1.37	1.36 ^{b}	1.54 ^{ab}	1.84
Mannoseoligosaccharide (MOS)	1.14 ^{ab}	1.35	1.38 ^{ab}	1.55 ^{a}	1.91
Mannoseoligosaccharide + Zinc Bacitracin	1.14 ^{ab}	1.32	1.36 ^{b}	1.58 ^{a}	1.94
Standard error of the mean (SE)	± 0.010	± 0.019	± 0.014	± 0.024	± 0.066

^{ab} Column means with the same superscript do not differ ($P > 0.05$)

Table 3.8 indicates the cumulative FCR over the 35-day trial period. For 0-14 days, birds on the ZB treatment had a lower (P ≤0.05) FCR compared to birds on the mixture of Enviva Pro + ZB. For 0-21 days, birds on the Biacid Nuclease treatment had a higher (P ≤0.05) FCR compared to the birds receiving the negative control, ZB and a mixture of MOS + ZB, with no significant difference between the other treatments. Over the 28-days cumulative period, birds on the mixture of Biacid Nuclease + ZB had a lower (P ≤0.05) FCR compared to birds on the Biacid Nuclease treatment and the mixture of Enviva Pro + ZB and MOS. There was no significant difference between the rest of the treatments. No significant difference was observed in FCR over the 35-day cumulative period.

Table 3.8 Cumulative FCR of broilers over a 35-day period receiving 8 different dietary treatments

Treatments	Days of age				
Negative control	0-7	0-14	0-21	0-28	0-35
Negative control	1.13 ^{ab}	1.27 ^{ab}	1.31 ^{bc}	1.41 ^{ab}	1.60
Zinc Bacitracin (ZB)	1.12 ^{ab}	1.25 ^{b}	1.31 ^{bc}	1.42 ^{ab}	1.62
Biacid Nuclease	1.12 ^{b}	1.28 ^{ab}	1.34 ^{a}	1.43 ^{a}	1.59
Biacid Nuclease + Zinc Bacitracin	1.13 ^{ab}	1.28 ^{ab}	1.33 ^{abc}	1.40 ^{b}	1.62
Enviva Pro	1.12 ^{ab}	1.27 ^{ab}	1.32 ^{abc}	1.42 ^{ab}	1.63
Enviva Pro + Zinc Bacitracin	1.15 ^{a}	1.29 ^{a}	1.33 ^{abc}	1.43 ^{a}	1.63
Mannoseoligosaccharide (MOS)	1.14 ^{ab}	1.28 ^{ab}	1.33 ^{ac}	1.43 ^{a}	1.65
Mannoseoligosaccharide + Zinc Bacitracin	1.14 ^{ab}	1.26 ^{ab}	1.31 ^{bc}	1.43 ^{ab}	1.64
Standard error of the mean (SE)	± 0.010	± 0.012	± 0.009	± 0.011	± 0.019

$^{a-c}$ Column means with the same superscript do not differ (*P* >0.05)

Table 3.9 indicate the organ weights of the broilers sacrificed at day 35 as well as the organ weight ratio to the 35-day body weight for each treatment. No difference (*P* ≤0.05) in proventriculus weight was observed between any of the treatments. Birds on both the Mannoseoligosaccharide (MOS and MOS + ZB) treatments had a lower proventriculus weight expressed as % of BW compared to those on the Biacid Nuclease treatment. No differences (*P* ≤0.05) in gizzard weight and gizzard weight as % of BW were observed.

Table 3.9 Average proventriculus and gizzard weight of broilers at 35 days of age that received different feed additives supplements as an absolute value (g) and expressed as % of BW

Treatments	Proventriculus weight (g)	Proventriculus weight as % of BW	Gizzard weight (g)	Gizzard weight as % of BW
Negative control	6.49	0.30^{ab}	26.92	1.23
Zinc Bacitracin (ZB)	6.58	0.30^{ab}	26.42	1.21
Biacid Nuclease	6.81	0.31^{a}	25.93	1.18
Biacid Nuclease + Zinc Bacitracin	6.49	0.30^{ab}	26.52	1.23
Enviva Pro	6.45	0.30^{ab}	26.27	1.22
Enviva Pro + Zinc Bacitracin	6.63	0.30^{ab}	27.21	1.23
Mannoseoligosaccharide (MOS)	6.40	0.29^{b}	26.26	1.20
Mannoseoligosaccharide + Zinc Bacitracin	6.42	0.29^{b}	27.19	1.24
Standard error of the mean (SE)	± 0.146	± 0.0067	± 0.57	± 0.028

^{ab} Column means with the same superscript do not differ (*P* >0.05)

3.3.2 Histomorphological data

In table 3.10 the effect of the different dietary treatments on the duodenal villi height, crypt depth and villi height: crypt depth at 35 days of age were reported. The mixture of Enviva Pro + ZB resulted in longer ($P \leq 0.05$) villi compared to the negative control, ZB, Biacid Nuclease, Enviva Pro and MOS + ZB combination. Enviva Pro resulted in shorter ($P \leq 0.05$) villi compared to the rest of the treatments except the negative control. Enviva Pro resulted in shallower ($P \leq 0.05$) crypt depths compared to the rest of the treatments. There was no difference ($P > 0.05$) in the villi height: crypt depth between the treatments.

Table 3.10 Effects of different feed additives on the histomorphological parameters of the duodenum at 35 days of age

Treatments	Villi height (µm)	Crypt depth (µm)	Villi/crypt ratio
Negative control	1863.12^{bc}	183.99^{a}	10.32
Zinc Bacitracin (ZB)	1929.32^{c}	180.14^{a}	10.89
Biacid Nuclease	1911.41^{c}	181.74^{a}	10.57
Biacid Nuclease + Zinc Bacitracin	1992.74^{ac}	185.97^{a}	11.07
Enviva Pro	1748.57^{b}	152.37^{b}	11.64
Enviva Pro + Zinc Bacitracin	2097.66^{a}	188.07^{a}	11.39
Mannoseoligosaccharide (MOS)	1982.30^{ac}	189.91^{a}	10.55
Mannoseoligosaccharide + Zinc Bacitracin	1943.44^{c}	193.78^{a}	10.70
Standard error of the mean	± 48.44	± 8.53	± 0.55

$^{a-c}$ Column means with the same superscript do not differ ($P > 0.05$)

Table 3.11 indicates the effect of the different dietary treatments with and without ZB on the duodenal villi height at 35 days of age. Birds receiving dietary treatments with ZB had longer ($P \leq 0.05$) villi compared to those treatments that did not contain any ZB.

Table 3.11 Effects of different feed additives on the duodenum villi height (µm) at 35 days of age with and without Zinc Bacitracin

Treatments	Without Zinc Bacitracin (µm)	With Zinc Bacitracin (µm)	Average (µm)
Control	1863.12	1929.32	1896.22
Biacid Nuclease	1911.41	1992.74	1952.08
Enviva Pro	1748.57	2097.66	1923.12
Mannoseoligosaccharide	1982.30	1943.44	1962.87
Average (µm)	1876.35^{B}	1990.79^{A}	

Column means with the same superscript do not differ ($P > 0.05$)

^{AB} Row means with the same superscript do not differ ($P > 0.05$)

Table 3.12 indicates the effect of the different dietary treatments with and without ZB on the duodenal crypt depth at 35 days of age. There was no difference (P >0.05) between treatments containing and not containing ZB. Enviva Pro, either supplemented alone or with ZB, resulted in shallower (P ≤0.05) crypt depths compared to the rest of the treatments at 35 days of age.

Table 3.12 Effects of different feed additives on the duodenum crypt depth (µm) at 35 days of age with and without Zinc Bacitracin

Treatments	Without Zinc Bacitracin (µm)	With Zinc Bacitracin (µm)	Average (µm)
Control	183.99	180.14	182.07[ab]
Biacid Nuclease	181.74	185.97	183.86[ab]
Enviva Pro	152.37	188.07	170.22[b]
Mannoseoligosaccharide	189.91	193.78	191.84[a]
Average (µm)	177.00	186.99	

[ab] Column means with the same superscript do not differ (P >0.05)

Row means with the same superscript do not differ (P >0.05)

Table 3.13 indicates the effect of the different dietary treatments on the jejunum villi height, crypt depth and villi height: crypt depth at 35 days of age. There was no difference (P >0.05) between the dietary treatments on villi height, crypt depth and villi height: crypt depth in the jejunum.

Table 3.13 Effects of different feed additives on the histomorphological parameters of the jejunum at 35 days of age

Treatments	Villi height (µm)	Crypt depth (µm)	Villi/crypt ratio
Negative control	1410.36	143.98	9.96
Zinc Bacitracin (ZB)	1340.43	149.87	9.21
Biacid Nuclease	1355.33	153.08	8.90
Biacid Nuclease + Zinc Bacitracin	1275.28	149.89	8.60
Enviva Pro	1328.11	146.61	9.30
Enviva Pro + Zinc Bacitracin	1380.87	149.50	9.40
Mannoseoligosaccharide (MOS)	1380.87	157.92	8.64
Mannoseoligosaccharide + Zinc Bacitracin	1352.92	149.30	9.40
Standard error of the mean (SE)	± 52.61	± 7.04	± 0.49

Column means with the same superscript do not differ (P >0.05)

There were no significant differences between treatments for jejunum villi height and crypt depth when given with or without ZB (Results not shown). Table 3.14 indicates the effect of the 8 dietary treatments on the villi height and crypt depth in the ileum. Enviva Pro resulted in shorter (P ≤0.05) villi compared to the Biacid Nuclease + ZB mixture. Biacid Nuclease + ZB resulted in longer (P ≤0.05) villi than the negative control, with no difference (P >0.05) between the rest of the treatments. There was no difference (P >0.05) between any treatments for crypt depth or villi height: crypt depth.

Table 3.14 Effects of different feed additives on the histomorphological development of the ileum at 35 day of age

Treatments	Villi height (µm)	Crypt depth (µm)	Villi/crypt ratio
Negative control	692.71[b]	139.84	5.51
Zinc Bacitracin (ZB)	731.37[ab]	140.78	5.25
Biacid Nuclease	742.05[ab]	142.84	4.91
Biacid Nuclease + Zinc Bacitracin	876.12[a]	152.11	5.82
Enviva Pro	676.87[b]	143.09	4.80
Enviva Pro + Zinc Bacitracin	798.89[ab]	141.47	5.67
Mannoseoligosaccharide (MOS)	744.22[ab]	135.43	5.95
Mannoseoligosaccharide + Zinc Bacitracin	756.03[ab]	143.49	5.42
Standard error of the mean (SE)	± 52.75	± 8.04	± 0.45

[ab] Column means with the same superscript do not differ (*P* >0.05)

Table 3.15 indicates the effect of the different dietary treatments with and without ZB on the ileal villi height at 35 days of age. Feed supplemented with ZB resulted in longer (P ≤0.05) villi than those not supplemented with ZB.

Table 3.15 Effects of different feed additives on the ileum villi height (µm) at 35 days of age with and without Zinc Bacitracin

Treatments	Without Zinc Bacitracin (µm)	With Zinc Bacitracin (µm)	Average (µm)
Control	692.71	731.37	736.71
Biacid Nuclease	742.05	876.12	784.41
Enviva Pro	676.87	798.89	771.56
Mannoseoligosaccharide	744.22	756.03	716.45
Average (µm)	713.96[B]	790.6[A]	

Column means with the same superscript do not differ (*P* >0.05)

[AB] Row means with the same superscript do not differ (*P* >0.05)

There were no significant differences between treatments for ileal crypt depths when given with or without ZB (Results not shown).

3.4 Discussion

3.4.1 Broiler performance

During Trial 1, the birds did not receive any pathogenic challenge. There were, however, significant differences in feed intake. A weekly difference in feed intake was observed, but cumulatively over the whole 35-day trial period, there was no difference in feed intake. No difference in the weekly BW was noted in this trial. This is in agreement with a study done by Gunal *et al.* (2006) who determined the effects of AGP, organic acids and probiotics on the performance, intestinal microflora and tissue of broilers over a 42-day trial period. They reported no significant difference in live weight gain, feed intake, FCR and mortality. Similar results were verified in another study who also evaluated the effects of different feed additives and found no significant

difference on weight gain and FCR of broilers between 1 and 21 days of age (Pelicano *et al.*, 2004). Several researchers found that well-nourished chicks raised under clean conditions did not respond to growth promoters. There are, however, some reports of beneficial effects of these additives on the weight gain and FCR of broilers (Gunal *et al.*, 2006). Generally, the results in literature seems to be inconsistent when it comes to additives such as AGP, probiotics, prebiotics and EO. There are numerous factors that can influence responses to probiotics such as age, diet type, environment and management, all of which contribute to the contrasting results (Yang *et al.*, 2009). The cumulative FCR results are inconsistent with no real difference between any of the treatments over the 35-day period. None of the additives could provide a better feed conversion compared to the control diet during this period. It appears that the well established *in vitro* bacteriostatic and bactericide mode of actions of these additives might not reach its full potential through practical field applications due to low stressed environmental conditions.

The Enviva Pro treatment consisted of a three-strain combination of *Bacillus* spp*.,* which is suitable for use in animal feed as a live microbial product due to its long shelf life and retained viability during distribution, processing and storage of feed. The spores are able to germinate rapidly within the animal, and vegetative cells were detected in the GIT of broilers only 20 hours after dosing when the product was administered to day old chicks (Cartman *et al.*, 2008). In this trial there was a consistent lower feed intake of the treatment containing the probiotic over 35 days. This effect on feed intake could be due to rearing the birds in optimum prophylactic conditions and therefore not constituting a challenging environment. The intestinal flora may become unbalanced when supplementing microorganisms present in the probiotic at amounts above those usually found in the digestive tract and, thereby, the microorganisms can actually become an infective agent to the bird, disrupting the metabolism and consequently reducing feed intake (Nunes *et al.*, 2012). An alternative reason could be that probiotics increase nutrient digestion and that the birds will meet their nutrient requirement with less feed, consequently resulting in a lower feed intake. The fact that there is no significant difference in FCR between the Enviva Pro treatment and the rest of the treatments over the 35-day period makes the latter speculation more probable, especially since a disturbed microbiome would reduce feed intake with a significant reduction in BW, which was not observed. Loddi *et al.* (2000) found similar results in their study when feeding broilers probiotics and antibiotics. Broilers fed the diets containing probiotics had lower feed intake in the initial rearing phase compared to the rest of the treatments.

