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Improving gut health and performance of broilers by adding *Bacillus subtilis* to the diet

by

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DECLARATION

I, **Gerhard Coertze**, declare that the dissertation/thesis, which I hereby submit for the degree Bachelor of Science Master in Animal Science at the University of Pretoria, is my own work and not previously been submitted by my for a degree at this or any other tertiary institution.

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ABSTRACT

The purpose of the study was to evaluate the benefits, if any, of adding a probiotic product containing *Bacillus subtillus* to the diet of broilers. The study was conducted in an open-sided house, with curtains and a coal boiler (HEATCO) attached to a heat sock. The house contained 64 pens in total, divided into 2 rows of 32 pens each over the length of the house. The pens were covered with used chicken litter (4cm deep). Three thousand six hundred Ross 308 broiler birds were feather-sexed and randomly distributed throughout the pens, 60 broilers of the same sex per pen at a stocking density of 20 birds/ m^2. All broilers received similar typical South African maize-soya diets throughout the study. Diets were treated with antibiotic growth promoters (AGP) and / or direct-fed-microbials (DFM) to create six treatments as follows: Negative Control: Basal diet (without AGP), Positive Control: Basal diet (with AGP), DFM at 500g/ton (without AGP), DFM at 250g/ton (without AGP), DFM at 500g/ton (with AGP), and DFM at 250g/ton (with AGP). Broiler performance was measured weekly in terms of body weight (BW), feed intake (FI) and feed conversion ratio (FCR) from day old to 35 days of age. Birds were culled at day old, as well as 22 days of age for the isolation of Avian Pathogenic *E. coli* (APEC), *E. coli* and *Lactobacillus* from the gastrointestinal tract.

Males showed a higher feed intake from 14 to 35 days of age compared to females, even though the FCR remained similar between sexes ($P > 0.05$). There were no dose response between DFM 500g/ton and DFM 250g/ton, as well as between DFM 500g/ton + AGP and DFM 250g/ton + AGP ($P > 0.05$) in terms of BW, FI and FCR. Positive Control had a higher body weight, no difference in feed intake and lower FCR compared to Negative Control at 35 days ($P < 0.05$). A lower dose of DFMs (250g/ton) in the diets of broilers revealed a significantly lower FCR compared to diets without DFMs (Negative Control), and DFM (500g/ton and 250g/ton) + AGP, and no significant difference in FCR compared to DFM (500g/tom) and Positive Control. However, when combining a DFM with AGP in the diet, the FCR of broilers increased due to a lower weight obtained with a higher feed intake at 35 days. The DFM (500g/ton) + AGP had a significantly lower BW compared to Positive Control at 35 days, although, revealed no significant difference in terms of FI ($P > 0.05$), but a significantly higher FCR at 35 days compared to Positive Control ($P < 0.05$).

DFM (250g/ton) without AGP tend to lower the feed intake ($P < 0.05$) of broilers compared to AGPs, but when combining a DFM with AGP, the feed intake increased significantly ($P < 0.05$), revealing a higher FCR ($P < 0.05$) compared to AGPs and DFM (250g/ton). The recommendation will be to use a lower dose of DFM (250g/ton) as an alternative for AGP in broiler diets.

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TABLE OF CONTENT

DECLARATION ... i
ACKNOWLEGMENTS ... ii
ABSTRACT ... iii
LIST OF TABLES ... vi
LIST OF FIGURES ... vii
CHAPTER 1 ... 1
1. INTRODUCTION ... 1
CHAPTER 2 ... 4
2. LITERATURE REVIEW ... 4
2.1 The microflora of a healthy gastrointestinal tract (GIT) ... 4
2.2 Functions of microbial species in the gut ... 5
2.3 The GIT of a day-old chick ... 7
2.4 The role of microflora in the GIT of a young chick ... 9
2.5 The cost associated with microflora in the gut of broilers ... 10
2.6 Factors affecting the intestinal microflora ... 11
2.7 Establishment of sufficient DFM cultures ... 12
2.8 Antibiotic growth promoters (AGPs) ... 24
2.9 Alternatives to antibiotics to manipulate the intestinal microflora ... 25
2.10 Models to study the impact of gut microflora ... 28
2.11 Gut microflora quantification ... 29
2.12 Conclusion ... 29
CHAPTER 3 ... 30
3. MATERIALS AND METHODS ... 30
3.1 Housing and birds ... 30
3.2 Diets and treatments ... 31
3.3 Feed samples ... 34
3.4 Avian Pathogenic *E. coli* isolation (APEC) and *E. coli* and *Lactobacillus* Enumeration ... 34
3.5 DNA isolation and Multiplex PCR ... 35
3.6 Statistical design ... 36
3.7 Statistical Methods ... 36
CHAPTER 4 ... 37
4. RESULTS ... 37

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4.1 Body weight of broilers from 0 to 35 days of age ... 37
4.2 Cumulative feed intake of broilers from 0 to 35 days of age ... 38
4.3 Cumulative feed conversion ratio of broilers from 0 to 35 days of age ... 39
4.4 Cumulative mortality for broilers from 0 to 35 days of age ... 40
4.5 APEC Counts of the GIT of broilers at 21 days of age, both pooled and individual samples ... 41
CHAPTER 5 ... 44
5. DISCUSSION ... 44
5.1 Effect of sex on broiler performance ... 44
5.2 The effect of zinc bacitracin on broiler performance ... 44
5.3 The effect of DFM on broiler performance ... 44
5.4 The effect of a combination of DFM with AGP on broiler performance ... 45
5.5 The potential of a DFM to replace AGPs in the diet of broilers ... 46
5.6 The effect of DFM alone or a combination of DFM + AGP on the Avian Pathogenic *E. coli* colony levels in the gut of broilers ... 46
CHAPTER 6 ... 48
6. CONCLUSION ... 48
CHAPTER 7 ... 49
7. RECOMMENDATIONS ... 49
APPENDIX A ... 55
APPENDIX B ... 56

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LIST OF TABLES

Table 3.1. Composition of the basal diet ………………………………………………………..……33

Table 4.1: The effect of DFM and zinc bacitracin on body weight of broilers from 0 to 35 days of age…………………………………………………………………………………………………37

Table 4.2. The effect of DFM and zinc bacitracin on cumulative feed intake of broilers from 0 to 35 days…………………………………………………………………………………………………38

Table 4.3 The effect of DFM and zinc bacitracin on feed conversion ratio of broilers from 0 to 35 days…………………………………………………………………………………………………39

Table 4.4. The effect of DFM and zinc bacitracin on cumulative mortality for broilers from 0 to 35 days…………………………………………………………………………………………………40

Table 4.5 Average Avian Pathogenic *E. coli* colonies (APEC) counts (log^{10} *cfu*/g) in the gastro intestinal tract by treatment as relates to the Negative Control……………………………………43

Table 1: Lighting program followed during the experimental period……………………………55

Table 2: Temperature profile of the houses during the experimental period………………………56

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LIST OF FIGURES

Figure 4.1: Effect of DFM and zinc bacitracin on the percentage of *E. coli* isolates with corresponding number of Avian Pathogenic *E. coli* colonies (APEC) and without the corresponding number of Avian Pathogenic *E. coli* genes……………………………………………………………41

Figure 4.2 The effect of Avian Pathogenic *E. coli* colonies (APEC) on gene category by treatment at 22 days of age………………………………………………………………………………42

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CHAPTER 1

1. INTRODUCTION

The population of intestinal microflora is a complex ecosystem, consisting of a large variety of bacteria. The microflora metabolic capacity is extremely diverse and can have positive and/or negative effects on the gut physiology and well-being of the host animal as a whole (Miles *et al*., 2006; Onderci *et al*., 2008). A bacterial population is present in the small intestine of the avian species within 24 hours after hatch (Miles *et al*., 2006). Studies from various *in vivo* and *in vitro* trials have shown that the commensal intestinal microflora inhibits pathogens, but any disturbances of the intestinal microflora can increase susceptibility to infection, and that the oral supplementation of a direct-fed microbial (DFM) species, as well as prebiotics (inactive microbial species) increases resistance to infection (Patterson & Burkholder, 2003).

DFMs have gained a lot of attention around the world due to their health promoting effects, their ability to improve feed utilisation and maintain performance in growth of broilers and because they are a natural alternative to antibiotics for growth promotion in poultry (Patterson & Burkholder, 2003; Novoa Garrido *et al*., 2004; Bai *et al*., 2013; Salim *et al*., 2013; Tabidi *et al*., 2013). DFMs are live non-pathogenic microbial supplements, which beneficially affect the health of the host animal by restoring intestinal microbial balance, thereby improving intestinal functions (Chapman *et al*., 2012; Tabidi *et al*., 2013; Waititu *et al*., 2014). DFMs normally consist of a single strain or combination of several strains of bacteria and yeast species (Waititu *et al*., 2014). Single-strain DFMs may be less effective than multi-strain DFMs, which could amplify the protective spectrum against microbial infections (Zhang & Kim, 2014).

The microbial species most commonly used as DFMs are *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Escherichia coli*, *Aspergyllus*, *Candida*, *Lactococcus*, *Saccharomyces*, *Leuconostoc*, *Pediococcus*, *Propionibacterium* and *Kluyveromyces*. A variety of yeast species and undefined mixed cultures have beneficial effects on broiler performance (Patterson & Burkholder, 2003; Mountzouris *et al*., 2007; Kabir, 2009; Gaggia *et al*., 2010; Lee *et al*., 2010; Waititu *et al*., 2014). These commonly used species function by maintaining the presence of beneficial microorganisms and competitively exclude pathogenic bacteria from the intestine, enhancing production performance. They develop and stimulate the immune response as well as reducing bird mortalities by inhibiting pathogens from colonising the gut via competition for nutrients and binding sites on the intestinal epithelium, thus inhibiting the production of toxic conditions and compounds. These microbial species also promote acid fermentation to reduce the gut pH, increasing production of short-chain fatty acids and reducing epithelial cell apoptosis

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(Patterson & Burkholder, 2003; Bai *et al*., 2013; Salim *et al*., 2013 & Tabidi *et al*., 2013). The intestinal microflora, epithelium and immune system provide resistance to enteric pathogens (Patterson & Burkholder, 2003).