In contrast, Correa *et al.* (2003) found no difference in feed intake of broilers when feeding diets containing probiotics and antibiotics during the rearing phase. Likewise, Pelicano *et al.* (2004) did not observe any significant difference in weight gain, feed intake and FCR of broilers fed probiotics during a 42-day experimental period. The different responses in probiotic trials with broilers might be due to factors such as the concentration and microorganisms used, the animal health, diets, temperature, strain, sex of the bird, stocking density and facility (Faria *et al.*, 2009).

The use of Enviva Pro during this trial promoted a reduction in feed intake without compromising performance. In terms of performance, there was synergy when Enviva Pro was combined with ZB, which is in agreement with Wealleans *et al.* (2017). In fact, these two treatment groups had the same FCR over a 35-day period, with the Enviva Pro + ZB combination resulting a numerically higher BW (2182.4 g vs 2138.9 g) compared to Enviva Pro alone at 35 days. It is important to keep in mind that that the production environment has a major influences on the improvements in growth performance when supplementing AGPs or other

performance-enhancing agents (Hathaway *et al.*, 1996; Turner *et al.*, 2001). This might explain the non-significant effects of the dietary treatments on the 35-day BW or FCR over the 35-day period. In unchallenged situations such as this study where birds were housed on clean litter and not subjected to any artificial challenge, it will be unlikely to observe one full effect of the probiotic or probiotic + AGP additives on growth performance.

The MOS treatment consisted out of mannoseoligosaccharides, which is derived from the outer cell wall of a selected strain of *Saccharomyces cerevisiae*. The aim of MOS is to bind to pathogenic bacteria such as *Salmonella* or *E. coli*, preventing the colonisation of these pathogenic bacteria in the gut (Bozkurt *et al.*, 2008). Since the birds did not receive any artificial challenge while reared under a clean environment, there were no difference between either the MOS + ZB treatment or MOS treatments and the control diet. MOS can thus only contribute to the performance of the birds under an environmental condition that diverts nutrients away from growth, such as a pathogenic challenge.

The addition of Biacid Nuclease + ZB to the broiler diet improved the feed conversion over the 28-day cumulative period compared to Biacid Nuclease alone. These results are in agreement with Mandal *et al.* (2000) and Jamroz *et al.* (2003) who observed that birds receiving EO had a lower FCR compared to the control. The addition of the combination of Biacid Nuclease + ZB resulted in a lower FCR ($P \leq 0.05$) and numerically higher BW over the 28-day period. Several other studies have described a synergistic or additive effect between antibiotics and EO. For example, thymol was found to be synergistic with *Penicillin* against *E. coli*, where thymol was also synergistic with *Penicillin* against both *E. coli* and *S. typhimurium* (Gallucci *et al.*, 2006). There are, however, contrasting results, for example, *Penicillin* combined with whole oregano oil or carvacrol did not show synergistic effects against *E. coli* (Gallucci *et al.*, 2006; Si *et al.*, 2008). It is therefore important to find the right synergistic relationship between the AGP and EO used. It is therefore believed that the mixture of EO (Biacid Nuclease) covers a bigger frame of this synergistic relationship. For example, thymol is synergistic with ZB where eugenol does not show the same synergistic results in other studies (Langeveld *et al.*, 2014). It has also been reported that the combination of AGPs and plant extracts such as EO can prevent the growth of pathogens by competitive elimination. During the trial, the use of a growth promoter combination may have led to a desirable effect on the bird's performance which was confirmed by Biacid Nuclease + ZB combination outperforming the Biacid Nuclease individual treatment. One might also speculate that when using this combination of Biacid Nuclease + ZB, that ZB reduces the pathogenic load to a point where less energy is diverted towards an immune response and more energy is available for growth and nutrient uptake which is further facilitated by Biacid Nuclease.

The birds during this trial were not challenged, therefore an alternative mode of action should be considered to the welfare and performance of the bird. Biacid Nuclease can positively influence the immune system due to its anti-oxidant effects. The antioxidant activity is mainly due to its redox properties and chemical structure (Brenes & Roura, 2010). EO are also known for improving nutrient absorption by increasing the membrane translocation of sodium-glucose contransporter-1 that controls glucose uptake from the gut. Furthermore, improved digestibility of nutrients can lead to a more balanced microflora with the potential not to only reduce the proportion of pathogenic bacteria, but to also improve the efficiency of feed utilisation which results in enhanced growth (Langhout, 2000; Williams & Losa, 2001). Increased feed intake and digestive secretion were observed in animals offered EO supplemented feed (Yang *et al.*, 2009).

3.4.2 Histomorphological data

An increase in villi height suggests an increase in the surface area that is capable of greater absorption of available nutrients (Samanya & Yamauchi, 2002). The villi crypt, however, is considered to be the villus factory which is responsible for renewal of the villi as needed in response to sloughing or inflammation from pathogens and their toxins that can create a higher demand for tissue (Yason *et al.*, 1987). Shorter villi with deeper crypts may lead to poor nutrient absorption, increased secretion in the GIT and consequently lower performance (Xu *et al.*, 2003). Longer villi and villi height: crypt depth thus result in a slower turnover of intestinal mucosa. This slower turnover of intestinal epithelial cells results in a lower maintenance requirement, which leads to higher growth rates and improved efficiency (Van nevel *et al.*, 2005).

The Enviva Pro treatment resulted in the shortest duodenal villi compared to the rest of the treatments, together with the shallowest duodenal crypt depth, which resulted in the numerically largest villi height: crypt depth. It can be speculated that the lower feed intake of this treatment is correlated with the shortest villi and shallowest crypt depth. Since the 35-day FCR of the Envivo Pro treatment showed no significant difference from the rest of the treatments, there seems to be efficient digestion and utilisation of nutrients that can be portioned towards growth. The shorter villi and shallow crypt depth, together with no difference in broiler performance could be an indication of the good health status of the bird. Shorter villi with deeper crypt depths are an indication of pathological colonisation (Samadian *et al.*, 2013), but since these birds were not challenged and the villi height: crypt depth is numerically higher, it can be concluded that the birds had no pathological threat and was able to meet its nutrient requirement with a shorter villi height. These results are contrary to other studies (Samanya & Yamauchi, 2002; Gunal *et al.*, 2006; Awad *et al.*, 2009), which found that probiotics increased the villi height compared to the basal control. Although there was no significant difference between the negative control and the Enviva Pro treatment, the Enviva Pro treatment had numerically shorter villi compared to the negative control. The villi height: crypt depth in the ileum was the smallest, contrary to what was found in the duodenum, but then again there were no significant differences between the rest of the treatments.

The combination of Enviva Pro + ZB resulted in the longest duodenal villi, significantly longer than all of the treatments except the Biacid Nuclease + ZB and MOS treatment. Although the duodenal villi were the longest for the Enviva Pro + ZB treatment, there was still no significant difference between the villi height: crypt depth, due to the deeper crypt depths of this treatment. This Enviva Pro + ZB interaction is in agreement with another study (Wealleans *et al.*, 2017) which found that their probiotic + AGP treatments significantly increased the villi height compared to the negative control and AGP treatment alone. These improvements in gut histology parameters with the Enviva Pro + ZB treatments are likely to be multifactorial. This synergy might be due to AGP directly changing the microbiome community structure from pathogenic to commensal which has been shown to increase villi heights (Xu *et al.*, 2003; Pan & Yu, 2014), while probiotics aids in establishing a beneficial microbial population, in the presence of AGP suppressing the microbiome. The ability of *Bacillus* spp. probiotic to increase lactobacilli counts have been documented (Lee *et al.*, 2010; Mallo *et al.*, 2010), which can increase butyrate production, an important energy source for the epithelial cells. Lactobacilli however do not produce the butyrate, instead they increase butyric concentration indirectly by stimulating the proliferation of butyrate producing bacteria through a mechanism known as cross feeding (Harimurti & Hadisaputro, 2015).

There were no significant effects of the Enviva Pro + ZB combination on the villi height and crypt depth of the ileum. In fact, there were no significant difference between the combination of Enviva Pro + ZB and the Enviva Pro and ZB individual treatments.

The Biacid Nuclease + ZB combination resulted in the longest villi in the ileum, being significantly longer than the negative control and Enviva Pro treatment. The crypt depth, however, did not differ significantly between any of these treatments, resulting in no significant difference for the villi height: crypt depth. When analysing the performance data, the same treatment group with the lowest FCR over a 28-day period had the longest ileal villi height. Biacid Nuclease can increase the turnover of the gut lining and prevent a coccidial attack by maintaining a more healthy population of gut cells (Ferket, 2004). For this increased turnover of the gut lining, there has to be deeper crypt depths, which might explain why this treatment had the deepest ileal crypts. In addition to increasing epithelial cell turnover, intestinal microvilli enzymes such as disaccharidase, alkaline phosphatase and leucine aminopeptidase secretion is also increased, which is important constituents of the microvillous membrane associated with degradation and absorption of nutrients (Jang *et al.*, 2007). The combination with ZB could be synergistic on the histopathological development of the gut since ZB together with Biacid Nuclease can suppress the growth of undesirable microbes, allowing for longer villi. Samadian *et al.* (2013) reported that the supplementation of EO might have reduced the number of harmful bacteria and its adhesion to epithelial cells, hence reducing the production of toxic compounds that damage the epithelial cells, resulting in shorter villi. Longer villi will increase the number of microvilli, thus more enzymes are secreted to aid in digestion and absorption of nutrients which could consequently improve the growth rate and FCR as seen in the current trial.

The MOS treatment had no effect on the histomorphological development of the intestine, even when combined with ZB. MOS offers a binding site for pathogenic bacteria, so instead of binding to the epithelial cells, pathogens absorb the MOS and therefore moves through the intestine without colonising the gut (Bozkurt *et al.*, 2008). The birds in this study probably did not have any significant pathogen challenge and therefore the treatment will not exert its intended potential, resulting in no difference between the treatment and the negative control.

In this trial, the treatment additives were either given alone or with ZB. When recording the effect of the absence or presence of ZB on histomorphological development in the duodenum, a difference (P <0.05) in villi height for treatments combined with ZB (1990.79 µm) was reported compared to treatments given without ZB (1876.35 µm). The same effect on the histomorphological development of the ileum in the bird was reported, with those treatments combined with ZB increasing the villi height significantly longer (790.6 µm) than those treatments given alone (713.96 µm). Naturally, it seems reasonable to assume that AGP act on the microflora in the intestine, inhibiting certain bacterial species and consequently preventing them from producing toxins that destroy epithelial cells (Bozkurt *et al.*, 2008), including those of villi. The synergy between ZB and the alternative treatments seems to be prominent and might be explained by the different modes of action of each treatment being complimented in the presence of ZB.

3.5 Conclusion

The effects of the alternative feed additives under non-challenging conditions are difficult to quantify due to the intended mode of action of these supplements. When given in combination with AGP, the alternative feed additives resulted in longer duodenal and ileal villi heights compared to the individual treatment. However, the alternative feed additives under non-challenging conditions showed no direct increase in performance when given alone or in combination with AGP, which again is in agreement with other research that showed well-nourished chicks raised under clean conditions do not always respond to growth promoters. In some cases where an effect on performance under non-challenging conditions was noted, it could be attributed to the effect of the supplement on appetite and immune stimulating properties. EOs are an excellent example of this, where apart from having anti-microbial properties, it can improve digestion and absorption of nutrients.

Chapter 4

Trial 2: The effects of antibiotic growth promoters and alternative feed additives on performance and gut morphology of broilers challenged with necrotic enteritis.

4.1 Introduction

The second trial was conducted under conditions where the birds were challenged with necrotic enteritis (NE) in a broiler facility on the Hillcrest Experimental Farm, University of Pretoria. All procedures used in this trial were approved by the Animal Ethics Committee (AEC) of the University of Pretoria (EC008-16).

4.2 Materials and methods

4.2.1 Housing and care

Housing and care of the birds were done in such a way as to represent as far as possible commercial broiler production conditions. Prior to placing the day-old chicks, the broiler house was washed, disinfected, and pre-heated to the comfort zone of the chicks of 36ºC ambient temperature and at least 34ºC litter (floor) temperature. Pine shavings was spread on the floor of the pens to absorb waste and to assist with insulation from the floor. A total of 2208 day-old male chicks were obtained from a commercial hatchery. Only male Ross birds from the same breeder flock were used for these trials to minimise variation between birds. On arrival, all birds were selected randomly and weighed as a group of 23 to determine the average body weight per pen. Weighed groups were then placed in floor pens of the environmentally controlled broiler house. Automatic heaters provided the optimum temperature to keep the birds in their desired comfort zone. Ventilation was controlled automatically to ensure optimum oxygen supply and removal of ammonia and carbon dioxide. A lighting program consisting of 23 hours of light and 1 hour of dark was provided during the first week of life to the chicks to stimulate normal daily feed and water intake. Thereafter, the length of daylight was reduced to 16 hours of light according to the Ross' Broiler Management Guide (Broiler management manual Ross-308, 2014). Birds were monitored on a daily basis by the principal investigators and supervisor as well as students and staff on the farm to ensure optimum growing conditions and bird comfort throughout the 35-day trial period. Any variation from the individual's normal behaviour or from its general appearance or appearance of the excreta was noted. Special attention was given to individual birds that were sick or in poor condition. Any bird judged to be suffering pain, distress, or appearing to be unlikely to survive was humanly euthanised and subjected to necropsy to determine the cause. Culled or dead birds were weighed to correct for feed conversion ratio (FCR).

4.2.2 Pen design

Pens treatment designation followed a completely randomised block design to minimise the influence of variations in the house environment (Figure 4.1). There were 96 pens of 1.5 meters x 1.5 meters with 23 birds housed per pen to simulate typical stocking densities used in the broiler industry. Pens were divided into 8 treatment groups consisting of 12 replicate pens per treatment to provide sufficient statistical power to the study. All birds had free access to feed and water at all times, provided by a tube feeder and 5 nipple drinkers

per pen. During the first week after chick placement one extra pan feeder and 1 fountain drinker were provided per pen in order to encourage and assist chicks to eat and drink.

Block 6

7*	3
1	5
8	4
2	6

Block 3

8	4
2	6
1	5
3	7

Block 5

1	5
3	7
2	6
4	8

Block 2

2	6
4	8
3	7
5	1

Block 4

3	7
5	1
4	8
6	2

Block 1

4	8
6	2
5	1
7	3

* Numbers indicate the dietary treatment applied to the specific pen

Figure 4.1 Pen arrangements of treatments and replications for one of the two experimental houses, demonstrating the blocking of treatments

4.2.3 Dietary treatments

The birds received a nutrient dense broiler ration mixed at a small-scale feed mill (Pennville Animal Nutrient Solutions) according to nutrient specifications recommended by the breeder company (Ross Breeders). Feed consumption was monitored and the feeders refilled when needed on a daily basis to ensure *ad libitum* feed intake. A four-phase feeding program was implemented with pre-starter, starter, grower and finisher feeds. The pre-starter was fed from day 0-7 as crumbles and starter was also fed as crumbles from day 7-21. The grower was fed as pellets from day 21-28 and finisher also fed as pellets from day 28-35. All groups received identical feed with the only difference between treatments being the specific additive supplemented in the feed. The dietary feed additives were blended with the premix to be used for the relevant treatments. The negative control consisted out of the base diet without any feed additives blended with the premix. The same basal diet was mixed for all 8 treatments after which the premixes with the different additives listed below were added to the basal feed and remixed to form the 8 different dietary treatments shown in Table 4.1. The additives used during Trial 2:

1. Zinc Bacitracin 15 % m/m (ZB; an antibiotic growth promoter) (Ceva Animal Health, South Africa, Reg. No. G1070 (Act 36/1947)
2. Clostat Dry (a three strain Bacillus probiotic product) (Kemin, South Africa; Reg. No. V21583)
3. Biacid (an essential oil (EO) and organic acid blend) (Cargill, Minnesota, USA).
4. Biacid Nucleus (an essential oil (EO) product) (Cargill, Minnesota, USA). DAFF import permit (no: 11/1/391) for trial purposes (date of permit: 08/02/2016)

The 8 dietary treatments in (Table 4.1) consisted of a negative control (basal), a positive control (ZB), EO (Biacid Nucleus), EO + Organic acids (Biacid), Probiotic (Clostat), EO + probiotic (Biacid nucleus + Clostat), EO + Positive control (Biacid nucleus + ZB), Positive control + EO + organic acids (ZB + Biacid).

Table 4.1 Eight dietary treatments containing different inclusion levels of feed additives

Treatment name	Inclusion level (g/ton)			
	ZnBac®	Biacid Nuclease®	Clostat®	Biacid®
Negative control	0	0	0	0
Zinc Bacitracin (ZB)	333	0	0	0
Biacid Nuclease	0	100	0	0
Biacid	0	0	0	1000
Clostat	0	0	500	0
Biacid Nuclease + Clostat	0	100	500	0
Biacid Nuclease + Zinc Bacitracin	333	100	0	0
Biacid + Zinc Bacitracin	333	0	0	1000

The rations were mixed to specifications shown in Table 4.2.

Table 4.2 Raw material inclusion (%) and calculated nutrient composition of the basal broiler diet for each of the four phases

Ingredients	Phase			
	Pre-starter	Starter	Grower	Finisher
Maize yellow	58.50	64.00	69.63	73.73
Soya oilcake (46.5%)	33.63	28.50	20.27	16.70
Sunflower oilcake (36%)	2.00	2.50	3.00	3.00
Gluten 60	1.00	0.93	3.00	3.00
Lysine (Sint 78%)	0.28	0.28	0.33	0.36
Methionine (DL 98%)	0.26	0.24	0.16	0.15
Threonine (98%)	0.06	0.05	0.03	0.04
Oil crude soya	0.67	0.50	0.97	0.80
Feed lime (50:50 Mix)	1.74	1.55	1.42	1.28
Mono-dicalcium phosphate (Ws>70%)	0.93	0.61	0.45	0.24
Salt (Fine)	0.25	0.25	0.16	0.16
Sodium bicarbonate	0.36	0.28	0.33	0.33
Phytase (Axtra Phy 1000 FTU's)	0.01	0.01	0.01	0.01
Broiler starter premix (3kg/t)	0.30	0.30	0.00	0.00
Broiler grower premix (2.5kg/t)	0.00	0.00	0.25	0.00
Broiler finisher premix (2kg/t)	0.00	0.00	0.00	0.20
Calculated nutrient values			(g/kg)	
Moisture	106.50	106.89	105.73	106.05
Metabolisable energy, MJ/kg	11.50	11.75	12.30	12.50
Crude protein	220.37	201.86	182.84	169.78
Crude fat	35.74	35.29	40.71	39.93
Crude fibre	34.43	35.36	35.44	35.36
Ash	59.36	51.90	44.23	39.18
Calcium	10.40	9.06	8.14	7.22
Total phosphorus	5.93	5.15	4.58	4.04
Total lysine	13.60	12.28	10.70	10.02

4.2.4 Proximate and mineral analyses of final feed

Representative feed samples of each phase (pre-starter, starter, grower and finisher) were sampled during production of the basal diet. The feed samples (4 samples) were analysed according to the proximate analysis system for their nutritional content at Nutrilab (Department of Animal and Wildlife Science, University of Pretoria). This system determines seven fractions in food including dry matter (AOAC, 2000, Official method of analysis 942.05), ash (AOAC, 2000, Official method of analysis 942.05), crude protein, crude fat (AOAC, 2000, Official method of Analysis 920.39), crude fibre (AOAC, 2000, Official method of Analysis 962.09), calcium (AOAC, 2000, Official method of Analysis 935.13) and total phosphorus (AOAC, 2000, Official method of Analysis 965.17). The Leco FP-428 (Leco Corporation, 3000 Lakeview Avenue, St. Joseph, MI 49085-2396) was used to analyse the nitrogen content of the feed and the method used was according to the AOAC's official method of analysis (AOAC, 2000, Official method of Analysis 988.05). Results of the analysis are presented in Table 4.3.

Table 4.3 Analysed nutrient values (on "as fed" basis) of the pre-starter, starter, grower and finisher feeds

Nutrient	Pre-starter	Starter	Grower	Finisher
Dry Matter (%)	89.1	89.3	88.6	89.8
CrudeProtein (%)	22.4	20.1	18.5	18.0
CrudeFibre (%)	3.60	3.27	3.50	3.00
CrudeFat (%)	3.72	3.80	4.32	4.22
Ash (%)	5.50	4.55	4.12	3.68
Calcium (%)	0.82	0.78	0.70	0.66
Phosphorus (%)	0.62	0.50	0.42	0.40

4.2.5 Performance parameters

Body weight (BW) of broiler chickens was measured on a weekly basis. The chicks were weighed before they were placed (day 0) and then on day 7, 14, 21, 28 and 35. Feed intake was measured weekly on the same day that the chicks were weighed. To measure feed intake, each pen was allocated a bin of known weight. Feed was weighed into the bin at the beginning of the week and weighed again, together with any orts from the pen's feeder, at the end of the week. The total amount of consumed feed was divided by the amount of birds in each pen to determine feed intake per bird over the week period. During two daily inspections in the broiler house dead birds were collected and the weight of dead birds recorded. The weekly FCR was calculated as feed intake (g)/ body weight (g). The FCR was corrected for BW of mortalities.

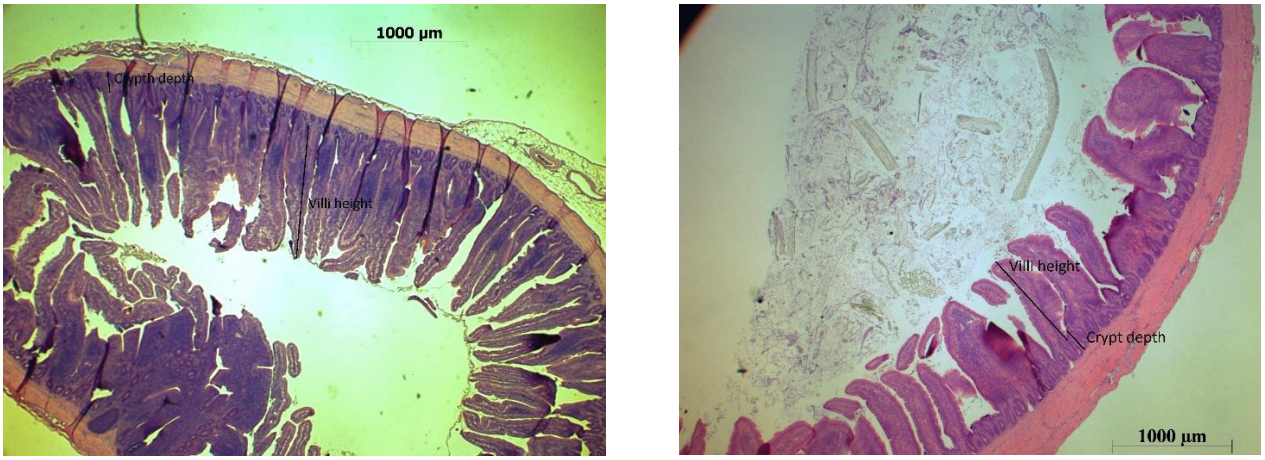
4.2.6 Method for inducing necrotic enteritis

During this trial, all the birds were subjected to conditions prescribed by Lensing *et al.* (2010) and M'Sadeq *et al.* (2015) to induce a NE challenge. At 10 days of age the broilers received a coccidial vaccine (Immunocox, Ceva), which consisted of attenuated oocysts of *Eimeria acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella*, at 10 x times the dosage prescribed by the manufacturer. Birds were orally inoculated 4 days later on day 14 with *C. perfringens* type A culture (10^8 CFU/µL, 1 mL liver broth). A gastrointestinal infection like coccidiosis will increase mucus production, which is a predisposing factor for

the enteric pathogen *C. perfringens* to proliferate and cause NE. This model was used to produce subclinical NE without increasing mortality, with only a slight decrease in performance.

4.2.7 Histomorphology

At the end of the trial (Day 35), two birds per pen (24 birds per treatments) were selected, based on the average weight of the pen and sacrificed for sample collection. During sample collection the small intestine was immediately opened after killing, after which the duodenum, jejunum and ileum were inspected and scored for lesions caused by necrotic enteritis. The ileum was defined as extending from Meckel's diverticulum to a point 4 cm to distal. The jejunum was defined as midway between the end of the duodenum and Meckel's diverticulum. Gut lesions were scored in different intestinal sections for macroscopical lesions typical for coccidial infections according to Johnson and Reid (1970) and for NE according to Prescott *et al.* (1978) with scores ranging from 0 to 4 (0: no lesions, 1+: thin-walled or friable small intestine, 2+: focal necrosis or ulceration, 3+: larger patches of necrosis and 4: extensive necrosis). For histopathologic and morphometric analysis, 1-cm tissue samples from the duodenum, jejunum and ileum were obtained and fixed in 10% buffered formalin (100 mL of 40% formaldehyde. Four grams phosphate, 6.5g dibasic sodium phosphate and 900 mL of distilled water) for 48 hours. Tissues were then dehydrated by transferring it through a series of alcohols with increasing concentrations over a 24-hour period after which it was placed into xylol and embedded in paraffin wax. A microtome was used to make cuts that were 4µm thick. The cuts were stained with hematoxylin-eosin on microscope slides by the Department of Pathology, University of Pretoria, Onderstepoort. The values were measured using a Zeiss Axiovert 2000 with Zeiss Axiovision 4.8.2.0 software at the Microscopy Department, University of Pretoria. The morphometric indices evaluated were villi height from the tip of the villi to the crypt, crypt depth from the base of the villi to the submucosa, and the villus height to crypt depth ratio. Measurements of the villi height and crypt depth were determined at a magnification of 5X.



a) Jejenum b) Ileum

Figure 4.2 Villi height and Crypt depth measurements of the jejenum (a) and ileum (b)

4.2.8 Statistical analysis

Statistical analysis was done with the statistical software program SAS (Statistical Analysis System, 2014). The significance between treatments was determined by analysis of variance (ANOVA) with the general linear model (GLM). Means, standard error and significance of differences between means were determined by Fischers test (Samuals, 1989) at the 95% confidence level. In all cases the level of statistical significance was $P \leq 0.05$. Repeated Measures Analysis of Variance with the GLM model (SAS, 2014) were used for repeated period measures. Means and standard error of means for the different treatments were calculated and significant differences ($P \leq 0.05$) between means were determined by Fischers test (Samuels, 1989). The linear model used is described by the following equation:

$$Y_{ij} = \mu + T_i + H_j + TH_{ij} + e_{ij}$$

Where Y_{ij} = variable studied during the period

μ = overall mean of the population

T_i = effect of the i^{th} treatment

H_j = effect of the j^{th} house

TH_{ij} = effect of the ij^{th} interaction between treatment and house

e_{ij} = error associated with each Y

e_{ij} = error associated with each Y

4.3 Results

4.3.1 Broiler performance

Table 4.4 indicates the average weekly feed intake of broilers over a 35-day period. During the first 7 days, feed intake of the birds was lower ($P \leq 0.05$) on the feed containing the ZB treatment compared to those birds on the treatments containing Biacid Nuclease, Biacid, Biacid Nuclease + Clostat as well as Biacid Nuclease + ZB. During days 7-14, feed intake of birds on the Biacid Nuclease + Clostat treatment was lower ($P \leq 0.05$) compared to those on the Clostat and Biacid Nuclease + ZB treatments, with no differences ($P > 0.05$) between the rest of the treatments. During days 14-21, the feed intake of birds on the Biacid + ZB treatment was lower ($P \leq 0.05$) compared to the feed intake of birds on Clostat, with no difference ($P > 0.05$) between the rest of the treatments. There was no difference in feed intake ($P > 0.05$) between any of the treatments during days 21-28. During days 28-35, the feed intake of birds on the negative control was higher ($P \leq 0.05$) compared to those on the Biacid Nuclease, Biacid and Clostat treatments. The feed intake of birds on the ZB treatment was higher ($P \leq 0.05$) compared to those on the Biacid Nuclease and Clostat treatments. Feed intake of birds on the Biacid Nuclease treatment was lower ($P \leq 0.05$) compared to those on the Biacid Nuclease + ZB and Biacid + ZB treatments during days 28-35.