In modern broiler production systems, stressors from various production practices may weaken birds' immune functions and predispose broilers to pathogen colonisation of the gastrointestinal tract, which would pose a threat to their health and, ultimately, compromise food safety (O'Dea *et al*., 2006; Baffoni *et al*., 2012). Recent studies showed that a direct-fed microbial (DFM) product of *Lactobacillus fermentum* and *Saccharomyces cerevisiae* stimulated the T-cell immune system in the intestine without sacrificing growth performance in broilers during the first 21 days (Bai *et al*., 2013).

The main benefit of DFMs is improving broiler intestinal microflora balance (Jin et al., 1998; Waititu *et al*., 2014). One-day-old chicks that received aerobic caecal microflora cultures were healthier and more efficient than untreated chicks, and showed lower caecal colonisation of *Salmonella enteritidis*, followed by mixed caecal microflora culture (Andreatti *et al*., 2003). Facultative anaerobic bacteria are first needed to remove the oxygen from the caecum, favouring the establishment of strict anaerobic bacteria (Andreatti *et al*., 2003). Zhang & Kim (2014) reported that high inclusion levels of DFMs in the diet did not always result in better performance in animals, whereas in contrast, other research obtained beneficial effects with supplementation of 10^5 to 10^9 cfu/ kg of probiotics in the diet.

Antibiotics are used for their therapeutic, preventative or additive effects, provided through the feed. Antibiotics are substances produced by certain species of probiotic bacteria and fungi that have the ability to inhibit or kill bacterial and microbial growth (Tabidi *et al*., 2013). Sub-therapeutic antibiotics not only influence the intestinal microbial populations, but also affect animal metabolism and specifically alter intestinal function (Patterson & Burkholder, 2003). In some parts of the world, antibiotics are still used in the poultry industry as growth stimulants and therapeutic agents (Tabidi *et al*, 2013).

The increased risk of development of resistance in animal pathogens is due to the extensive use and misuse of antibiotics in animal production units (Salim *et al*., 2013). Consumer pressure still grows, demanding the reduction of antibiotics/antimicrobial agents in feed and also elimination of *Salmonella* and other harmful bacteria (food-borne pathogens). The eradication of disease from poultry and their products, and improved intestinal health are important concerns for the production of safe meat and meat products (Novoa Garrido *et al*., 2004; Hammons *et al*., 2010; Burgain *et al*., 2011; Chambers & Gong, 2011; Giannenas *et al*., 2012, Baffoni *et al*., 2012; Tabidi *et al*., 2013). Since the beginning of the 1990s, strains of *Salmonella* have emerged that are resistant to a range of antimicrobials, including first-choice agents for the treatment of humans, and this is becoming a

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serious health problem over the whole world (Chambers & Gong, 2011). The threat of antibiotic-resistant pathogens, therefore, has forced the poultry industry to consider various alternatives (Novoa Garrido *et al*., 2004; Dahiya *et al,* 2006).

The ban on sub-therapeutic antibiotic usage in Europe, and a potential ban in the United States have had a profound effect on the incidence of necrotic enteritis, cholangiohepatitis, gangrenous dermatitis and botulism in broiler chickens throughout Europe and also in the USA (Patterson & Burkholder, 2003; Dahiya *et al*., 2006). This has resulted in an increasing interest in finding alternatives to antibiotics for poultry production, and methods to control and prevent pathogenic bacterial colonisation (Patterson & Burkholder, 2003; Gaggia *et al*., 2010). More research is required on the efficiency of DFMs to replace antibiotics in broiler feeds without negatively affecting broiler performance. From a marketing point of view, it is also necessary to test such DFMs under typical South African conditions for broiler production.

The aim of this trial was to evaluate the effect of a DFM product (Enviva™ Pro, DUPONT™) comprising strains of *Bacillus subtilis*, on *E.coli* colonisation in the gut of broilers, and subsequent broiler performance. Further aims were to determine if Enviva™ Pro can replace antibiotics in broiler feeds and what level of supplementation of the probiotic is most beneficial. The effect of a combination of antibiotic growth promoters (AGPs) and DFMs was also evaluated in the current study because South African broiler producers typically include AGPs in feed as it is still allowed in the country. The aim was to test whether AGPs and DFMs act in either an antagonistic or synergistic way when combined in the same feed.

The null hypothesis (H_0) of this study was that broilers fed a standard broiler diet with DFMs (Enviva™ Pro) will not perform better than broilers fed a standard diet that does not contain any DFM. The alternative hypothesis (H_1) was that broilers fed a standard broiler diet with DFMs (Enviva™ Pro) will perform better than broilers fed a standard diet that does not contain any DFM.

A second null hypothesis was that broilers fed a standard broiler diet with DFM (Enviva™ Pro) will perform at a lower level than broilers fed a standard diet that contains AGP (Zn Bacitracin). The alternative hypothesis was that broilers fed a standard diet with DFM (Enviva™ Pro) will perform the same than broilers fed diets containing AGP (Zn Bacitracin).

A third null hypothesis was that broilers fed a standard broiler diet with a DFM (Enviva™ Pro) and AGP (Zn Bacitracin) combination will perform the same as broilers fed a standard diet with only DFM (Enviva™ Pro), or only AGP (Zn Bacitracin). The alternative hypothesis was that broilers fed a standard diet with a DFM (Enviva™ Pro) and AGP (Zn Bacitracin) combination will perform better or at a lower level than broilers fed a standard diet with only DFM (Enviva™ Pro), or only AGP (Zn Bacitracin), and therefor acting in either an antagonistic or synergistic way when combined in a feed.

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CHAPTER 2

2. LITERATURE REVIEW

2.1 The microflora of a healthy gastrointestinal tract (GIT)

Gastrointestinal flora play a significant role in the health and performance of poultry as well as providing "colonisation resistance" to bacterial pathogens (*Salmonella, Clostridia, Campylobacter* or *Colibacilla*) in chickens (Novoa Garrido *et al*., 2004; Tabidi *et al*., 2013). About 90% of the intestinal digestive tract bacteria of chickens have not been previously identified using conventional culture methods (Miles *et al*., 2006; Chambers & Gong, 2011), and only 20 to 50% of the bacterial species present in the intestinal tract have been cultured (Patterson & Burkholder, 2003). This is why the microflora of the gut remain largely unexplored, especially regarding the effects of AGPs upon these mostly unknown species (Miles *et al*., 2006). Intestinal microflora as defined by Chambers & Gong (2011) comprise a complex mixture of bacterial populations colonising a given area of the gastrointestinal tract (GIT) in animal hosts that have not been affected by or exposed to medical or experimental intervention or disease. In 60 species of mammals, studies conducted on a survey of the gut bacterial populations based on 16S rRNA-analysis indicated that the diet, host phylogeny and gut morphology influence the microbial ecology of the GIT (Gaggia *et al*., 2010). Some practices applied in commercial poultry production systems inhibit the normal development of microflora in the gut, leaving the chicken vulnerable to colonisation and possible infection by pathogenic bacteria, including *Salmonella* species (Chambers & Gong, 2011). In the GIT of vertebrates, a highly diverse group of microflora, especially gram-positive bacteria, predominates in the gut (Dibner & Richards, 2005), whereas *Ruminococcus* and *Streptococcus* tend to predominate in the chicken GIT (Patterson & Burkholder, 2003).

In the GIT of normal, healthy, non-stressed poultry, there is an unsteady balance of beneficial and non-beneficial bacteria. Bird performance will maximise when such a balance exists, but when birds are stressed, the beneficial flora, especially *Lactobacilli*, will decrease in number, allowing an overgrowth of non-beneficial microflora (Kabir, 2009). If a disturbance in the GIT microflora population occurs, it may predispose the birds to frank diseases (e.g., diarrhoea), or present subclinically and decrease production in terms of growth rate, feed efficiency and mortality (Kabir, 2009). Bacterial species that exist in the gastrointestinal microflora can number as many as 500, with up to 10^{10} to 10^{12} bacterial cells/g in colonic content or faeces. According to Gaggia *et al*. (2010), the host's physiological status can be predicted through the composition of the faecal microflora of that host. In monogastric animals (e.g. pig, chicken, rabbit and man), the major

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microbial groups are *Bacteroides*, *Clostridium*, *Bifidobacterium*, *Eubacterium*, *Lactobacillus*, *Enterobacteriacea*, *Streptococcus*, *Fusobacterium*, *Peptostreptococcus* and *Propionibacterium*. The percentage of the different microbial groups differs between individuals, depending on age and health or physiological state of the animal (Gaggia *et al*., 2010).

2.2 Functions of microbial species in the gut

The intestinal microflora, epithelium and immune system provide resistance to enteric pathogens, which also encounter a multifaceted defence system composed of low gastric pH and rapid transit through sections of the intestinal tract (Patterson & Burkholder, 2003). A variety of immunological, physiological, nutritional and protective processes of the GIT are influenced by the bacterial population present, which has profound effects on the overall health, development and performance of monogastric animals (Dibner & Richards, 2005). Commensal bacteria play a crucial role in organ, tissue and immune system development, as well as providing a variety of nutritional compounds, but they do come with a cost to the animal (Dibner & Richards, 2005; Gaggia *et al*., 2010).

There are two types of non-immune-evoking innocuous antigenic molecules in chickens that confront the gut-associated lymphoid tissue. These are nutrients and antigens resulting from intestinal or external pathogens that induce protective immune responses (Waititu *et al*., 2014). The mucosal barrier, formed by the interaction of various mucosal secretions, separates the internal milieu from the luminal environment. The mucosal secretions include mucin glycoproteins, trefoil peptides, and surfactant phospholipids (Gaggia *et al*., 2010). The mucus and the intestinal epithelium together provide the first adaptive defence mediating the active sampling of resident bacteria, pathogens and other antigens. There are three main types of immune-sensory cells involved, i.e. surface enterocytes, M-cells, and intestinal dendritic cells (Gaggia *et al*., 2010). A correlation exists between the composition of the colonising microflora and variations in immunity.

The dual function of the resident bacteria is to stimulate the mucosal mechanisms of defence and to maintain the homeostasis of the immune response (Gaggia *et al*., 2010). The commensal bacteria compete with the host for nutrients, secrete toxic compounds, and induce an on-going immune / inflammatory response in the GIT, which negatively impacts the health and performance of the animal. Intestinal microflora inhibit pathogens by mechanisms called bacterial antagonism, bacterial interference, barrier effect, colonisation resistance and competitive exclusion (CE). Indigenous intestinal bacteria inhibit pathogens through mechanisms including competition for colonisation sites, production of toxic compounds, competition for nutrients, or stimulation of the immune system (Patterson & Burkholder, 2003). The integrity of the intestinal barrier can be

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positively affected by gut microflora with its metabolic, trophic and protective functions. Intestinal barrier dysfunction leads to a progressive rise in intestinal permeability, resulting in pathological inflammation that is characteristic of diseases like intestinal bowel disease (IBD). These intestinal pathogens interfere with epithelial metabolism by producing toxins and other substances like mucinases, adhesins and invasions (Gaggia *et al.*, 2010). The gut microbial composition and susceptibility to enteric pathogens can be severely affected due to physiological and psychological stressors, such as excessive hygiene, antibiotic therapy and stress, which lead to dysfunction of the intestinal barrier, and an increase of intestinal permeability. These stressors will also result in a decrease in beneficial bacteria such as *Lactobacilli* and *Bifidobacteria* (Kabir, 2009).

Bifidobacteria are gram-positive polymorphic rods that are non-filamentous, non-spore-forming and non-motile, with either club-shaped or spatulated extremities (Russel *et al.*, 2011). These bacteria can occur singly, in chains or in clumps (Russel *et al.*, 2011). *Bifidobacteria* are strain and media-dependent, chemo-organotrophic obligated anaerobes (Russel *et al.*, 2011), and mainly acid producing saccharolytic bacteria, fermenting a variety of carbohydrates without producing gas. These bacteria also degrade glucose via fructose-6-phosphate metabolic pathways, producing mainly acetic and lactic acid in a molar ratio of 3:2. *Bifidobacteria* can tolerate acidic conditions and will provide health benefits in such an environment (Russel *et al.*, 2011).

Lactobacilli are gram-positive bacilli that are non-spore-forming and grow optimally under anaerobic conditions (Tannock, 1997). *Lactobacilli* produce lactic acid, which is a major product of glucose fermentation. *Lactobacilli* have the longest record of use of DFM products given to farm animals (Tannock, 1997; Zhang *et al.*, 2012). *Lactobacillus* seems to be the most appropriate because the digestive tract microflora in pigs and poultry are found to contain high levels of *Lactobacilli* when kept under optimal conditions (Tannock, 1997). Lactic acid bacteria can be sourced by isolation from the intestinal microflora of healthy animals; alternatively, non-animal strains can be obtained from fermented products (Zhang *et al.*, 2012). Over the last decade, disease infections and immune disorders have been treated and prevented by the use of *Lactobacilli* and *Bifidobacterium* (Zhang *et al.*, 2012). Certain species of *Lactobacillus* (*L. reuteri ATCC 55730*) and glycerol will reduce *E.coli* populations in an *in vitro* colonic fermentation model. In the presence of glycerol, *Lactobacillus reuteri* produced a broad-spectrum antimicrobial substance (3-hydroxy-propionaldehyde) during anaerobic growth (Zhang *et al.*, 2012). Furthermore, the intestinal pH will decrease to a level that is unfavourable to most pathogenic bacteria. A study was conducted with two strains of *Lactobacillus* isolated from healthy chickens and previously characterised as antimicrobial producers (Zhang *et al.*, 2012). The study showed that *Lactobacillus* can serve as a therapeutic alternative to counter multidrug-resistant pathogens by suppressing the proliferation and virulence of bacterial pathogens (Zhang *et al.*, 2012). *Lactobacilli* contain S-proteins binding non-

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covalently to the outer surface of the cell wall, representing an important component associated with adhesion to intestinal epithelial cells (IECs) and mammalian extracellular matrix (Sun *et al*., 2012).

Escherichia coli are gram-negative rod-like organisms that function optimally in facultative anaerobic conditions and do not utilise citrate (Tannock, 1997). *E. coli* will ferment carbohydrates into lactic, acetic and formic acids where most strains ferment lactose into an energy source. Formic acid is then split by a complex hydrogenase system to give equal amounts of carbon dioxide and hydrogen (Tannock, 1997). *E. coli* are either motile (via peritrichous flagella) or non-motile (Tannock, 1997).

2.3 The GIT of a day-old chick

The dominant bacterial species found in the crop, duodenum and ileum of chicks during the first week of life are *Enterococci* and *Lactobacillus*. Also high numbers of *coliforms*, *Enterococci* and *Lactobacilli* are present in the caeca. A highly complex group of obligate anaerobes begins to take over the caeca after the first week, whereas the crop, duodenum and ileum are taken over by *Lactobacilli* (Chambers & Gong, 2011). After 2 to 3 weeks, the intestinal microflora is established and stable. The intestinal microflora performs an important role in controlling enteric bacterial pathogens. Of prime importance is its ability to resist colonisation by pathogens (i.e. colonisation resistance), especially in young chicks (Chambers & Gong, 2011). Some practices in commercial poultry production inhibit the normal development of the microflora, leaving chickens highly vulnerable to colonisation and possible infection by pathogenic bacteria, including *Salmonella* spp. These pathogenic bacteria enter the digestive tract by ingestion, and become the major component of the intestinal microflora (Chambers & Gong, 2011).

The mature microflora is highly diverse, with a few hundred bacterial species and extremely dense counts in the digesta. The alimentary tract of the healthy chick is thought to be sterile at hatch, but recent evidence suggests that some bacteria may colonise the caecum of the late embryo. Microbial colonisation occurs soon after hatch via ingestion of microorganisms through food, faeces, and also contact with microorganisms occurring on the eggshell (O'Dea *et al*., 2006; Hammons *et al*., 2010). After hatch, it is very important that chicks establish a healthy microbial population in the GIT for protection against undesirable organisms. If chicks fail to establish a healthy gut microflora population, their GIT will be vulnerable to colonisation by pathogens (O'Dea *et al*., 2006; Hammons *et al*., 2010). A healthy gut may be established by administration of a saline suspension of the alimentary tract content of adult chickens containing a mature microflora to newly hatched chicks with a deficient microflora. This action is comparable to microflora

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transfer. The treatment material that most effectively controls *Salmonella* colonisation of chicks is obtained from the caeca or colon of mature birds free from *Salmonella*. Competitive exclusion (CE) develops rapidly and is apparently not affected by breed, sex or immune status of the recipient, and is active against all host non-specific serotypes of *Salmonella enteric* (Kabir, 2009; Chambers & Gong, 2011). The mechanisms of action of CE agents are to prevent or reduce colonisation by pathogens, involving competition for nutrients, occupation of attachment sites on the mucosal surface of the intestine that may include specific interference towards pathogen adhesion or other physiological activities, and production of hydrogen sulphide (H_2S) and bacteriocins by elements of the microflora (Hammons *et al*., 2010; Chambers & Gong, 2011). Commensal bacteria found in the intestinal lumen provide non-inflammatory protection of the mucosal membrane through immune modulation (Chambers & Gong, 2011).

Following hatch, microbial colonisation of the digestive tract evolves very rapidly through ecological succession; noteably a few genera of *Enterobacteriaceae,* species of *Enterococci* and even *Clostridium* can occur throughout the digestive tract (Chambers & Gong, 2011). *Enterococci* are mainly facultative anaerobes, and can tolerate a 6.5% NaCl broth and also a normal broth at pH 9.6 (Tannock, 1997). Studies show that *Staphylococcus* spp., *Escherichia coli*, *Clostridium tertium* (a potential pathogen), *Klebsiella* spp. and *Enterobacter* spp. were present in the embryo caecum (Dahiya *et al.,* 2006; Chambers & Gong, 2011). When diets were fed as either mash or pellets, remarkable differences were observed in microflora populations. Pellet-fed birds showed larger numbers of *Coliform* bacteria and *Enterococci* in the ileum, and a lower number of *Clostridium perfringens* and *Lactobacilli* in the distal end of the digestive tract (caeca and rectum). Diets consisting of meat and bone meal reduced the size of the *L. salivarius* population in the ileum (Hammons *et al*., 2010). Studies by Dahiya *et al*. (2006) indicated a 70% reduction in the frequency of *C. jejuni* shedding and a 27% reduction in jejunal colonisation when day-old chicks were given *Lactobacillus acidphilus* and *Streptococcus faecium* as probiotics. Caecal microflora continues to evolve after 40 days and becomes dominated by obligate non-sporing anaerobic bacteria, including species of *Bacteroides, Fusobacterium, Peptostreptococcus, anaerobic Streptococcus, Eubacterium* and budding bacteria such as *Gemminger* (Chambers & Gong, 2011). Competitive exclusion of microflora can be seen when the intestinal content of healthy adult birds is administered orally to one-day-old chicks, and their sensitivity to infection with *Salmonella* spp. is subsequently reduced (Van der Wielen *et al*., 2000; Andreatti, 2003). In modern production processes of the poultry industry, young birds do not receive their microflora from their mothers as they would under normal conditions in the wild, because commercially reared chickens are hatched in incubators, which are clean and do not usually contain organisms commonly found in the chickens' gut (Kabir, 2009).