Table 4.4 Mean weekly feed intake (g) of broilers over a 35-day feeding period receiving 8 different dietary treatments

Treatments	Days of age				
	0-7	7-14	14-21	21-28	28-35
Negative control	0.17 [ab]	0.41 [ab]	0.68 [ab]	0.91	1.18 [a]
Zinc Bacitracin (ZB)	017 [b]	0.40 [ab]	0.68 [ab]	0.92	1.15 [ab]
Biacid Nuclease	0.18 [a]	0.42 [ab]	0.71 [ab]	0.90	1.02 [c]
Biacid	0.18 [a]	0.41 [ab]	0.71 [ab]	0.91	1.08 [bc]
Clostat	0.18 [ab]	0.42 [a]	0.72 [a]	0.90	1.05 [cd]
Biacid Nuclease + Clostat	0.18 [a]	0.39 [b]	0.70 [ab]	0.90	1.09 [abc]
Biacid Nuclease + Zinc Bacitracin	0.18 [a]	0.42 [a]	0.71 [ab]	0.94	1.11 [abd]
Biacid + Zinc Bacitracin	0.18 [ab]	0.41 [ab]	0.67 [b]	0.91	1.13 [abd]
Standard error of the mean (SE)	±0.02	± 0.008	± 0.013	± 0.033	± 0.034

[a-d] Column means with the same superscript do not differ (*P* >0.05)

Table 4.5 indicates the average cumulative feed intake of broilers over a 35-day period. Over the 14-day cumulative period, the feed intake of birds on the ZB treatment was lower (P ≤0.05) compared to those on the Biacid Nuclease, Clostat and Biacid Nuclease + ZB treatments. The feed intake of birds on the Biacid Nuclease + Clostat treatments was lower (P ≤0.05) compared to those on the Biacid Nuclease + ZB treatments. The feed intake of birds on the ZB treatment was lower (P ≤0.05) compared to those on the Biacid Nuclease, Clostat and Biacid Nuclease + ZB treatments for the first 21-days. The feed intake of birds on the Biacid + ZB treatments was also lower (P ≤0.05) compared to those on the Biacid Nuclease, Clostat and Biacid Nuclease + ZB treatments for the first 21-days. There were no significant differences between the treatments over the 28- day or 35-day cumulative period.

Table 4.5 Cumulative feed intake (g) of broilers over a 35-day feeding period receiving 8 different dietary treatments

Treatments	Days of age				
	0-7	0-14	0-21	0-28	0-35
Negative control	0.17 [ab]	0.58 [ab]	1.27 [ab]	2.17	3.36
Zinc Bacitracin (ZB)	0.17 [b]	0.57 [b]	1.25 [b]	2.17	3.32
Biacid Nuclease	0.18 [a]	0.59 [ac]	1.30 [a]	2.20	3.22
Biacid	0.18 [a]	0.59 [ab]	1.30 [ab]	2.21	3.29
Clostat	0.18 [ab]	0.60 [ac]	1.31 [a]	2.22	3.27
Biacid Nuclease + Clostat	0.18 [a]	0.57 [bc]	1.27 [ab]	2.17	3.26
Biacid Nuclease + Zinc Bacitracin	0.18 [a]	0.60 [a]	1.31 [a]	2.24	3.36
Biacid + Zinc Bacitracin	0.18 [ab]	0.58 [ab]	1.26 [b]	2.16	3.29
Standard error of the mean (SE)	± 0.002	± 0.009	± 0.180	± 0.038	± 0.058

[ab] Column means with the same superscript do not differ (*P* >0.05)

In table 4.6 the weekly body weights of broilers over the 35-day trial period were summarized. Biacid and Biacid Nuclease + Clostat resulted in higher ($P \leq 0.05$) 7-day body weights compared to the ZB treatment. At 14 days of age, birds receiving Biacid had higher ($P \leq 0.05$) body weights compared to those on the Clostat and ZB treatments. At 21 days of age, Biacid resulted in higher body weights ($P \leq 0.05$) compared to the negative control, with no significant difference between the other treatments. At 28 days of age, Biacid and Biacid Nuclease + ZB resulted in higher ($P \leq 0.05$) body weights compared to Biacid Nuclease and the negative control.

Table 4. 6 Mean weekly body weights (g) of broilers over a 35-day feeding period receiving 8 different dietary treatments

Treatments	Days of age					
	0	7	14	21	28	35
Negative control	45.84	181.36 ^{ab}	456.61 ^{ab}	904.40 ^{b}	1384.81 ^{b}	1907.90
Zinc Bacitracin (ZB)	45.57	176.32 ^{b}	446.16 ^{b}	912.34 ^{ab}	1408.27 ^{ab}	1973.68
Biacid Nuclease	45.14	182.48 ^{ab}	458.89 ^{ab}	918.72 ^{ab}	1377.93 ^{b}	1904.17
Biacid	45.48	187.84 ^{a}	475.58 ^{a}	940.70 ^{a}	1437.94 ^{a}	1984.82
Clostat	45.32	179.07 ^{ab}	450.60 ^{b}	914.93 ^{ab}	1405.31 ^{ab}	1929.48
Biacid Nuclease + Clostat	45.26	186.78 ^{a}	454.73 ^{ab}	928.25 ^{ab}	1408.11 ^{ab}	1995.11
Biacid Nuclease + Zinc Bacitracin	45.35	184.09 ^{ab}	466.85 ^{ab}	933.19 ^{ab}	1475.85 ^{a}	1997.39
Biacid + Zinc Bacitracin	45.45	181.81 ^{ab}	461.96 ^{ab}	913.31 ^{ab}	1399.88 ^{ab}	1957.93
Standard error of the mean (SE)	± 0.259	± 3.522	±7.869	±12.577	± 30.207	± 38.913

^{ab} Column means with the same superscript do not differ ($P > 0.05$)

Table 4.7 indicates the mean weekly FCR over the 35-day trial period. During the first 7 days, the FCR of birds on Biacid was lower compared to those on Clostat, with no significant difference between the rest of the treatments. During 7-14 days the FCR of birds on Biacid was lower ($P \leq 0.05$) compared to those on the Clostat treatment. During 14-21 days the FCR of birds on Clostat was higher ($P \leq 0.05$) compared to those on the ZB, Biacid Nuclease + Clostat and Biacid + ZB treatments. During the last week of the trial, the FCR of birds on the negative control was higher ($P \leq 0.05$) compared to those on the other treatments except for those on the ZB treatment. It was however numerically different than ZB (2.35 vs 2.18).

Table 4.8 indicates the cumulative FCR, and during 0-14 days the FCR of birds on Clostat was higher ($P \leq 0.05$) compared to the birds on Biacid and Biacid Nuclease + ZB treatments. Over a 21-day period, the FCR of birds on the Clostat treatment was higher ($P \leq 0.05$) compared to all of the treatments, except for the birds on the negative control and Biacid Nuclease treatment. Over the whole 35-day period the negative control resulted in a higher ($P \leq 0.05$) FCR compared to Biacid, with no difference in FCR between the rest of the treatments ($P > 0.05$).

Table 4.7 Mean FCR of broilers over a 35-day feeding period receiving 8 different dietary treatments

Treatments	Days of age				
	0-7	7-14	14-21	21-28	28-35
Negative control	1.29 [ab]	1.48 [ab]	1.54 [ab]	1.94	2.35 [a]
Zinc Bacitracin (ZB)	1.32 [ab]	1.47 [ab]	1.49 [b]	1.90	2.18 [ab]
Biacid Nuclease	1.30 [ab]	1.51 [ab]	1.57 [ab]	1.98	1.97 [b]
Biacid	1.26 [b]	1.43 [b]	1.53 [ab]	1.86	2.06 [b]
Clostat	1.33 [a]	1.55 [a]	1.59 [a]	1.90	2.00 [b]
Biacid Nuclease + Clostat	1.28 [ab]	1.47 [ab]	1.50 [b]	1.85	2.03 [b]
Biacid Nuclease + Zinc Bacitracin	1.31 [ab]	1.48 [ab]	1.54 [ab]	1.89	2.03 [b]
Biacid + Zinc Bacitracin	1.30 [ab]	1.45 [ab]	1.50 [b]	1.87	2.12 [b]
Standard error of the mean (SE)	± 0.024	± 0.036	± 0.028	± 0.074	± 0.089

[ab] Column means with the same superscript do not differ (*P* >0.05)

Table 4.8 Cumulative FCR of broilers over a 35-day feeding period receiving 8 different dietary treatments

Treatments	Days of age				
	0-7	0-14	0-21	0-28	0-35
Negative control	1.29 [ab]	1.43 [ab]	1.49 [abc]	1.65	1.94 [a]
Zinc Bacitracin (ZB)	1.32 [ab]	1.44 [ab]	1.46 [bc]	1.63	1.87 [ab]
Biacid Nuclease	1.30 [ab]	1.44 [ab]	1.52 [ac]	1.69	1.87 [ab]
Biacid	1.26 [b]	1.36 [b]	1.46 [b]	1.62	1.83 [b]
Clostat	1.33 [a]	1.48 [a]	1.54 [a]	1.69	1.87 [ab]
Biacid Nuclease + Clostat	1.28 [ab]	1.40 [ab]	1.46 [b]	1.64	1.84 [ab]
Biacid Nuclease + Zinc Bacitracin	1.31 [ab]	1.41 [b]	1.49 [b]	1.63	1.84 [ab]
Biacid + Zinc Bacitracin	1.30 [ab]	1.40 [ab]	1.46 [b]	1.62	1.85 [ab]
Standard error of the mean (SE)	± 0.024	± 0.028	± 0.020	± 0.030	± 0.037

[a-c] Column means with the same superscript do not differ (*P* >0.05)

Table 4.9 indicate the organ weights of the broilers sacrificed at day 35 as well as the organ weight ratio to the 35-day body weight for each treatment. It also indicates the ratio between each organ and the 35-day BW. There was no difference (P ≤ 0.05) between treatments on the weight of either the proventriculus or the gizzard. No differences (P ≤ 0.05) in gizzard weight and proventriculus weight as % of BW were observed.

Table 4.9 Average proventriculus and gizzard weight of broilers at 35 days of age that received different feed additives supplements as an absolute value (g) and expressed as % of BW

Treatments	Proventriculus weight (g)	Proventriculus weight as % of BW	Gizzard weight (g)	Gizzard weight as % of BW
Negative control	6.08	0.32	31.99	1.69
Zinc Bacitracin (ZB)	5.93	0.31	30.21	1.60
Biacid Nuclease	6.26	0.32	29.94	1.52
Biacid	6.12	0.30	31.18	1.56
Clostat	5.99	0.32	30.01	1.61
Biacid Nuclease + Clostat	6.22	0.31	31.66	1.59
Biacid Nuclease + Zinc Bacitracin	6.24	0.31	32.60	1.63
Biacid + Zinc Bacitracin	6.08	0.31	31.01	1.59
Standard error of the mean (SE)	± 0.14	± 0.0081	± 1.00	± 0.064

4.3.2 Histomorphological data

Table 4.10 indicates the influence of the 8 dietary treatments on the villi height, crypt depth and villi height: crypt depth of the duodenum at 35 days of age. Clostat resulted in shallower ($P \leq 0.05$) crypt depths compared to the negative control, Biacid Nuclease, Biacid Nuclease + ZB and Biacid + ZB treatments. Biacid Nuclease resulted in deeper ($P \leq 0.05$) crypt depths compared to Biacid Nuclease + Clostat treatment. There was a significantly smaller villi height: crypt depth for the negative control and Biacid + ZB than Biacid, Clostat and Biacid Nuclease + Clostat.

Table 4.10 Effects of different feed additives on the histomorphological parameters of the duodenum at 35 days of age

Treatments	Villi height (µm)	Crypt depth (µm)	Villi/crypt ratio
Negative control	1804.25	295.83^{ab}	6.13^{b}
Zinc Bacitracin (ZB)	1851.34	270.81^{abc}	6.91^{ab}
Biacid Nuclease	1872.91	296.16^{a}	6.35^{ab}
Biacid	1876.13	269.37^{abc}	7.06^{a}
Clostat	1787.20	253.17^{c}	7.18^{a}
Biacid Nuclease + Clostat	1868.26	265.59^{bc}	7.18^{a}
Biacid Nuclease + Zinc Bacitracin	1784.34	281.98^{ab}	6.33^{ab}
Biacid + Zinc Bacitracin	1732.85	295.76^{ab}	5.95^{b}
Standard error of the mean (SE)	± 63.61	± 10.38	± 0.33

$^{a-c}$ Column means with the same superscript do not differ ($P > 0.05$)

Table 4.11 indicates the influence of the 8 dietary treatments on the villi height, crypt depth and villi height: crypt depth of the jejunum at 35 days of age. There was no difference (P >0.05) between any of the treatments on the histomorphological parameters of the jejunum.