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Cultures of fresh faeces and full caecal content are more successful in preventing colonisation by *Salmonella* spp. compared to products that provide a pure culture in reduced amounts (Andreatti *et al*., 2003). Gut microfloral characteristics may also be influenced by microbiological contamination of the shell, and gastric secretion of HCl, starting at 18 days of incubation, can have a profound impact on microflora selection (Kabir, 2009). Facultative anaerobic bacteria are necessary to reduce oxygen levels in the caecum and to produce a favourable environment for the strictly anaerobic bacteria to establish themselves and so reduce caecal colonisation by *Salmonella* spp. (Mead, 2000; Andreatti *et al*., 2003).

Microflora obtained from the bedding of adult broiler chickens stored for 50 days exposed to oxygen provided good protection against *Salmonella* colonisation in the intestine (Andreatti *et al*., 2003). DFMs consisting of both facultative and strict anaerobic bacteria were effective in decreasing infection with *Salmonella* spp. (Andreatti *et al*., 2003). It is also important to note that pure frozen or lyophilized cultures will lose their initial effectiveness after a certain period of time especially when the storage conditions and/or handling of stock cultures were not optimal, compared to fresh caecal material (Andreatti *et al*., 2003). It is beneficial to use aerobic cultures for the preparation of DFMs.

DFM cultures in poultry are used to target enteric bacterial pathogens, as shown through research done by Chambers and Gong (2011) using faeces from healthy birds to control *Salmonella* infection in broiler chickens (Lee *et al.*, 2011). Exposing day-old chicks to old or used poultry litter stimulated humoral and cell-mediated immune responses, mainly due to contact with contaminating enteric pathogens (Lee *et al*., 2011). The expression and development of avian immune cells can therefore be influenced by the quality of litter used during chicken growth (Lee *et al*., 2011). Birds raised on used litter systems showed significant improvement in growth performance and composition of the intestinal microflora (Lee *et al*., 2011). The microbial population of fresh litter-raised chicks was dominated by *Lactobacillus* spp*.,* and the intestinal microflora of chicks raised on used litter systems was predominated by unidentified *Clostridials* (Lee *et al*., 2011) at 7 days of age.

2.4 The role of microflora in the GIT of a young chick

Colonisation resistance is achieved through competitive exclusion and immune modulation of the young chick (Chambers & Gong, 2011). The microflora also stimulate development of the digestive tract to in turn influence functions such as the following: nutrient digestion, production of digestive enzymes and digestive tract development, gut mucosal proliferation, vitamin synthesis and utilisation, and utilisation of fermentation and endogenous products (Lan *et al*., 2005; Gabriel

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et al., 2006; Chambers & Gong, 2011). Manipulation of the intestinal microflora has become a strategy of importance for the avoidance of intestinal infection and promotion of host health and performance in chicken production (Chambers & Gong, 2011). Microflora in the gut produce short-chain fatty acids via fermentation, and stimulate digestive system development by increasing the size and amount of gut tissue. Acetate, propionate and butyrate are major short-chain fatty acids produced in the caecal digesta (Chambers & Gong, 2011). These acids accelerate gut epithelial cell proliferation and so increase intestinal tissue weight, and through these acids the microflora also represses the expression of pathogenic virulence genes of *Salmonella*. Diet composition and microflora (as well as their interaction) affect the intestinal development, mucosal architecture and mucus composition of the lower digestive tract, and also enhance both fractional and absolute rates of protein synthesis in the intestines (Chambers & Gong, 2011). Indigenous bacterial populations are established in a complex relationship within the host environment, including the mucosa and luminal contents of the digestive tract, so forming the microflora (Chambers & Gong, 2011).

2.5 The cost associated with microflora in the gut of broilers

Despite their many benefits, microbes can impose a variety of costs on the animal in addition to the immunological disadvantages mentioned above. These costs include competition for nutrients, involving both energy and amino acids, with a consequent reduction in host nitrogen utilisation (Dibner & Richards, 2005; Amerah *et al*., 2013;) when these amino acids are incorporated into bacterial protein. In addition, certain bacteria can ferment amino acids, producing toxic catabolites that can have an impact on the intestinal cell turnover and growth performance of the animal (Van der Wielen *et al*., 2000; Dibner & Richards, 2005; Chambers & Gong, 2011). These catabolites, examples of which include a variety of amines, phenols and indoles, have a negative impact on animal health and performance (Dibner & Richards, 2005). Amino acid deamination and urea hydrolysis will result in the production of ammonia (Dibner & Richards, 2005). Excess ammonia depresses growth, partly due to an increase in the gut epithelial cell turnover under high ammonia conditions (Dibner & Richards, 2005; Bai *et al*., 2007; Amerah *et al*., 2013; Salin *et al*., 2013). Decarboxylation of amino acids will produce toxic amines (Dibner & Richards, 2005). Several different bacterial species mediate these reactions, including *Bacteriodes, Clostridium, Enterobacterium, Lactobacillus* and *Streptococcus*. The resulting products include histamine, cadaverine and many others (Dibner & Richards, 2005). The breakdown of aromatic amino acids, which is mediated by *Bacteroides, Lactobacillus, Clostridium* and *Bifidobacterium* leads to the production of phenols and indoles. Growth performance and flavour characteristics of

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the meat can be negatively affected by these compounds (Dibner & Richards, 2005; Russel *et al*., 2011).

Microflora in the gut also reduce fat digestibility. For proper fat digestion and absorption, bile acids and their salts are required. A variety of bacterial species, but primarily *Lactobacillus*, will catabolise the bile acids and their salts entering the gut (Jin *et al*., 1998; Salin *et al*., 2013). Lipid absorption will be reduced due to this catabolism, and growth performance will also be inhibited, due to the production of toxic degradation products (Dibner & Richards, 2005). Mucus secretion and gut epithelial cell turnover are increased tremendously due to the resident microflora, because many bacterial species enzymatically digest away the mucus layer, and the host must constantly secrete more (Dibner & Richards, 2005). The major function of the mucus layer is simply to lubricate the GIT, and it serves to prevent the microflora from attaching to and invading the intestinal epithelial cells of the host (Dibner & Richards, 2005; Onderci *et al*., 2008; Salin *et al*., 2013). Commensal microflora increases the energy and amino acids required for cell turnover. This is because the mucus secreting goblet cells and absorptive enterocytes on the intestinal villi have a short lifespan. The fastest rate of renewal of any tissue in the body occurs in the gut epithelium, and this high cell turnover is accompanied by an extremely high rate of metabolism and protein synthesis, accounting for 23 to 36% of the body's total energy expenditure (Dibner & Richards, 2005). An hypothesis of Miles *et al.* (2006), states that gut microflora reduces nutrient absorption by increasing GIT thickness and digesta passage rate; they also enhance nutrient requirements of the host by increasing turnover of the gut mucosa and by competing with the host for a portion of the dietary protein and energy (Miles *et al*., 2006).

2.6 Factors affecting the intestinal microflora

The factors with the most significant effect on intestinal microflora are diet (including antibiotics), age and major stresses (Chambers & Gong, 2011). In young birds, the number of microorganisms comprising the microflora, organism diversity, and their specificity to digestive tract segments, increase rapidly with age (Chambers & Gong, 2011). Dietary ingredients are also nutrients for bacterial growth; therefore, the intestinal microflora is a reflection of the diet itself (Chambers & Gong, 2011). Diet composition and host microflora**,** and their interaction can affect the mucosal architecture as well as the mucus composition of the intestinal tract (Chambers & Gong, 2011). If the direct-fed microbials (DFM) in poultry in combination with diet are not compatible with the eco-physiology of the microbes, then the functionality of the preparations will be affected. Therefore, the efficiency of "competitive exclusion" cultures, including DFMs, could

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be enhanced by using a compatible diet, and formulators need to take the feed composition into consideration when developing such products (Hammons *et al*., 2010).

Dietary antibiotics will also influence the digestive microflora (Chambers & Gong, 2011). A common practice for promoting growth and preventing disease includes the use of sub-therapeutic doses of antibiotics in a broiler diet. However, such an approach reduces both the stability of the microflora and also the *Lactobacillus* population in the intestines. The dietary antibiotic effects on the microflora composition are dose and age dependent (Chambers & Gong, 2011).

Major stressors, for example, starvation, have a serious effect on the microflora, manifesting in a higher incidence of *Salmonella* in layer hens when they are forced to moult through feed withdrawal. Housing density, as well as thermal extremes give rise to stressors that increase adverse bacteria at the expense of the beneficial bacteria (Chambers & Gong, 2011).