Table 4.11 Effects of different feed additives on the histomorphological parameters of the jejunum at 35 days of age

Treatments	Villi height (µm)	Crypt depth (µm)	Villi/crypt ratio
Negative control	1232.90	220.70	5.72
Zinc Bacitracin (ZB)	1410.18	226.19	6.23
Biacid Nuclease	1279.56	241.26	5.32
Biacid	1312.37	228.31	5.87
Clostat	1329.86	215.25	6.37
Biacid Nuclease + Clostat	1252.53	222.66	5.80
Biacid Nuclease + Zinc Bacitracin	1403.17	222.14	6.48
Biacid + Zinc Bacitracin	1251.27	230.57	5.47
Standard error of the mean (SE)	± 68.61	± 10.44	± 0.43

Column means with the same superscript do not differ ($P > 0.05$)

Table 4.12 indicates the influence of the 8 dietary treatments on the villi height, crypt depth and villi height: crypt depth of the ileum on 35 days of age. Clostat resulted in longer ($P \leq 0.05$) villi compared to other treatments except Biacid Nuclease and Biacid. Biacid Nuclease resulted in longer ($P \leq 0.05$) villi than Biacid Nuclease + Clostat.

Table 4.12 Effects of different feed additives on the histomorphological parameters of the ileum at 35 days of age

Treatments	Villi height (µm)	Crypt depth (µm)	Villi/crypt ratio
Negative control	762.61^{bc}	164.13	4.73
Zinc Bacitracin (ZB)	764.37^{bc}	168.24	4.57
Biacid Nuclease	825.44^{ab}	178.65	4.86
Biacid	786.72^{abc}	164.57	4.85
Clostat	872.71^{a}	172.23	5.08
Biacid Nuclease + Clostat	732.12^{c}	162.58	4.52
Biacid Nuclease + Zinc Bacitracin	763.27^{bc}	171.42	4.50
Biacid + Zinc Bacitracin	771.44^{bc}	172.18	4.58
Standard error of the mean (SE)	± 32.71	± 8.99	± 0.28

$^{a-c}$ Column means with the same superscript do not differ ($P > 0.05$)

Table 4.13 indicates the mean NE score over the 35-day trial period. Biacid Nuclease and Biacid resulted in lower ($P \leq 0.05$) NE scores than the negative control.

Table 4.13 Mean NE score of birds at 35-days of slaughter after receiving 8 different dietary treatments

Treatments	Mean NE Score
Negative control	0.65^{a}
Zinc Bacitracin (ZB)	0.49^{ab}
Biacid Nuclease	0.22^{b}
Biacid	0.20^{b}
Clostat	0.38^{ab}
Biacid Nuclease + Clostat	0.27^{ab}
Biacid Nuclease + Zinc Bacitracin	0.52^{ab}
Biacid + Zinc Bacitracin	0.45^{ab}
Standard error of the mean (SE)	± 0.13

^{ab} Column means with the same superscript do not differ ($P > 0.05$).
0: no lessions
1+: thin-walled or friable small intestine
2+: focal necrosis or ulceration
3+: larger patches of necrosis
4+: extensive necrosis

4.4 Discussion

4.4.1 Broiler performance

During this trial the birds were superdosed with a coccidial vaccine, receiving ten times the normal dose at 10 days of age. Four days later, at 14 days of age, the birds were inoculated with *C. perfringens* type A to induce NE. The feed intake over the 35-day trial period did not differ between any of the treatments (P >0.05). This is in agreement with Gunal *et al.* (2006), who reported no significant difference in feed intake when supplementing broiler diets with a probiotic, AGP and organic acid mixture. The BW of the broilers did not differ significantly (P >0.05) over the 35-day period, but for the first 28 days the Biacid treatment resulted in the highest weekly and cumulative BW, significantly higher than the negative control and EO only treatments over the 28-day cumulative period.

The Biacid mixture consisted of citric acid, calcium butyrate, calcium lactate and calcium formate, which was mixed with the EOs thymol, eugenol, carvacrol and cinnamaldehyde. The amount of acids and oils allows for a synergistic effect between the organic acids and EO. This synergy has been recognised by other studies (Bozkurt *et al.*, 2012; Basmacioğlu-Malayoğlu *et al.*, 2016) who found that the EO and organic acid treatment resulted in a higher body weight gain and feed efficiency when compared to the control. It was interesting to note that even though there was no difference between the 35-day body weights, there were differences between treatments on the 35-day cumulative FCR of the birds, with the Biacid treated birds being the only ones having a significantly lower FCR than the control, which is also in agreement with Basmacioğlu-Malayoğlu *et al.* (2016). Explaining this synergy starts by evaluating the mode of action of EO and organic acids individually. Both these feed additives are multifactorial, meaning they have more than one function in the gut. EO improve feed digestion by increasing bile salt secretion while stimulating the enzymatic activity of the mucosa and pancreas (Wallace *et al.*, 2010; Papatsiros *et al.*, 2013). Additionally, EO are known to have an effect on the membrane of pathogenic bacteria. In principle, these EO compounds disturb the integrity and function of the bacterial cell membranes, and is therefore likely that the antibacterial activity of EO are attributed to the interaction between the EO chemical structure and a variety of targets in the bacterial cell (Bento *et al.*, 2013).

Organic acids reduce the pH of the intestinal tract, creating an unsuitable habitat for pathogenic bacteria to ensure a balanced intestinal microbiome (Bozkurt *et al.*, 2012; Dittoe *et al.*, 2018). It might be that the EO increases the permeability of the pathogenic bacteria's membrane, allowing the undissociated acid to diffuse through the membrane easier, disrupting the ATPase H^+ pump. This allows for the combination of the two additives to have a more potent antibacterial mechanism, which explains why the combination results in better performance than the additives given alone, especially in a challenging environment. In this study when the EO treatment (Biacid Nuclease) was given alone, it resulted in a significantly lower body weight than the combination of EO with organic acids (Biacid), while the FCR was numerically higher. This is supported by another study which reported that an organic acid and EO blend significantly reduced the FCR of broiler chickens, compared to the individual use of an EO blend (Zhang *et al.*, 2005). It seems that the combination of EO and organic acids results in a stronger product and improves the digestive process throughout the digestive tract, with organic acids being particularly active in the crop and gizzard, while EO appear to work more in the latter segments of the intestine (Langhout, 2000).

4.4.2 Histomorphological data

The effects of the feed additives on the histomorphological development of the gut was quantified, giving us a better understanding of how these additives contributes towards gut health. In the duodenum, there were no significant differences between the treatments on the villi height. The treatments did however have an effect on the crypt depth, with Clostat resulting in the shallowest crypt depth, significantly shallower than the control. The consequence of this is that both Clostat and Clostat + ZB had significantly larger villi height: crypt depth, compared to the control and Biacid + ZB treatment. This higher ratio indicates longer villi in which the epithelium is sufficiently matured and active, in combination with the shallow crypt depth (Jayaraman *et al.*, 2013). Birds that suffer from a clostridial infection is in a constant mode of intestinal inflammation (Jayaraman *et al.*, 2013), which can lead to shortened villi with deeper crypts. A shallow crypt indicates faster tissue turnover, permitting the renewal of the villi as needed in response pathogens and the toxins produced by them. The consequence would be poor nutrient absorption and increased secretion in the GIT which results in lower performance (Xu *et al.*, 2003). The probiotic (Clostat) used during this trial is a *Bacillus subtilis* strain isolated from a healthy chicken gut that has been shown *in vitro* to be anti-clostridial (Teo & Tan, 2005). This *Bacilus subtilis* is known to secrete surfactins that are amphipathic cyclic lipoheptapeptides. These surfactins have emulsifying capabilities, as well as antibacterial, antiviral and antitumor properties (Vollenbroich *et al.*, 1997; Heerklotz & Seelig, 2001).The results from a study done by Jayaraman *et al.* (2013) is in agreement with the results from this study. They used the same probiotic (Clostat) as in this study and found that Clostat increased the villi height: crypt depth over the negative control. This in turn means that there is an increased surface area which improves the absorption of the available nutrients.

The Biacid treatment resulted in a significantly larger villi height: crypt depth compared to the control and the same treatment combined with ZB (Biacid + ZB). In fact, combining Biacid with ZB resulted in a villi height: crypt depth lower than the control. It might be that the combined anti-microbial action of the Biacid + ZB suppressed the microbial population to a state where there were not enough beneficial bacteria to produce the necessary SCFA for epithelial growth. At the same time this combination could have suppressed the growth of pathogenic bacteria which prevented any negative effects on performance. The synergistic effect of combining EO with organic acids can be traced back to their individual mode of actions being combined, where EO increases the permeability of pathogen's membrane, allowing the organic acid to penetrate the cell.

As mentioned earlier, a blend of EO provide a larger frame of synergistic effects, especially under conditions where the birds are challenged with *C. perfringens*. A *C. perfringens* challenge will require the alternative feed additives to act as antimicrobials together with their improved digestibility characteristics. One of the characteristics of EO is their hydrophobicity, which enables them to partition lipids in the bacterial cell wall and mitochondria which disturbs the structures and renders them more permeable (Brenes & Roura, 2010). EO are slightly more active against gram-positive than gram-negative bacteria (Trombetta *et al.*, 2005). This is mainly due to the structure of gram-positive bacteria, which allows hydrophobic molecules to easily penetrate the cells and act on both the cell wall and within the cytoplasm. Gram negative bacteria on the other hand is more complex, with a peptidoglycan layer that is thinner than the one in gram positive bacteria.

This peptidoglycan layer is surrounded by an outer membrane which is firmly linked to the inner peptidoglycan layer by Braun's lipoproteins. The outer membrane is one of the features that differentiate gram-

negative bacteria from gram-positive bacteria, and allows the gram-negative bacteria to be more resistant to EO and other additives with antimicrobial activity (Nazzaro *et al.*, 2013). Fortunately, *C. perfringens* is a gram-positive bacteria and numerous studies have found EO to be effective against this pathogen (Jamroz *et al.*, 2003; Mitsch *et al.*, 2004; Griggs & Jacobs, 2005; Brenes & Roura, 2010). Generally, the EO possessing the strongest antibacterial properties against pathogens contain phenolic compounds such as carvacrol, eugenol and thymol (Juliano *et al.*, 2000; Lambert *et al.*, 2001). Thymol and carvacrol have similar antimicrobial effects, which is mainly related to their functional groups, the hydroxyl group of the phenolic terpenoids, and the presence of delocalised electrons. These two EO have prominent outer membrane disintegrating properties, making them effective against gram-negative bacteria as well. This causes a release of lipopolysaccharides and increase the permeability of the cytoplasmic membrane to ATP and depolarise the cytoplasmic membrane (Helander *et al.*, 1998; Xu *et al.*, 2008).

In a study done by Lambert *et al.* (2001), they found an additive effect when testing carvacrol and thymol against *S. aureus* and *P. aeruginosa*. Eugenol and cinnamaldehyde forms part of the most studied phenylpropenes in which the antimicrobial activity is conferred mainly by their free hydroxyl groups (Laekeman *et al.*, 1990). The antibacterial activity of eugenol is due to the presence of a double bond in the α,ß positions of the side chain and to a methyl group located in the γ position (Jung & Fahey, 1983). Cinnamaldehyde is generally less potent than eugenol, but at low concentrations it is able to inhibit enzymes involved in cytokine interactions whereas at higher concentrations, it acts as an ATPase inhibitor (Nazzaro *et al.*, 2013). The mechanism of action of each individual EO in Biacid Nuclease depends on its chemical composition and its antimicrobial activity is not only explained by a unique mechanism, but rather to a cascade of reactions involving the bacterial cell (Burt, 2004).

By combining the different EO with their unique chemical composition with a blend of organic acids, a synergistic effect is expected, that outperform the EO blend alone. This is mainly due to the fact that as the EO increases the permeability of the pathogen's cell membrane, the non-dissociated organic acids are able to diffuse through the lipophilic membrane where it disrupts the enzymatic reactions and transport system of the bacteria (M'Sadeq *et al.*, 2015). The blend of organic acids allows the feed additive to cover a broader spectrum activity and combines the good qualities of the different acids (Huyghebaert *et al.*, 2011; Khan & Iqbal, 2015). This additive effect of a blend of EO and organic acids will create a more potent antimicrobial environment. This not only improves performance as already discussed, but prevents the *C. perfringens* from producing toxins that destroys the epithelial cells, including the villi. This might explain why there are larger duodenal villi height: crypt depth in the Biacid treatment compared to the control.

There were no significant differences between the treatments on the jejunum villi height or crypt depth. This might be explained by the lower concentration of microflora in this part of the intestine. In the ileum, the villi height of the Clostat treatment was significantly longer than the rest of the treatments, except for the Biacid Nuclease and Biacid treatments. This improvement in villi height in the Clostat treatment indicates a matured and functionally active epithelium in spite of the *C. perfringens* challenge. Although there is no significant difference between the treatments on the villi height: crypt depth ratio, Clostat did result in a numerically larger ratio, which is the same effect that was observed in the duodenum. The supplementation with Clostat did not only control the *C. perfringens* infection, but also improved the gut health and integrity by increasing the villi height and villi height: crypt depth ratio (Jayaraman *et al.*, 2013).

4.4.3 Lesion scores

There was a significant difference between the negative control (0.65) and the Biacid Nuclease (0.22) and Biacid treatments (0.2) on the NE score. However, the lesions were not as severe as expected. In fact, the only lesions observed was small red hemorrhages and it was scored accordingly. The lower lesion score of the Biacid Nuclease and Biacid treatment can be explained by their pathogen inhibiting mode of action. A study done by Mitsch *et al.* (2004) showed that blending EO containing thymol, eugenol and carvacrol can reduce *C. perfringens* colonisation and proliferation in the broiler gut. They attributed this effect not only to the direct inhibition of pathogens, but also to the digestive enzymes induced by EO that could increase nutrient digestibility and stabilise the gut microflora. There is also the possibility that digestive enzymes such as trypsin, stimulated by EO, can inactivate *C. perfringens* and reduce the colonisation of these bacteria (Arbuckle, 1972; Baba *et al.*, 1992). Similar studies reported a reduction in coliform counts of *E. coli* and *C. perfringens* after birds were supplemented with EO containing thymol, cinnamaldehyde and carvacrol (Jamroz *et al.*, 2003; Jang *et al.*, 2007; Brenes & Roura, 2010). Studies have also shown the effect of EO on the control of coccidiosis in broilers, which is one of the major precursors for the development of NE. One of these studies reported a reduced oocyte excretion in chicks fed diets supplemented with EO compared to those fed control diets (Evans *et al.*, 2001). All these effects are in agreement with results from this study, and it can be concluded that the Biacid Nuclease and Biacid treatment inhibited the growth of *C. perfringens* to a point where the pathogen no longer produced enough toxins to cause any lesions in the gut. Although there were no significant differences between the other treatments, they were numerically lower compared to the negative control and it is possible that these additives were also able to suppress the growth of the pathogen to a point where the lesion scoring was lower than the negative control.

4.4.4 Conclusion

The beneficial effects of alternative feed additives have been reported previously. From this study it can be concluded that the dietary addition of feed additives under challenging conditions did improve broiler performance and gut morphology. The supplementation of a Biacid to the diet significantly improved 28-day body weight, cumulative FCR and intestinal health of broilers when compared to broilers that received no feed additive supplementation. Biacid may be considered as an alternative to antibiotic growth promoters, with the potential to achieve an environmentally friendly broiler production system. It is encouraging to know that the other feed alternatives reviewed in the context also exerted growth-promoting effects, comparable to Zinc Bacitracin. These results can however be variable when compared between studies, and therefore these facts require careful examination of the modes of action of the alternative supplements and under which conditions to use them.

Chapter 5: General conclusion

There are many factors that are associated with infectious agents that negatively affect the chicken gut and consequently the health status, which inevitably causes a drop in performance. The removal of in-feed antibiotics is a reality South Africa will one day face, and it might be sooner than anticipated. This could cause a great challenge for the South African poultry industry, especially since studies from Denmark and Sweden have confirmed that the main problem of removing AGPs is the inability to effectively control NE (Casewell et al., 2003; Jacobsen & Jensen, 2004). Many similar studies to the one discussed have been done to alleviate the problems associated with antibiotic withdrawal from poultry diets, but to date no therapy has been established to completely substitute AGP. Several feed additives, including probiotics, prebiotics, EO and organic acids, have been used to reduce the incidence of NE, but no product has been as effective as AGP in terms of controlling NE. Although the use of these additives has been proven not to effectively substitute AGP in poultry diets, recent investigations are in agreement with results of this study (Hassan *et al.*, 2018; Bortoluzzi *et al.*, 2019).

These investigations have shown that the use of these antibiotic alternatives have improved GIT health and reduced the intestinal colonisation of pathogenic bacteria, including *Clostridium perfringens*. Therefore, using alternative feed additives with a better understanding of the relationship between the environment, nutrition and NE, and by limiting the exposure of birds to infectious agents through better biosecurity, might help to reduce the incidence of NE in an antibiotic free environment. In cases where birds are not subjected to adverse environmental conditions, these alternative feed additives have additional properties that improve performance and immunity, allowing the broiler industry to further exploit the genetic potential of the birds. These properties could increase the return on investment under normal commercial conditions, which is in most cases a prerequisite for alternative feed additives, especially in an industry that is still allowed to use AGP in feed.

By comparing the effect of feed additives between two trials, it is necessary to keep the variation between the two trials as low as possible. One shortcoming of the study that prevented us from comparing the results between the two trials was the fact that the treatments differed between the two trials. It would have been interesting to see how the same feed additives performed under the two different environments. It is recommended that future studies keep the treatments between two trials the same, to allow for the opportunity to compare results.

Future studies will benefit from a more robust necrotic enteritis model. Although the model followed did have an effect on the performance of the bird as well as the histomorphological development of the gut, the intestinal lesions were not to the extend we expected from birds exposed to conditions that stimulates NE. It is recommended to follow a model in which the birds receive a challenge from the diet as well, and not only by inoculating them with the pathogen after the high dosage of coccidiosis vaccine. Moore *et al.* (2016) suggested that high protein diets that include fishmeal can alter the composition of the GIT microbiota and favour certain species such as *Clostridium perfringens*.

References

Aarestrup, F. M., 1999. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. Int. J. Antimicrob. Ag. 12, 279-285.

Al-Sheikhly, F., Al-Saieg, A., 1979. Role of Coccidia in the Occurrence of Necrotic Enteritis of Chickens. Avian Dis. 24(2).

Alakomi, H. L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K. & Helander, I. M., 2000. Lactic Acid Permeabilizes Gram-Negative Bacteria by Disrupting the Outer Membrane. Appl. Environ. Microb. 66(5), 2001-2005.

Alçiçek, A., Bozkurt, M. & Çabuk, M., 2004. The effect of a mixture of herbal essential oils, an organic acid or a probiotic on broiler performance. S. Afr. J. Anim. Sci. 34(4), 217-222.

Alloui, M. N., Szczurek, W. & Świątkiewicz, S., 2013. The Usefulness of Prebiotics and Probiotics in Modern Poultry Nutrition a Review. Ann. Anim. Sci. 13(1), 17-32. doi:10.2478/v10220-012-0055-x

Antonissen, G., Van Immerseel, F., Pasmans, F., Ducatelle, R., Haesebrouck, F., Timbermont, L., Verlinden, M., Janssens, G. P., Eeckhaut, V., Eeckhout, M., De Saeger, S., Hessenberger, S., Martel, A. & Croubels, S., 2014. The mycotoxin deoxynivalenol predisposes for the development of Clostridium perfringens-induced necrotic enteritis in broiler chickens. Plos one 9(9). doi:10.1371/journal.pone.0108775

Ao, Z. & Choct, M., 2013. Oligosaccharides affect performance and gut development of broiler chickens. Asian-Australas. J. Anim. Sci. 26(1), 116-121. doi:10.5713/ajas.2012.12414

Ao, Z., Kocher, A., & Choct, M., 2012. Effects of Dietary Additives and Early Feeding on Performance, Gut Development and Immune Status of Broiler Chickens Challenged with Clostridium perfringens. Asian-Australas. J. Anim. Sci. 25(4), 541-551. doi:10.5713/ajas.2011.11378

AOAC, 2000. Official method of analysis 920.39 (17th Edition) Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

AOAC, 2000. Official method of analysis 935.14 (17th Edition) Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

AOAC, 2000. Official method of analysis 942.05 (17th Edition) Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

AOAC, 2000. Official method of analysis 943.01 (17th Edition) Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

AOAC, 2000. Official method of analysis 962.09 (17th Edition) Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

AOAC, 2000. Official method of analysis 965.17 (17th Edition) Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

AOAC, 2000. Official method of analysis 988.05 (17th Edition) Volume I. Association of Official Analytical Chemists, Inc., Maryland, USA.

Arbuckle, J. B. R., 1972. The attachment of Clostridium welchii (Cl. perfringens) type C to intestinal villi of pigs. J. Pathol. 106(2), 65-72. doi:10.1002/path.1711060202

Awad, W. A., Ghareeb, K., Abdel-Raheem, S. & Bohm, J., 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poultry Sci. 88(1), 49-56. doi:10.3382/ps.2008-00244

Baba, E., Fuller, A. L., Gilbert, J. M., Thayer, S. G. & McDougald, L. R., 1992. Effects of Eimeria brunetti Infection and Dietary Zinc on Experimental Induction of Necrotic Enteritis in Broiler Chickens. Avian Dis. 36(1), 59-62. doi:10.2307/1591716

Barton, M. D., 2000. Antibiotic use in animal feed and its impact on human health. Nutr. Res. Rev. 13(2), 279-299. doi:10.1079/095442200108729106

Basmacioğlu-Malayoğlu, H., Ozdemir, P. & Bağriyanik, H. A., 2016. Influence of an organic acid blend and essential oil blend, individually or in combination, on growth performance, carcass parameters, apparent digestibility, intestinal microflora and intestinal morphology of broilers. Br. Poultry Sci. 57(2), 227-234. doi:10.1080/00071668.2016.1141171

Bbosa, G. S., Mwebaza, N., Odda, J., Kyegombe, D. B. & Ntale, M., 2014. Antibiotics/antibacterial drug use, their marketing and promotion during the post-antibiotic golden age and their role in emergence of bacterial resistance. Sci. Res. Essays 6(5), 410-425. doi:10.4236/health.2014.65059

Bento, M. H. L., Ouwehand, A. C., Tiihonen, K. S. L., Nurminen, P., Saarinen, M. T., Schulze, H., Mygind, T. & Fischer, J., 2013. Essential oils and their use in animal feeds for monogastric animals - Effects on feed quality, gut microbiota, growth performance and food safety. Vet. Med-Czech. 9(58), 449-458.

Black, J. L., 2000. Bioavailability: the energy component of a ration for monogastric animals. In: Feed evaluation: principles and practice. Ed's: Moughan, P.J., Verstegen, M.W.A. & Visser-Reyneveld, M.I. Wageningen Pers, Wageningen, Netherlands. pp 133-152.

Bolla, J. M., Alibert-Franco, S., Handzlik, J., Chevalier, J., Mahamoud, A., Boyer, G., Kieć-Kononowicz, K. & Pagès, J.M., 2011. Strategies for bypassing the membrane barrier in multidrug resistant Gram-negative bacteria. FEBS Letters. 585(11), 1682-1690. doi:10.1016/j.febslet.2011.04.054

Bolton, T., 2015. The state of the South African Broiler industry. Supermarket & Retailer. pp. 17-23.

Bortoluzzi, C., Vieira, B.S., de Paula Dorigam, J.C., Menconi, A., Sokale, A., Doranalli, K., Applegate, T.J., 2019. Bacillus subtilis DSM 32315 Supplementation Attenuates the Effects of Clostridium perfringens Challenge on the Growth Performance and Intestinal Microbiota of Broiler Chickens. Microorganisms 7(3), 71.

Bozkurt, M., Küçükyilmaz, K., Çatli, A. U. & Çinar, M., 2008. Growth Performance and Slaughter Characteristics of Broiler Chickens Fed with Antibiotic, Mannan Oligosaccharide and Dextran Oligosaccharide Supplemented Diets. Int. J. Poultry Sci. 7(10), 969-977.

Bozkurt, M., Küçükyilmaz, K., Çatli, A. U., Çinar, M., Cabuk, M. & Alçiçek, A., 2012. Effects of administering an essential oil mixture and an organic acid blend separately and combined to diets on broiler performance. Arch. Poultry 76(2) 81-87.

Brenes, A. & Roura, E., 2010. Essential oils in poultry nutrition: Main effects and modes of action. Anim. Feed Sci. Tech. 158(1-2), 1-14. doi:10.1016/j.anifeedsci.2010.03.007

Broom, L. J., 2015. Organic acids for improving intestinal health of poultry. World Poultry Sci. J. 71(4), 630-642. doi:10.1017/s0043933915002391

Broiler Management Manual Ross-308, pp.1-132, 2014, Home page address: www.aviagen.com

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. Int. J. Food Microbiol. 94(3), 223-253. doi:10.1016/j.ijfoodmicro.2004.03.022

Caly, D. L., D'Inca, R., Auclair, E. & Drider, D., 2015. Alternatives to Antibiotics to Prevent Necrotic Enteritis in Broiler Chickens: A Microbiologist's Perspective. Front Microbiol. 6, 1336. doi:10.3389/fmicb.2015.01336

Cartman, S. T., La Ragione, R. M. & Woodward, M. J., 2008. Bacillus subtilis Spores Germinate in the Chicken Gastrointestinal Tract. Appl. Environ. Microb. 74(16), 5254-5258. doi:10.1128/AEM.00580-08

Casewell, M., Friis, C., Marco, E., McMullin, P. & Phillips, I., 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52(2), 159-161. doi:10.1093/jac/dkg313

Cogliani, C., Goosens, H. & Greko, C. 2011. Restricting Antimicrobial Use in Food Animals: Lessons from Europe. Microbe 6, 6.

Cooper, K. K., 2007. Necrotic Enteritis in Broiler Chickens: Studies in Desease Reproduction and Pathogenesis. Ed: Cooper, K.K., The University of Arizona, Arizona, United States of America. pp 11-144.

Cooper, K. K. & Songer, J. G., 2009. Necrotic enteritis in chickens: a paradigm of enteric infection by Clostridium perfringens type A. Anaerobe 15, 55-60. doi:10.1016/j.anaerobe.2009.01.006

Corrêa, G. S. S., Gomes, A. V. C., Corrêa, A. B., Salles, A. S., & Mattos, E. S., 2003. Effect of antibiotic and probiotics on performance and carcass yield of broilers. Braz. Arch. Vet. Med. Anim. Sci. 55(4), 467-473. doi:10.1590/S0102-09352003000400013

Cox, L. M., 2016. Antibiotics shape microbiota and weight gain across the animal kingdom. Animal Frontiers 6, 8. doi:10.2527/af.2016-0028

Crittenden, R. & Playne, M. J., 2008. Prebiotics. In: Handbook of Probiotics and Prebiotics. Eds: Lee, Y. K. & Salminen, S., John Wiley & Sons, Inc., Hoboken, New Jersey. pp 533-581.

Dahiya, J. P., Wilkie, D. C., Van Kessel, A. G. & Drew, M. D., 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Anim. Feed. Sci. Tech. 129(1-2), 60-88. doi:10.1016/j.anifeedsci.2005.12.003

Das, A., Mazumder, Y., Dutta, B. K., Shome, B. R., Bujarbaruah, K. M., & Kumar, A. 2008. Clostridium perfringens type A from broiler chicken with necrotic enteritis. Int. J. Poultry Sci. 7(6), 601-609.