It is important to understand the microflora-host relationship, because while the concept of intestinal microflora enhancing resistance to infection is well-known, resistance will reduce when the intestinal microflora is disturbed (Patterson & Burkholder, 2003). Factors like stress have detrimental effects on the immune system and intestinal epithelium, and the neuro-endocrine system is closely involved in the response of immune and epithelial systems to stress (Patterson & Burkholder, 2003). Extensive rearrangement of epithelial cells upon colonisation by pathogens is a result of cross-talk between pathogens and epithelial tissues (Patterson & Burkholder, 2003). Cross-talk among bacteroides and the epithelium results in epithelial secretion of specific glycans, which are utilised by the bacterium. Other intestinal bacteria, including probiotic bacteria, may interact with the epithelium in a related manner to improve the ability of these microorganisms to colonise the mucosal lining (Patterson & Burkholder, 2003). The conditions creating the balance and disturbing the populations of microflora in the gut are not clear; however, *Lactobacilli* and *Bifidobacterial* classes seem to be sensitive to stress, as these populations tend to reduce when a bird is under stress (Patterson & Burkholder, 2003; Gaggia *et al*., 2010).

2.7 Establishment of sufficient DFM cultures

Direct-fed microbials (DFMs), also known as probiotics, are live microorganisms providing health benefits to their host when administered in adequate amounts by means of improvement in intestinal balance (Dahiya *et al.,* 2006; Mountzouris *et al*., 2007; Kabir, 2009; Chambers & Gong, 2011; Lee *et al*., 2011; Chapman *et al*., 2012; Giannenas *et al*., 2012; Sun *et al*., 2012). DFMs can also be termed "normal gut flora" or defined as products composed of a single or small number of characterised bacterial strains. It is a preparation of live obligate and facultative anaerobic bacteria,

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originating from normal, healthy adult individuals of an avian species, which is free from specific pathogenic microorganisms and which is quality controlled.

DFM intake should give rise to the formation of gut micro-ecology conditions that overpower harmful microorganisms and favour beneficial bacterial micro-organisms, ultimately improving gut health (Mountzouris *et al*., 2007). It is possible to control and even eliminate intestinal pathogens such as *Salmonella* spp. via CE through oral administration of intestinal microflora from "healthy" adult broilers or cultures of such material to immature chicks raised without the presence of a mother hen (Mountzouris *et al*., 2007; Gaggia *et al*., 2010). The impact of the intestinal microflora on intestinal function and disease resistance can be demonstrated successfully by the competitive exclusion approach of inoculating one-day-old chicks with adult microflora (Patterson & Burkholder, 2003). The competitive exclusion approach instantly provides the chick with an adult intestinal microflora, instead of adding one or a few bacterial species to an established population (Patterson & Burkholder, 2003). This approach can also be used to determine the modes of action and efficacy of the microorganisms employed. Because the one-day-old chicks are highly susceptible to infection, this practice is of considerable commercial importance. The inclusion of DFMs in the diet has been found to improve growth performance and FCR in broilers; therefore, DFM microorganisms are sound alternatives to antibiotic growth promoters (Patterson & Burkholder, 2003; Mountzouris *et al*., 2007; Kabir, 2009; Giannenas *et al*., 2012).

The process of selecting DFMs as bio control agents in the poultry industry involves the screening of poultry and the ability to isolate microbial strains for *in vitro* assays and for pre-selection of probiotic strains. Each DFM culture can be evaluated on its ability to produce inhibitory compounds, resistance factors and adherence factors, and it should also be able to colonise inside the host of interest, allowing scientists to undertake histopathology of the gut. Birds can then be challenged experimentally with pathogenic strains using the DFM culture to evaluate its effectiveness and economic value (Kabir, 2009). Many *in vitro* assays have been developed for the pre-selection of DFM strains, and their competitiveness has been evaluated using *in vivo* studies to monitor their persistence in chickens (Kabir, 2009). Bifidobacteria are used in DFMs globally in many food products like yoghurt, milk, infant formula, cheese and dietary supplements. For a DFM to influence the host indigenous macrobiotics beneficially, the DFM should be able to survive passage through the GIT (Russel *et al*., 2011). Viable cells of this organism should be able to survive and colonise the GIT so as to maintain population sizes of sufficient magnitude to sustain their beneficial effects on the host without regular replenishment.

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2.7.1 The effect of DFM supplementation on broiler performance

Studies by Bai *et al*. (2013) investigated the effects of a DFM (*Lactobacillus fermentum* and *Saccharomyces cerevisiae*) on the growth performance and intestinal immune status in broiler chickens. Supplementing with lactic acid bacteria improved broiler performance in the starter phase, and feeding *Saccharomyces cerevisiae* products improved growth performance after the age of 21 days. Three treatments were compared with each other; a basal diet supplemented with an antibiotic or probiotic, and a basal diet (BD) as control. Compared to the BD, dietary supplementation of either antibiotics or probiotics improved the body weight of 21-day-old chicks. In addition, average daily gain (ADG) and average daily feed intake (ADFI) increased where the feed to gain ratio during the starter period up to 21 days was decreased. Body weight at 42 days, ADG, ADFI, and feed to gain ratio during the grower phase (22 to 42 days) and overall period were not significantly affected by dietary treatments compared to the BD (Bai *et al*., 2013). Feeding a DFM supplemented diet, therefore, improved the growth performance in the starter phase but no dose response to increasing DFM inclusion levels was noted.

Research by Salim *et al*. (2013) also indicated that feeding a DFM resulted in improved growth performance in broilers and increased egg production in laying hens. Starter broilers that received a probiotic supplement had an improved body weight (BW) and also feed to gain ratio that was most likely due to increased feed consumption and improved nutrient digestibility. Bioavailability of both calcium and phosphorus was increased by supplementing with a yeast culture, and the digestibility of dry matter, energy, calcium, phosphorus, crude protein, and some amino acids were also increased by dietary supplementation with a mixture of yeast and other microbes during the starter period (Bai *et al*., 2013). *Bacillus subtilis*-based DFMs improved FCR in poultry, and decrease colonisation of avian pathogenic *E. coli* and *C. perfringens* type A in the GIT (Lee *at al*., 2010).

Higher inclusion levels of a DFM in the diet do not always result in improved animal performance (Bai *et al*., 2013; Zhang & Kim, 2014). The supplementation of single *L.acidophilus I 26* strain or mixture of 12 *Lactobacillus* cultures (2 strains of *L. acidophilus*, 3 strains of *L. fermentum*, 1 strain of *L.crispatus* and 6 strains of *L. brevis*) to a basal diet improved the feed to gain ratio, feed efficiency and BW of the broilers from 0 to 6 weeks, and decreased the number of coliforms in the caecum 10 and 20 days after feeding. It also increased the total volatile fatty acid production in the ileum and caecum, and lowered the caecal pH. The *Lactobacilli* population in the ileum and caecum did not increase significantly (except for 30 days after feeding) (Jin *et al*., 1998). Lactic acid bacteria given in the diet beneficially affected performance of broilers (Huyghebaert *et al*., 2011).

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Lactobacillus may fail to colonise or survive in the GIT, and so fail to enhance production in chickens due to the inability to antagonise or competitively exclude the pathogenic bacteria (Jin *et al*., 1998). Jin *et al*. (1998) stated that the efficacy of DFMs is related to two main factors, namely, the correct amount of living bacteria used and stress on the birds; also important is the ability of the *Lactobacillus* cultures to attach strongly to the intestine, antagonism towards pathogenic bacteria and competitive exclusion of some pathogenic bacteria. Broilers improved in their performance when *Lactobacillus* cultures were added to the diet, since they were reared in a hot (average temperature of 30.1 ºC) and humid climate, which is stressful for the birds.

2.7.2 Characteristics required by commercial DFM cultures

DFM cultures used in practice must have characteristics that exert a positive effect on animal performance before they can qualify as an ideal DFM. Ideal DFMs should possess the following characteristics:

1) They should be of host origin (isolated from gastrointestinal content, mouth and/or faeces) (Patterson & Burkholder, 2003);
2) they should be non-pathogenic and have non-toxic effects or substrates (Patterson & Burkholder, 2003; Gaggia *et al*., 2010);
3) accurate taxonomic identification (Gaggia *et al*., 2010);
4) they should be a standard inhabitant of the targeted species (Kabir, 2009; Gaggia *et al*., 2010);
5) they must have the ability to colonise the intestine and adhere to epithelium or mucus (Patterson & Burkholder, 2003; Kabir, 2009; Gaggia *et al*., 2010; Chambers & Gong, 2011);
6) they should be able to survive in the gut of the chicken; therefore it must resist a low pH in the stomach, resist the presence of bile acids as well as gastric acids in the intestines, survive competition in the GIT for binding sites and nutrients (Patterson & Burkholder, 2003; Kabir, 2009; Gaggia *et al*., 2010), have low nutrient requirements and high growth rate and be able to compete with the resident microflora (Chambers & Gong, 2011);
7) they must suppress enteric pathogens through either cells or metabolites (Chambers & Gong, 2011), produce antimicrobial substances (Gaggia *et al*., 2010), modulate the immune response, and alter microbial activities (Patterson & Burkholder, 2003; Gaggia *et al*., 2010), and produce inhibitory compounds (Patterson & Burkholder, 2003);
8) they should be able to antagonise pathogenic bacteria (Gaggia *et al*., 2010);

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9) they should be grown easily on a large scale under commercial conditions (Patterson & Burkholder, 2003; Chambers & Gong, 2011);
10) they must exhibit at least one scientifically-supported health-promoting property (Gaggia *et al*., 2010);
11) they must have a stable activity and survive in feed through the manufacturing and storage processes (Patterson & Burkholder, 2003; Gaggia *et al*., 2010; Chambers & Gong, 2011),
12) they must be active and stable at high populations (Gaggia *et al*., 2010), and have desirable organoleptic and technological properties when included in industrial processes (Gaggia *et al*., 2010).

DFM preparations can be provided orally to newly hatched chicks, promoting the rapid establishment of an adult-type intestinal microflora, and thereby producing almost immediate resistance to colonisation by pathogens that gain access to the rearing environment (Dahiya *et al.,* 2006; Kabir, 2009*)*. It is important to recognise that the commercially produced probiotic may be species-specific, and that one needs to take into consideration the origin of the bacterial strains used in the probiotic product (O'Dea *et al*., 2006). Some of the strains are host-specific and will limit or improve the ability to colonise and attach to the GIT epithelial cells. This will affect the choice of composition of the probiotic to be used (O'Dea *et al*., 2006). The main DFM properties that should be analysed to assess functionality and safety for use in animal feeding, and which can be improved by preliminary *in vitro* screening are the following: antimicrobial activity, adhesion properties, and the ability to survive in the GIT (Gaggia *et al*., 2010).

2.7.3 Microbial species used as DFMs

A variety of microbial species have been used as DFMs in broiler nutrition, and the following species exert beneficial effects on broiler performance:

1) *Lactobacillus* spp.(Patterson & Burkholder, 2003; Mountzouris *et al*., 2007; Kabir, 2009; Gaggia *et al*., 2010; Lee *et al*., 2010; Chambers & Gong, 2011; Salim *et al*., 2013; Waititu *et al*., 2014), *Lactobacillus bulgaricus* (Kabir, 2009)*, Lactobacillus acidophilus* (Kabir, 2009; Gaggia *et al*., 2010)*, Lactobacillus casei* (Kabir, 2009; Gaggia *et al*., 2010)*, Lactobacillus helveticus* (Kabir, 2009)*, Lactobacillus lactis* (Kabir, 2009)*, Lactobacillus salivarius* (Kabir, 2009; Gaggia *et al*., 2010)*, Lactobacillus plantarum* (Kabir, 2009; Gaggia *et al*., 2010),
2) *Streptococcus* spp. (Patterson & Burkholder, 2003; Mountzouris *et al*., 2007; Kabir, 2009; Gaggia *et al*., 2010; Lee *et al*., 2010; Salim *et al*., 2013; Waititu *et al*., 2014), *Streptococcus thermophilus* (Kabir, 2009),

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3) *Bacillus* spp. (Patterson & Burkholder, 2003; Mountzouris *et al*., 2007; Kabir, 2009; Gaggia *et al*., 2010; Lee *et al*., 2010; Salim *et al*., 2013; Waititu *et al*., 2014),
4) *Bifidobacterium* (Patterson & Burkholder, 2003; Mountzouris *et al*., 2007; Kabir, 2009; Gaggia *et al*., 2010; Lee *et al*., 2010; Salim *et al*., 2013; Waititu *et al*., 2014),
5) *Enterococcus* spp. (Patterson & Burkholder, 2003; Mountzouris *et al*., 2007; Kabir, 2009; Gaggia *et al*., 2010; Lee *et al*., 2010; Salim *et al*., 2013; Waititu *et al*., 2014), *Enterococcus faecium* (Kabir, 2009; Gaggia *et al*., 2010) and *Enterococcus faecalis* (Kabir, 2009; Gaggia *et al*., 2010),
6) *Aspergyllus* (Mountzouris *et al*., 2007; Kabir, 2009; Gaggia *et al*., 2010),
7) *Candida* (Mountzouris *et al*., 2007; Kabir, 2009),
8) *Escherichia coli* (Patterson & Burkholder, 2003; Kabir, 2009; Lee *et al*., 2010),
9) Lactococcus (Lee *et al*., 2010; Salim *et al*., 2013),
10) *Saccharomyces* (Mountzouris *et al*., 2007; Kabir, 2009; Gaggia *et al*., 2010; Salim *et al*., 2013; Waititu *et al*., 2014),
11) *Leuconostoc, Pediococcus, Propionibacterium* and *Kluyveromyces*, a variety of yeast species (Patterson & Burkholder, 2003), and undefined mixed cultures (Patterson & Burkholder, 2003).

2.7.4 Functions of a DFM in the gut of broiler birds

The major functions and beneficial effects of a DFM on the host include the following:

- stimulating the development of (modifying) a healthy microflora where beneficial bacteria dominates (Patterson & Burkholder, 2003; Kabir, 2009; Gaggia *et al*., 2010; Chambers and Gong, 2011; Amerah *et al*., 2013; Waititu *et al*., 2014)
- reducing/prevention of enteric pathogen colonisation through competitive exclusion (Patterson & Burkholder, 2003; Dahiya *et al*., 2006; Mountzouris *et al*., 2007; Kabir, 2009; Gaggia *et al*., 2010; Lee *et al*., 2010; Chambers & Gong, 2011; Giannenas *et al*., 2012; Salim *et al*., 2013; Tabidi *et al*., 2013)
- improving mucosal immunity via increased antibody production (Lee *et al*., 2010; Chambers & Gong, 2011)
- improving cell-mediated immunity (Lee *et al*., 2010), promotion of epithelial barrier integrity (Kabir, 2009; Gaggia *et al*., 2010; Lee *et al*., 2010; Chambers & Gong, 2011; Salim *et al*., 2013; Waititu *et al*., 2014), reducing epithelial cell apoptosis (Lee *et al*., 2010; Salim *et al*., 2013)
- enhancing dendritic T-cell interaction/ hypo-responsiveness (Lee *et al*., 2010)

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- augmenting toll-like receptor signalling (Lee *et al*., 2010)
- enhancing T-cell homing to mesenteric lymph nodes (Lee *et al*., 2010)
- stimulating the intraepithelial lymphocytes (Salim *et al*., 2013)
- stimulating the immune system associated with the gut (Patterson & Burkholder, 2003; Dahiya *et al*., 2006; Kabir, 2009; Gaggia *et al*., 2010; Giannenas *et al*., 2012; Salim *et al*., 2013; Amerah *et al*., 2013; Tabidi *et al*., 2013; Waititu *et al*., 2014)
- increasing digestive capacity by increased digestive enzyme activity and decreased bacterial enzyme activity (Dahiya *et al*., 2006; Kabir, 2009; Gaggia *et al*., 2010; Chambers & Gong, 2011; Giannenas *et al*., 2012; Waititu *et al*., 2014)
- decreasing ammonia production and urea excretion (Patterson & Burkholder, 2003; Dahiya *et al*., 2006; Kabir, 2009; Giannenas *et al*., 2012)
- reducing pH via acid production (Chambers & Gong, 2011; Salim *et al*., 2013)
- improving feed intake and digestion (Dahiya *et al*., 2006; Kabir, 2009; Gaggia *et al*., 2010; Giannenas *et al*., 2012)
- producing toxic conditions for pathogens and producing compounds such as bacteroicins, and low pH conditions) (Patterson & Burkholder, 2003; Gaggia *et al*., 2010; Salim *et al*., 2013)
- neutralizing enterotoxins (Dahiya *et al*., 2006; Giannenas *et al*., 2012)
- reducing inflammatory reactions (Patterson & Burkholder, 2003)
- enhancing animal performance (Patterson & Burkholder, 2003; Waititu *et al*., 2014)
- reducing carcass contamination (Patterson & Burkholder, 2003)
- and competing for mucosal attachment and nutrients (Patterson & Burkholder, 2003;Gaggia *et al*., 2010; Amerah *et al*., 2013; Salim *et al*., 2013).

Mechanisms by which DFMs improve feed conversion efficiency include modification of intestinal flora (Kabir, 2009), enhancement of growth of nonpathogenic facultative anaerobic and gram positive bacteria that produce lactic acid and hydrogen peroxide (Kabir, 2009), suppression of growth of intestinal pathogens (Kabir, 2009), and improvement of digestion and utilisation of nutrients (Kabir, 2009). The major outcomes when using DFMs in feed include improvement in growth, reduction in mortality, and improvement in feed conversion efficiency (Kabir, 2009). In contrast to these findings, Amerah *et al*. (2013) reported no effect on intestinal morphology with probiotic supplementation, but broiler performance improved and probiotics could be used as an alternative to antibiotic growth promoters.

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Most of these mechanisms are not mutually exclusive, and some microorganisms may affect change with a single mechanism, although others may use several mechanisms (Patterson & Burkholder, 2003). DFMs used for their immune stimulation and antimicrobial activity are *Bacillus* spp. Linear improvements in growth performance, apparent nutrient retention, villus height, and villus height to crypt depth ratio in the duodenum and ileum, and reduced caecal *Clostridium* and *Coliform* count, were all observed in broilers fed diets supplemented with increasing levels of *Bacillus subtilis* (Waititu *et al*., 2014). Other evidence supports an improved weight gain when probiotics are fed through the diet (Kabir, 2009). In contrast to these findings, some research shows no influence on feed intake and weight gain through dietary treatments, but probiotic supplementation significantly improved 35 day FCR compared to diets containing antibiotics (Amerah *et al*., 2013). Results obtained by Amerah *et al*. (2013), also indicated that the number of mucosa-associated avian pathogenic *E.coli* (APEC) was influenced significantly by dietary treatments. Lee *et al*. (2010) also reported that strains of *Bacillus subtilis* exert an inhibitory effect on avian pathogenic *Escherichia coli* or *Clostridium perfringens*. Another preferred probiotic used for the production of propionic acid, bacteriocins, vitamin B_{12}, growth stimulation of other beneficial bacteria, and ability to endure harsh gastric digestion is *Propionic bacterium* spp. This DFM also showed improved growth performance and immune response benefiting the host animal (Waititu *et al*., 2014; Dibner & Richards, 2005). The exact mechanisms that mediate the immune-modulatory activities of probiotics are not clear. DFMs stimulate the immune system cells to produce cytokines, which in turn play a role in the induction and regulation of the immune response (Kabir, 2009). The mucus layer segregates both normal and pathogenic microbes away from the animal tissue, while a barrier to entry into the animal tissue is provided by the epithelium, where antibodies provide a network of immune cells, cytotoxic and helper T cells, as well as phagocytic cells (Dibner & Richards, 2005; Lee *et al*., 2010; Bai *et al*., 2013). Pathogenic bacteria that produce toxins as well as an overgrowth, or which promote inappropriate attachment by normal microflora, will be inhibited by these immune cells (Dibner & Richards, 2005). Studies have shown that antibody diversity development in poultry is inhibited by germ-free growth conditions (Dibner & Richards, 2005). The intestinal immune system develops in parallel with the development of normal microflora, and the introduction of even a single species of commensal bacteria into germ-free animals can stimulate the development of the secretory IgA system (Dibner & Richards, 2005).