Di Pasqua, R., Betts, G., Hoskins, N., Edwards, M., Ercolini, D. & Mauriello, G., 2007. Membrane Toxicity of Antimicrobial Compounds from Essential Oils. J. Agr. Food. Chem. 55(12), 4863-4870. doi0.1021/jf0636465

Dibner, J. J. & Buttin, P., 2002. Use of Organic Acids as a Model to Study the impact of gut microflora on Nutrition and Metabolism. J. Appl. Poultry Res. 11, 453-463.

Dittoe, D. K., Ricke, S. C. & Kiess, A. S., 2018. Organic Acids and Potential for Modifying the Avian Gastrointestinal Tract and Reducing Pathogens and Disease. Front. Vet. Sci. 216(5), 1-12. doi:10.3389/fvets.2018.00216

Engberg, R. M., Hedemann, M. S., Leser, T. D. & Jensen, B. B., 2000. Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. Poultry Sci. 79(9), 1311-1319. doi:10.1093/ps/79.9.1311

Erik, K. & Knudsen, B., 2007. Development of antibiotic resistance and options to replace antimicrobials in animal diets. Proc. Nutr. Soc. 60(03), 291-299. doi:10.1079/pns2001109

Evans, J., Plunkett, M. & Banfield, M., 2001. Effect of an essential oil blend on coccidiosis in broiler chicks. Poultry Sci. 80, 258.

Faria, D. E. D., Henrique, A. P. F., Franzolin Neto, R., Medeiros, A. A., Junqueira, O. M. & Faria Filho, D. E. D., 2009. Alternatives to the use of antibiotic growth promoters for broiler chickens. Probiotics 10(1), 11.

Fattah, A. S. A., El-Sanhoury, M. H., El-Mednay, N. M. & Azeem, A. F., 2008. Thyroid activity, some blood constituents, organs morphology and performance of broiler chicks fed supplemental organic acids. Int. J. Poultry Sci. 3(7), 215-222.

Ferket, P. R., 2004. Alternatives to antibiotics in poultry production responses, practical experience and recommendations. Nutritional Biotechnology in the feed and food industries. Ed's: Lyons, T. P. & Jacques K. A. Nottingham University Press, Nottingham, United Kingdom. pp 57-67.

Ferket, P. R., Parks, C. W. & Grimes, J. L., 2002. Benefits of dietary antibiotic and mannanoligosaccharide supplementation for poultry. Nutritional Biotechnology in the feed and food industries. Ed's: Lyons, T.P. & Jacques K.A., Nottingham University Press, Nottingham, United Kingdom. pp 43-64.

Fuller, R., 1989. Probiotics of man and animals. J. Appl. Bacteriol. 66, 365-378.

Furuya, E. Y. & Lowy, F. D., 2006. Antimicrobial-resistant bacteria in the community setting. Nat. Rev. Microbiol. 4(1), 36-45. doi:10.1038/nrmicro1325

Gaggia, F., Mattarelli, P. & Biavati, B., 2010. Probiotics and prebiotics in animal feeding for safe food production. Int. J. Food. Microbiol. 141, 15-28. doi:10.1016/j.ijfoodmicro.2010.02.031

Gallucci, M., Casero, C., Oliva, M., Zygadlo, J. & Demo, M., 2006. Interaction between terpenes and penicillin on bacterial strains resistant to beta-lactam antibiotics. Mol. Med. Chem. 10, 30-32.

Gill, A. O. & Holley, R. A., 2004. Mechanisms of Bactericidal Action of Cinnamaldehyde against Listeria monocytogenes and of Eugenol against L. monocytogenes and Lactobacillus sakei. Appl. Environ. Microb. 70(10), 5750-5755. doi:10.1128/AEM.70.10.5750-5755.2004

Gill, A. O. & Holley, R. A. 2006a. Disruption of Escherichia coli, Listeria monocytogenes and Lactobacillus sakei cellular membranes by plant oil aromatics. Int. J. Food. Microbiol. 108(1), 1-9. doi:10.1016/j.ijfoodmicro.2005.10.009

Gill, A. O. & Holley, R. A., 2006b. Inhibition of membrane bound ATPases of Escherichia coli and Listeria monocytogenes by plant oil aromatics. Int. J. Food. Microbiol. 111(2), 170-174. doi:10.1016/j.ijfoodmicro.2006.04.046

Gillor, O., Etzion, A. & Riley, M. A., 2008. The dual role of bacteriocins as anti- and probiotics. Appl. Microbiol. Biot. 81(4), 591-606. doi:10.1007/s00253-008-1726-5

Griggs, J. P. & Jacob, J. P., 2005. Alternatives to Antibiotics for Organic Poultry Production. J. Appl. Poultry Res. 14, 750-756.

Gunal, M., Yayli, G., Kaya, O., Karahan, N. & Sulak, O., 2006. Effects of AGP, Probiotic and Organic Acid Supplementation on performance, Intestinal Microflora and Tissue of broilers. J. Appl. Poultry Sci. 5(2), 149-155. doi:10.3923/ijps.2006.149.155

Hajati, H. & Rezaei, M., 2010. Application of prebiotics in poultry production. Int. J. Poultry. Sci. 3(9), 298-304.

Hamed, D. M. & Hassan, A. M. A., 2013. Acids supplementation to drinking water and their effects on Japanese quails experimentally challenged with Salmonella enteritidis. Res. Zool. 3(1). doi:10.5923/j.zoology.20130301.03

Harimurti, S. & Hadisaputro, W., 2015. Probiotics in Poultry. In: Microbiology Monograph. Volume 29. Ed: Steinbuchel, A., Springs, Munster, Germany. pp 1-19.

Hassan, H.M.A., Samy, A., Amani, W.Y., Mohamed, M.A., 2018. Using Different Feed Additives as Alternative to Antibiotic Growth Promoter to Improve Growth Performance and Carcass Traits of Broilers. Int. J. Poultry Sci. 17, 255-261.

Hathaway, M. R., Dayton, W. R., White, M. E., Henderson, T. L. & Henningson, T. B., 1996. Serum insulin-like growth factor I (IGF-I) concentrations are increased in pigs fed antimicrobials. J. Anim. Sci. 74(7), 1541-1547. doi:10.2527/1996.7471541x

Hawkey, P. M., 1998. The origins and molecular basis of antibiotic resistance. Br. Med. J. 317, 657-660.

Heerklotz, H. & Seelig, J., 2001. Detergent-like action of the antibiotic peptide surfactin on lipid membranes. Biophys. J. 81(3), 1547-1554.

Helander, I. M., Alakomi, H.L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E. J., Gorris, L. G. M. & von Wright, A., 1998. Characterization of the Action of Selected Essential Oil Components on Gram-Negative Bacteria. J. Agr. Food. Chem. 46(9), 3590-3595. doi:10.1021/jf980154m

Hemaiswarya, S. & Doble, M., 2009. Synergistic interaction of eugenol with antibiotics against gram negative bacteria. Phytomedicine 16, 997-1005.

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., Gordon, J.I., 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Sci. 291, 881–884.

Hur, J., Jawale, C. & Lee, J. H., 2012. Antimicrobial resistance of Salmonella isolated from food animals: A review. Food. Res. Int. 45(2), 819-830. doi:10.1016/j.foodres.2011.05.014

Huyghebaert, G., Ducatelle, R. & Van Immerseel, F., 2011. An update on alternatives to antimicrobial growth promoters for broilers. Vet. J. 187(2), 182-188. doi:10.1016/j.tvjl.2010.03.003

Jacobsen, L. B. & Jensen, H. G., 2004. Effects of termination of AGP use on antimicrobial resistance in food animals.In: Beyond Antimicrobial Growth Promoters in Food Animal Production. Working papers from the international symposium. Anim. Husbandry 57, 9-16.

Jamroz, D., Orda, J., Kamel, C., Wiliczkiewicz, A., Wertelecki, T. & Skorupińska, J., 2003. The influence of phytogenic extracts on performance, nutrient digestibility, carcass characteristics, and gut microbial status in broiler chickens. J. Anim. Feed. Sci. 12(3), 583-596. doi:10.22358/jafs/67752/2003.

Jang, I. S., Ko, Y. H., Kang, S. Y. & Lee, C. Y., 2007. Effect of a commercial essential oil on growth performance, digestive enzyme activity and intestinal microflora population in broiler chickens. Anim. Feed. Sci. Tech. 134(3-4), 304-315. doi:10.1016/j.anifeedsci.2006.06.009.

Jayaraman, S., Thangavel, G., Kurian, H., Mani, R., Mukkalil, R. & Chirakkal, H., 2013. Bacillus subtilis PB6 improves intestinal health of broiler chickens challenged with Clostridium perfringens-induced necrotic enteritis. Poultry Sci. 92(2), 370-374. doi:10.3382/ps.2012-02528.

Jin, L. Z., Ho, Y. W., Abdullah, N. & Jalaludin, S., 1997. Probiotics in poultry: modes of action. World Poultry Sci. J. 53(04), 351-368. doi:10.1079/wps19970028.

Johnson, J. & Reid, W. M., 1970. Anticoccidial drugs: Lesion scoring techniques in battery and floor-pen experiments with chickens. Exp. Pathol. 28(1) 30-36.

Juliano, C., Mattana, A. & Usai, M., 2000. Composition and in vitro Antimicrobial Activity of the Essential Oil of Thymus herba-barona Loisel Growing Wild in Sardinia. J. Essent. Oil. Res. 12(4), 516-522. doi:10.1080/10412905.2000.9699578.

Jung, H. G. & Fahey, G. C., 1983. Nutritional Implications of Phenolic Monomers and Lignin. J. Anim. Sci. 57(1), 206-219. doi:10.2527/jas1983.571206x.

Kabir, S. M. L., 2009. The Role of Probiotics in the Poultry Industry. Int. J. Mol. Sci. 10(8), 3531-3546. doi:10.3390/ijms10083531

Keyburn, A. L., Bannam, T. L., Moore, R. J. & Rood, J. I., 2010. NetB, a pore-forming toxin from necrotic enteritis strains of Clostridium perfringens.Toxins 2(7), 1913-1927. doi:10.3390/toxins2071913.

Khan, S. H. & Iqbal, J., 2015. Recent advances in the role of organic acids in poultry nutrition. J. Appl. Anim. Res. 44(1), 359-369. doi:10.1080/09712119.2015.1079527

Kleyn, R., 2015. The use of Antibiotic Growth Promoters in South Africa: Some Perspectives. Spesfeed, 10.

Knudsen, B. K. E., 2001. Development of antibiotic resistance and options to replace antimicrobials in animal diets. Animal Nutrition and Metabolism Group Symposium on Quality inputs for Quality foods. P. Nutr. Soc. 60, 291-299.

Laekeman, G., Van Hoof, L., Haemers, A., Vanden Berghe, D., Herman, A. & Vlietinck, A., 1990. Eugenol a valuable compound for in vitro experimental research and worthwhile for further in vivo investigation. Phytother. Res. 4, 90-96. doi:10.1002/ptr.2650040304

Lambert, R. J. & Stratford, M., 1999. Weak-acid preservatives: modelling microbial inhibition and response. J. Appl. Microbiol. 86, 157-164.

Lambert, R. J. W., Skandamis, P. N., Coote, P. J. & Nychas, G. J. E., 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. 91(3), 453-462. doi:10.1046/j.1365-2672.2001.01428.

Langeveld, W. T., Veldhuizen, E. J. & Burt, S. A., 2014. Synergy between essential oil components and antibiotics. Crit. Rev. Microbiol. 40(1), 76-94. doi:10.3109/1040841X.2013.763219

Langhout, P., 2000. New additives for broiler chickens. World Poultry 16, 22-27.

Lee, K. W., Lillehoj, H. & Siragusa, G., 2010. Direct-fed microbials and their impact on the intestinal microflora and immune system of chickens. J. Poultry Sci. 47, 106-114.

Lee, K. W., Lillehoj, H. S., Jeong, W., Jeoung, H. Y. & An, D. J., 2011. Avian necrotic enteritis: experimental models, host immunity, pathogenesis, risk factors, and vaccine development. Poultry Sci. 90(7), 1381-1390. doi:10.3382/ps.2010-01319

Lensing, M., van der Klis, J. D., Fabri, T., Cazemier, A. & Else, A. J., 2010. Efficacy of a lactylate on production performance and intestinal health of broilers during a subclinical Clostridium perfringens infection. Poultry Sci. 89(11), 2401-2409. doi:10.3382/ps.2010-00942.

Lin, J., 2014. Antibiotic growth promoters enhance animal production by targeting intestinal bile salt hydrolase and its producers. Front. Microbiol. 5, 33. doi:10.3389/fmicb.2014.00033

Loddi, M. M., Gonzales, E., Takita, T. S., Mendes, A. A. & Roça, R. D. O., 2000. Uso de probiótico e antibiótico sobre o desempenho, o rendimento e a qualidade de carcaça de frangos de corte. Rev. Bras. Zootec. 29, 1124-1131. (In Portugese, English abstract)

Lorenzoni, G. 2010. Poultry diseases influenced by gastrointestinal health. Ed: Lorenzoni, G., Nottingham University Press, Nottingham, United Kingdom. 25-62.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J. J. & Lee, M. D., 2003. Diversity and Succession of the Intestinal Bacterial Community of the Maturing Broiler Chicken. Appl. Environ. Microb. 69(11), 6816-6824. doi:10.1128/aem.69.11.6816-6824.2003

M'Sadeq, S. A., Wu, S., Swick, R. A. & Choct, M., 2015. Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. Anim. Nutr. 1(1), 1-11. doi:10.1016/j.aninu.2015.02.004

Mallo, J., Gracia, M., Honrubia, P. & Sedano, G., 2010. Use of a Bacillus amyloliquefaciens probiotic in broiler farms. Poultry Sci. 89.

Mandal L., Biswas, T. & Sarkar, S. K., 2000. Broiler perform well on herbs or enzymes in maize diet. World Poultry 16(5), 19-21.

Marchese, A., Orhan, I. E., Daglia, M., Barbieri, R., Di Lorenzo, A., Nabavi, S. F., Gortzi, O., Izadi, M., Nabavi, S. M., 2016. Antibacterial and antifungal activities of thymol: A brief review of the literature. Food Chem. 210, 402-414.