Lactobacillus has the ability to modulate the systemic antibody response to antigens in chickens (Kabir, 2009). Birds supplemented with *Lactobacillus* had significantly higher numbers of both *Lactobacillus* and *Bifidobacterium* in the ileum and caecum, when compared to birds supplemented with *Enterococcus* spp., and *Bacillus subtilis* (Giannenas *et al*., 2012).

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Competitive exclusion of pathogens may be accomplished by mechanisms like competition for mucosal binding sites and luminal nutrients, or production of inhibitory substances such as volatile fatty acids, low pH and bacteriocins, which are bacteriostatic or bacteriocidal for pathogenic bacteria (Dahiya *et al.,* 2006). Conventionally grown animals are far less susceptible to colonisation by pathogens than germ-free animals (Dibner & Richards, 2005). There is an antagonistic effect between *Lactobacilli* and entero-bacteria, and it was demonstrated that *Lactobacillus* reduced the severity of clinical disease in *E. tenella* infection (Tierney *et al*., 2004).

Normal microflora have a nutritional value, secreting nutrients that become available for use by the host (Dibner & Richards, 2005). Nutrients secreted by these microbes include short-chain fatty acids, amino acids and vitamins B and K. The fatty acids produced by commensal bacteria in broiler chickens are lactate, propionate and butyrate, all of which contribute significantly to the animal's energy requirements (Dibner & Richards, 2005). It should also be noted that the un-dissociated forms of short-chain fatty acids play important roles in decreasing the numbers of "unwanted" bacterial species in the caecum, as well as increasing the absorptive surface area by stimulating the gut epithelial cell proliferation and villus size (Dibner & Richards, 2005).

2.7.5 Factors affecting the level of DFM application in broiler diets

A direct comparison of studies using different DFMs is difficult, because the efficacy of a DFM application depends on a variety of factors (Mountzouris *et al*., 2007). These factors include species composition, dietary administration level, product viability, application method (e.g., water, feed or spraying), frequency of application (e.g., once, intermittent or continuous), age of the birds, overall diet, general farm hygiene and environmental stress factors (e.g., temperature, stocking density). An optimal intake level of DFMs does not exist, even though it is generally accepted that efficacy for most probiotic microorganisms is demonstrated with daily consumption of 10^8 to 10^9 organisms per day in animals (Mountzouris *et al*., 2007). Studies by Mountzouris *et al*. (2007) indicated that, based on the bacterial composition of weekly feed samples and respective FI, an average daily intake level of 2.5 x 10^8 *cfu* of DFM bacteria per bird, provided in the water or in the feed was necessary for efficacy. Higher body weights were also seen in broilers where the DFM was included in both the feed and water compared to only in the feed. Therefore, each bird exposed to DFMs in the feed and water had an additional daily intake of 10^8 *cfu* DFM bacteria. It is not a straight forward matter to optimize the probiotic administration level, and more research is required for multi-strain products in particular, because, apart from the factors affecting efficiency, the optimal concentration for administering DFMs depends on broiler age and DFM strain (Mountzouris *et al*., 2007). Research by O'Dea *et al*. (2006) indicated that the application of a

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DFM in the feed resulted in lower mortalities than when it was placed in the water. In contrast to previous findings, DFM used in this study did not protect the GIT from harmful pathogens like *Clostridium perfringens*. Past research supported the fact that a simple DFM culture or undefined culture consisting of adult caecal material is effectively able to prevent colonisation of the GIT by harmful pathogens.

Further studies conducted by Mountzouris *et al*. (2007) on the caecal microflora composition, indicated that a DFM product gave rise to beneficial modulation of the caecal microflora, as shown by a significant increase in the concentration of bacteria belonging to *Bifidobacterium* spp., *Lactobacillus* spp., and gram-positive cocci in the treatments containing DFMs in both feed and water, and feed only, compared to antibiotics in the feed.

2.7.6 The use of single vs. multiple strains of DFM cultures

Evidence has shown that DFM cultures have the ability to inhibit one another when incubated together *in vitro*, although they may be more effective at inhibiting pathogens when tested at more or less the same concentrations of biomass (Chapman *et al*., 2012). Using a mixture of DFMs can be more effective at reducing gastrointestinal infections, and if one can create a mixture of species that exert different effects against different pathogens, it may have a broader mode of action than a single strain (Chapman *et al*., 2012). Through combining DFM bacteria an even greater inhibition of growth of *E. coli* was obtained than through a single *Lactobacillus* strain, also reducing the risk of bacterial infection (Chapman *et al*., 2012). The DFM cultures that showed the greatest inhibition effect were *Lactobacillus*, followed by *Bifidobacterium* (Kabir, 2009). Other species either showed little or no inhibition. Possible reasons are that *Lactobacillus* may produce a greater quantity of antimicrobial substances, and these substances may also have a broader spectrum of activity (Kabir, 2009). *Lactobacillus* may also have a strong ability for competitive exclusion. However, other research has shown that a *Lactobacillus* strain does not have the ability to produce protection against *Salmonella* infections (Kabir, 2009).

Single-strain DFMs may be less effective than multi-strain DFMs, where multi-strain DFMs could amplify the protective spectrum against microbial infections (Zhang & Kim, 2014). The mode of action of a DFM will also depend on the type of pathogen. For instance, *Lactobacillus* as a single strain will be more effective in inhibiting *E. coli* than a mixture of strains. Therefore, a mixture of strains may not always be the most effective way to prevent gastrointestinal infection. Also there is limited evidence to support the theory that a mixing of strains results in synergistic or additive bioactive effects or maybe reduces efficacy due to mutual inhibition by the component

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strains (Chapman *et al*., 2012). It is very important to make sure that when a mixture of probiotic strains is prepared, no single strain should have an inhibitory effect on another probiotic strain.

Chapman *et al*. (2012) and Gaggia *et al*. (2010) reported that combinations of *Lactobacilli* were more effective than single strains in preventing pathogenic growth and decreasing the risk of bacterial infections. Multispecies DFM cultures were formulated using five DFM species isolated from the crop, jejunum, ileum, and caecum of healthy adult chickens. Infected chickens fed a multi-species DFM mix showed a higher growth performance than infected chickens consuming diets with single strains of *Enterococcus faecium*, *Bifidobacterium anamalis*, *Lactobacillus reuteri*, and *Bacillus sutilis* (Giannenas *et al*., 2012).

2.7.7 Immune stimulation by DFMs in the gut

Microflora in the gastro intestinal tract play a very important role in the immune response of chickens, which could lead to colonisation resistance. The health of the chickens is adversely affected if the communities of microflora are compromised (Chambers & Gong, 2011). Higher proportions of CD3+, CD4+ and CD8+ T-lymphocytes are seen in chicks fed DFMs and those fed an antibiotic diet decreased the proportion of CD8+ T-lymphocytes in the foregut at 21 and 42 days. Diets supplemented with yeast products and a *Lactobacillus* based DFM culture increased intestinal immunity in chickens (Bai *et al*., 2013). To reduce the incidence of poultry enteric disorders, the immune response gut-associated lymphoid tissue (GALT) is critical, because GALT is exposed to microflora from associated feed and the environment. The GALT immune system could be stimulated by lactic acid bacteria and yeast products (immune biotic) (Bai *et al*., 2013).

Gram-negative bacteria populations such as *Coliform* will be decreased by the DFMs in the rectum of broilers, which is beneficial (Bai *et al*., 2013). Recent studies showed that a DFM culture of *Lactobacillus fermentum* and *Saccharomyces cerevisiae* stimulates the T-cell immune system in the intestine of broilers and is useful to protect the chicks from disease without sacrificing growth performance in starter broilers (Bai *et al*., 2013). The development of the gut microflora will have a major effect on the development and activation of the humoral and cellular gut-associated immune system (Waititu *et al*., 2014). Homeostasis should be maintained between the immune response to pathogens and tolerance of the fed protein in the gut, and this depends on the relationship between immune cells and the gut parenchyma (Waititu *et al*., 2014).

The microbial communities can stimulate the gastrointestinal immune response to support the animal's defence against invasive pathogens (Waititu *et al*., 2014). *Bacillus subtilis*-based DFMs stimulated different aspects of the host's innate and adaptive immunity; for instance, humoral and cell-mediated immunity in broiler chickens. Serum levels of alpha-1-acid glycoprotein

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and inflammatory marker were reduced, whereas splenic lymphocyte proliferation, intestinal intraepithelial subpopulation numbers as well as cytokine mRNA levels in the intestinal intraepithelial subpopulations were affected, depending on the strain of probiotic used (Lee *et al*., 2010; Lee *et al*., 2011; Abdelqader *et al*., 2013).