Maron, D. F., Smith, T. J. & Nachman, E., 2013. Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. Globalization Health 9 (48), 11.

McDevitt, R. M., Brooker, J. D., Acamovic, T. & Sparks, N. H. C., 2007. Necrotic enteritis; a continuing challenge for the poultry industry. World Poultry Sci. J. 62(02), 221-247. doi:10.1079/wps200593.

Mikkelsen, L. L., Vidanarachchi, J. K., Olnood, C. G., Bao, Y. M., Selle, P. H. & Choct, M., 2009. Effect of potassium diformate on growth performance and gut microbiota in broiler chickens challenged with necrotic enteritis. Br. Poultry Sci. 50(1), 66-75. doi:10.1080/00071660802613252

Mitsch, P., Zitterl-Eglseer, K., Köhler, B., Gabler, C., Losa, R. & Zimpernik, I., 2004. The effect of two different blends of essential oil components on the proliferation of Clostridium perfringens in the intestines of broiler chickens. Poultry Sci. 83(4), 669-675. doi:10.1093/ps/83.4.669

Mohyla, P., Bilgili, S. F., Oyarzabal, O. A., Warf, C. C. & Kemp, G. K., 2007. Application of Acidified Sodium Chlorite in the Drinking Water to Control Salmonella serotype Typhimurium and Campylobacter jejuni in Commercial Broilers. J. App. Poultry Res. 16(1), 45-51. doi:10.1093/japr/16.1.45

Moore, R.J., 2016. Necrotic enteritis predisposing factors in broiler chickens. Avian Pathol. 45, 1-22. 10.1080/03079457.2016.1150587.

Nauerby, B., Pedersen, K., Madsen, M., 2003. Analysis by pulsed-field gel electrophoresis of the genetic diversity among Clostridium perfringens isolates from chickens. Vet. Microbiol. 94(3), 257-266.

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R. & De Feo, V., 2013. Effect of essential oils on pathogenic bacteria. Pharm. 6(12), 1451-1474. doi:10.3390/ph6121451.

Nisbet, D., 2002. Defined competitive exclusion cultures in the prevention of enteropathogen colonisation in poultry and swine. Anton. Leeuw. 81, 481-486.

Nunes, R. V., Scherer, C., Pozza, P. C., Eyng, C., Bruno, L. D. G. & Vieites, F. M., 2012. Use of probiotics to replace antibiotics for broilers. Rev. Bras. Zootec. 41, 2219-2224.

Nurmi, E. & Rantala, M., 1973. New Aspects of Salmonella Infection in Broiler Production. Nature 241(5386), 210-211.

Otutumi, L. K., Góis, M. B., Garcia, E. R. D. M. & Loddi, M. M., 2012. Variations on the efficacy of probiotics in poultry. In InTech. Ed: E. C. Rigobelo. 9, pp 203-230.
Paiva, D. & McElroy, A., 2014. Necrotic enteritis: Applications for the poultry industry. J. App. Poultry Res. 23(3), 557-566. doi:10.3382/japr.2013-00925
Pan, D. & Yu, Z., 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 5(1), 108-119. doi:10.4161/gmic.26945
Papatsiros, V. G., Katsoulos, P. D., Koutoulis, K. C., Karatzia, M., Dedousi, A. & Christodoulopoulos, G., 2013. Alternatives to antibiotics for farm animals. CAB Reviews: Perspectives in agriculture, veterinary science, nutrition and natural resources 8 (32), 1-40. doi:10.1079/pavsnnr20138032
Patterson, J. A. & Burkholder, K. M., 2003. Application of Prebiotics and Probiotics in Poultry Production. Poultry Sci. 82, 627-631.
Paul, S. K., Halder, G., Mondal, M. K. & Samanta, G., 2007. Effect of organic acid salt on the performance and
gut health of broiler chicken. J. Poultry Sci. 44, 389-395.
Pelicano, E., Souza, P. D., Souza, H. D., Leonel, F., Zeola, N. & Boiago, M., 2004. Productive traits of broiler chickens fed diets containing different growth promoters. Braz. J. Poultry Sci. 6, 177-182.
Popova, T., 2017. Effect of probiotics in poultry for improving meat quality. Curr. Opin. In Food Sci. 14, 72-77. doi:10.1016/j.cofs.2017.01.008
Prescott, J. F., Sivendra, R., Barnum, D.A., 1978. The use of bacitracin in the prevention and treatment of experimentally induced necrotic enteritis in the chicken. Can. Vet. J. 19,181–183.
Pritchard, S., 2016. Life Without Antibiotic Growth Promoters – A Uk Perspective. Aust. Poultry Sci. Symp. 27, 206-2012.
Qiu, J., Feng, H., Lu, J., Xiang, H., Wang, D., Dong, J., Wang, J., Wang, X., Liu, J. & Deng, X., 2010. Eugenol reduces the expression of virulence-related exoproteins in staphylococcus aureus. Appl. Environ. Microb. 76(17), 5846-5851. doi:10.1128/AEM.00704-10
Rafiullah, P. & Sajid, A., 2011. Effect of feed additives on the performance of broilers. ARPN J. Agric. Biol. Res. 6(9), 66-72.
Ricke, S. C., 2018. Impact of prebiotics on poultry production and food safety. Yale J. Biol. Med. 91(2), 151-159.
Salim, H. M., Huque, K. S., Kamaruddin, K. M. & Beg, M., 2018. Global restriction of using antibiotic growth promoters and alternative strategies in poultry production. Sci. Prog. 101(1), 52-75. doi:10.3184/003685018X15173975498947
Samadian, F., Zeinoaldini, S., Towhidi, A., Torshizi, M. A. K., Pirasaraei, Z. A. & Gholamzadeh, P., 2013. Evaluation of some phytogenic feed additives in growing chicks diet. Intern. J. Agric. 3(1), 35-43.
Samanya, M. & Yamauchi, K.E., 2002. Histological alterations of intestinal villi in chickens fed dried Bacillus subtilis var. natto. Comp. Biochem. Physiol. A. Mol. Integr. Phys. 133(1), 95-104. doi:10.1016/S1095-6433(02)00121-6
Samuals, M.L. 1989. Statistics for the life science. Collier MacMillan Publishers, Londen.
Schjorring, S. & Krogfelt, K. A., 2011. Assessment of bacterial antibiotic resistance transfer in the gut. Int. J. Microbiol. 2011, 1-10. doi:10.1155/2011/312956
Schneitz, C., 2005. Competitive exclusion in poultry–30 years of research. Food Control 16(8), 657-667. doi:10.1016/j.foodcont.2004.06.002
Seal, B. S., Lillehoj, H. S., Donovan, D. M. & Gay, C. G., 2013. Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. Anim. Health. Res. Rev. 14(1), 78-87. doi:10.1017/S1466252313000030
Shojadoost, B., Vince, A. R. & Prescott, J. F., 2012. The successful experimental induction of necrotic enteritis in chickens by Clostridium perfringens: a critical review. Vet. Res. 43 (74), 1-12.
Si, H., Hu, J., Liu, Z. & Zeng, Z. l., 2008. Antibacterial effect of oregano essential oil alone and in combination with antibiotics against extended-spectrum beta-lactamase-producing escherichia coli. Immunol. Med. Microbiol. 53(2),190-194.
Simon, O., Jadamus, A. & Vahjen, W., 2001. Probiotic feed additives - effectiveness and expected modes of action. J. Anim. Feed Sci. 10, 51-67. doi:10.22358/jafs/70012/2001
Singer, R. S., Finch, R., Wegener, H. C., Bywater, R., Walters, J. & Lipsitch, M., 2003. Antibiotic resistance-the interplay between antibiotic use in animals and human beings. Lancet Infect. Dis. 3(1), 47-51. doi:10.1016/s1473-3099(03)00490-0
Spring, P., Wenk, C., Dawson, K. A. & Newman, K. E., 2000. The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. Poultry Sci. 79(2), 205-211. doi:10.1093/ps/79.2.205
Statistical Analysis Systems, 2014. SAS User's Guide: Statistics Version 9.3. SAS Institute Inc. Cary, NC, USA.
Taheri, H. R., Moravej, H., Tabandeh, F., Zaghari, M. & Shivazad, M., 2009. Screening of lactic acid bacteria toward their selection as a source of chicken probiotic. Poultry Sci. 88(8), 1586-1593. doi:10.3382/ps.2009-00041
Teo, A. Y. L. & Tan, H. M., 2005. Inhibition of Clostridium perfringens by a Novel Strain of Bacillus subtilis Isolated from the Gastrointestinal Tracts of Healthy Chickens. Appl. Environ. Microb. 71(8), 4185-4190. doi:10.1128/AEM.71.8.4185-4190.2005
Tian, X., Shao, Y., Wang, Z. & Guo, Y., 2016. Effects of dietary yeast β-glucans supplementation on growth performance, gut morphology, intestinal Clostridium perfringens population and immune response of broiler chickens challenged with necrotic enteritis. Anim. Feed Sci. Tech. 215, 144-155.
Timbermont, L., Haesebrouck, F., Ducatelle, R. & Van Immerseel, F., 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathol. 40 (4), 341-347. doi:10.1080/03079457.2011.590967
Timbermont, L., Lanckriet, A., Dewulf, J., Nollet, N., Schwarzer, K., Haesebrouck, F., Ducatelle, R. & Van Immerseel, F., 2010. Control of Clostridium perfringens-induced necrotic enteritis in broilers by target-released butyric acid, fatty acids and essential oils. Avian Pathol. 39(2), 117-121. doi:10.1080/03079451003610586

Trombetta, D., Castelli, F., Sarpietro, M. G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G. & Bisignano, G., 2005. Mechanisms of Antibacterial Action of Three Monoterpenes. Antimicrob. Agents.Chemother. 49(6), 2474-2478. doi:10.1128/AAC.49.6.2474-2478.2005

Turner, J. L., Dritz, S. S. & Minton, J. E., 2001. Alternatives to conventional antimicrobials in swine diets. The Professional Animal Scientist 17(4), 217-226. doi:10.15232/S1080-7446(15)31633-8

Van Immerseel, F., Rood, J. I., Moore, R. J. & Titball, R. W., 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends Microbiol. 17(1), 32-36. doi:10.1016/j.tim.2008.09.005

Van Immerseel, F., Russell, J. B., Flythe, M. D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F. & Ducatelle, R., 2006. The use of organic acids to combat Salmonella in poultry: a mechanistic explanation of the efficacy. Avian Pathol. 35(3), 182-188. doi:10.1080/03079450600711045

Van Nevel, C. J., Decuypere, J. A., Dierick, N. A. & Molly, K., 2005. Incorporation of galactomannans in the diet of newly weaned piglets: Effect on bacteriological and some morphological characteristics of the small intestine. Arch. Anim. Nutr. 59(2), 123-138. doi:10.1080/17450390512331387936

Vollenbroich, D., Pauli, G., Ozel, M. & Vater, J., 1997. Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from Bacillus subtilis. Appl. Environ. Microb. 63(1), 44-49.

Wallace, R. J., Oleszek, W., Franz, C., Hahn, I., Baser, K. H., Mathe, A. & Teichmann, K., 2010. Dietary plant bioactives for poultry health and productivity. Br. Poultry Sci. 51(4), 461-487. doi:10.1080/00071668.2010.506908

Wang, J. P., Yoo, J. S., Lee, J. H., Zhou, T. X., Jang, H. D., Kim, H. J. & Kim, I. H., 2009. Effects of phenyllactic acid on production performance, egg quality parameters, and blood characteristics in laying hens. J. App. Poultry Res. 18(2), 203-209. doi:10.3382/japr.2008-00071

Wealleans, A. L., Sirukhi, M. & Egorov, I. A., 2017. Performance, gut morphology and microbiology effects of a Bacillus probiotic, avilamycin and their combination in mixed grain broiler diets. Br. Poultry Sci. 58(5), 523-529. doi:10.1080/00071668.2017.1349298

Wegener, H. C., 2003. Antibiotics in animal feed and their role in resistance development. Curr. Opin. Microbiol. 6(5), 439-445. doi:10.1016/j.mib.2003.09.009

Williams, P. & Losa, R., 2001. Technical crina the use of essential oils and their compounds in poultry nutrition. World Poultry 17(4), 14-15.

Williams, R. B., 2005. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathol. 34(3), 159-180. doi:10.1080/03079450500112195

Xu, J., Zhou, F., Ji, B. P., Pei, R. S. & Xu, N., 2008. The antibacterial mechanism of carvacrol and thymol against Escherichia coli. Lett. App. Microbiol. 47 (3), 174-179. doi:10.1111/j.1472-765X.2008.02407.

Xu, Z. R., Hu, C. H., Xia, M. S., Zhan, X. A. & Wang, M. Q., 2003. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poultry Sci. 82(6), 1030-1036. doi:10.1093/ps/82.6.1030

Yang, Y., Iji, P. A. & Choct, M., 2009. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. World Poultry Sci. J. 65(01). doi:10.1017/s0043933909000087

Yason, C., A Summers, B. & Schat, K., 1987. Pathogenesis of rotavirus infection in various age groups of chickens and turkeys. Am. J. Vet. Res. 48, 927-938.

Yegani, M. & Korver, D. R., 2008. Factors affecting intestinal health in poultry. Poultry Sci. 87(10), 2052-2063. doi:10.3382/ps.2008-00091

Youngsub, L., Woo, H.K., Sung-jin, L., Hyun, S.J., 2018. Detection of chicken interleukin-10 production in intestinal epithelial cells and necrotic enteritis induced by Clostridium perfringens using capture ELISA. Vet. Immunol. Immunop. 204, 52-58.

Zhang, K. Y., Yan, F., Keen, C. A. & Waldroup P. W., 2005. Evaluation of microencapsulated essential oils and organic acids in diets for broiler chickens. Int. J. Poultry Sci. 4(9), 612-619. doi:10.3923/ijps.2005.612.619