2.7.8 VFA production in the GIT

Anaerobic bacteria producing volatile fatty acids (VFA) and non-VFA in the intestine are bacteriostatic for pathogenic bacteria. VFA form the major end products of microbial fermentation and are efficiently absorbed by the colonic mucosa. The VFA profile and concentration was largely affected by the amount and type of fermentable substrates, especially carbohydrates, reaching the large intestine (Mountzouris *et al*., 2007). An *in vivo* digestibility trial indicated no significant differences between treatments for caecal VFA concentration and profile, which could be explained by considering the fact that there was no difference regarding caecal populations of total aerobes and anaerobes among the treatments (similar basal diets) containing probiotics in both feed and water, only in feed, and antibiotics in feed only (Mountzouris *et al*., 2007). VFA, especially acetate, propionate and butyrate play a major role in microflora development in the caeca of broiler chickens during growth and were present in high concentrations (Van der Wielen *et al*., 2000). The total VFA concentration in the ileum and caecum was increased in broilers fed a diet with *L. acidophilus*. A mixture of *Lactobacilli* in the diet also increased the VFA production in the ileum, but not in the caecum of broilers, and decreased the pH values in the caecum, but not in the ileum (Jin *et al*., 1998).

High VFA concentrations indicate that fermentation by obligate anaerobic bacteria is important and the numbers of anaerobic bacteria were found to be 10 to 50 times higher than aerobic bacteria (Van der Wielen *et al*., 2000). The VFA composition (acetic, propionic, iso-butyric, butyric and iso-valeric acids) in the caecum was not affected by the incorporation of any *Lactobacillus* cultures. There was a reduction in coliform bacteria in the intestine due to the increased VFA concentration in the ileum and caecum (Jin *et al*., 1998). When different bacterial mixtures were administered to one-day-old broilers, the increased propionic acid concentrations found at three days of age were negatively correlated with *Salmonella* numbers counted in the caeca of broilers at ten days of age (Van der Wielen *et al*., 2000).

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2.8 Antibiotic growth promoters (AGPs)

After the ban on AGPs in Denmark in the late 20th Century, productivity (kg meat/ m^2) and liveability were not affected, but feed conversion ratio did increase by 0.016kg/ kg from November 1995 to May 1999 (1.78 to 1.796) (Dibner & Richards, 2005). Immediately after the ban on AGPs, feed efficiency increased to heights of 1.83 and 1.84 in late 1999 (Dibner & Richards, 2005). Mortality records have shown that fatalities due to necrotic enteritis did not increase significantly. Farmers began to increase the usage of salinomycin in 1996 from 4,500kg to 11,213kg in 2002 to control necrotic enteritis (Dibner & Richards, 2005). Ionophore antibiotics are still widely used as feed additives to control coccidiosis, even though these substances are also considered to have growth-promoting effects (Engberg *et al*., 2000).

2.8.1 The effects of AGPs on animal growth performance

Dietary antibiotics promote growth and efficiency of poultry as well as other animals (Miles *et al*., 2006). Weight gain is enhanced, but feed conversion ratio is increased (Miles *et al*., 2006). Some of the antibiotics fed will not be absorbed and, therefore, the mechanisms of action are centered in the gut (Miles *et al*., 2006). Not all antibiotics control growth and proliferation by the same mechanism of action, and they differ with regard to their ability to influence certain disease states or improve growth and feed efficiency (Miles *et al*., 2006). Research is lacking on the effects of dietary antibiotics on physical changes to the GIT, because the focus of studies during the past few years has been on the effects of easily cultured bacterial populations such as *Lactobacilli* and *Clostridium perfringes* on poultry health (Miles *et al*., 2006).

AGPs have direct effects on the microflora in germ-free animals. These effects can be used to explain a decreased competition for nutrients and reduction in microbial metabolites that depress growth. Gut size may be reduced due to a loss of mucosa cell proliferation in the absence of luminal short chain fatty acids derived from microbial fermentation due to thinner intestinal villi and overall gut wall. The enhanced nutrient digestibility observed using AGPs is linked to the reduction in gut wall and villus lamina propria (Miles *et al*., 2006). AGPs also reduce opportunistic pathogens and subclinical infections in the birds. Results obtained from Engberg (2000) showed a significant growth-promoting effect on birds receiving a combination of zinc bacitracin and salinomycin in their diet. *Coliform* bacteria in the ileum were significantly reduced by zinc bacitracin in the diet, although the activities of both amylase and lipase increased in the pancreas homogenates (Engberg *et al*., 2000). *C. perfringens* along with *Lactobacillus salivarius* decreased significantly when the diet was supplemented with salinomycin and zinc bacitracin, alone or in combination, even though

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it was the dominant lactic acid producing bacterium found in broiler intestinal contents (Engberg *et al*., 2000). Broiler growth can be depressed due to high numbers of *Lactobacilli* related to competition in nutrient uptake or impaired fat absorption due to bile acid de-conjugation (Engberg *et al*., 2000). Indigenous bacteria that are able to catalyze bile acid deconjugation are *Lactobacilli*, *Enterococci*, *Bifidobacteria*, *Clostridium* and *Bacteroides* (Engberg *et al*., 2000). Indigenous bacteria that can cause growth depression in chickens are *Streptococcus faecium*, and *C. perfringens* (Stutz *et al*., 1983; Engberg *et al*., 2000).

It is important to include growth promoting antibiotics as a Positive Control treatment in DFM studies, because antibiotics are more effective when the animal is producing below its genetic potential and thus not always cause statistically significant improvements in performance. It is also important to include growth promoting antibiotics as a Positive Control treatment in DFM studies because stress status is important in detecting growth performance responses (Patterson & Burkholder, 2003). If there is no response to the growth promoting antibiotic, it should not be considered negative for the DFM treatment.

Studies by Miles *et al*. (2006) indicate that feeding an AGP (virginiamycin or bacitracin methylene disalicylate) in the diet resulted in an increase in the number of villi per unit length in the duodenum of birds given virginiamycin and not bacitracin methylene disalicylate. Both antibiotics increased body weight and decreased intestinal length and weight. Birds fed virginiamycin and bacitracin methylene disalicylate at one to three weeks of age showed greater decreases in intestinal length and weight compared to five and seven weeks of age (Miles *et al*., 2006). The muscularis mucosa was thinner in birds given virginiamycin compared to a corn-soya bean meal based diet (Miles *et al*., 2006). Virginiamycin outperformed both the control and the bacitracin methylene disalicylate diet, showing a smaller total villus area and shorter villus height and crypt depth in the ileum. Both antibiotics increased the performance of the birds (Miles *et al*., 2006).

2.9 Alternatives to antibiotics to manipulate the intestinal microflora

Fully understanding the effect of antibiotics on microflora changes and related functions is highly relevant for poultry production, and can provide a clear guide to the development of viable alternatives to dietary antibiotics (Chambers & Gong, 2011). With respect to animal production, an important goal is to determine the optimal microflora for the animal to maximise the benefits and minimise the cost, and then to be able to manipulate the microfora through diet, supplements and other means to obtain the desired microflora population (Dibner & Richards, 2005). Many products exist in the market, including antibiotics, organic acids, prebiotics, DFMs, trace minerals, spices, herbs and enzymes, which are sold with the aim of altering the microflora for the benefit of animal

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health and production. More research is required to identify the optimum microflora, and also to develop quantitative methods (Dibner & Richards, 2005). The use of antibiotics is the most commonly used dietary intervention to modulate the gut microflora but is prohibited by lawin several countries. However, research has shown that antibiotics do not promote growth of germ-free animals (Dibner & Richards, 2005). An alternative to AGPs would have to provide an improvement in feed efficiency that is economically viable. If the alternative product lacks antimicrobial properties, the incidence of, for instance, enteric diseases and airsacculitis should be controlled by using ionophores (Dibner & Richards, 2005).

2.9.1 The effects of enzyme supplementation with a DFM

Onderci *et al*. (2008) evaluated the efficiency of *Escherichia coli* DH5-α strain, with β-glucanase produced from *Streptococcus bovis* on growth performance, diet utilisation and gut morphology of broiler chickens. Barley is high in non-starch polysaccharides (NSP), and replacing maize with barley leads to a reduction in growth despite having equal amounts of protein and energy provided. Disadvantages of barley and wheat are that water-soluble non-starch polysaccharides (NSP), which increase the viscosity of digesta in the GIT, also limit contact between enzymes and substrates. The resulting excreta is sticky, the unstirred water layer in the mucosa is thicker, and consequently the growth performance and nutrient absorption of the birds are both depressed (Onderci *et al*., 2008).

Chickens that consumed a barley-based diet and a bacteria culture producing β-glucanase provided via water had a lower feed conversion ratio, consumed more feed and had a higher growth rate than those birds eating the barley diet only. Therefore supplementing β-glucanase was beneficial to the host by causing a reduction in the negative effects of barley β-glucan. Giving *E. coli* with the diet via water improved the coefficient of the total tracts' apparent digestibility of dry matter, organic matter, crude protein and ether extract. Therefore, by including an *E. coli* strain with β-glucanase in the broiler diets, the nutritive value of barley was improved by altering the digestibility and intestinal morphology.

The morphology of the GITs of chickens was improved by the colonisation of the bacteria and increased β-glucanase activity in the intestines, and this may explain the improved feed conversion observed (Onderci *et al*., 2008). The inclusion of α-glucanase in the ration increased the villus size, height ratio, length and width that were associated with an increase in absorption capacity and improved nutrient digestibility (Onderci *et al*., 2008). Similar findings were made by Baurhoo *et al*. (2007) where the villi height in jejunum and duodenum increased when supplemented with a prebiotic (purified lignin and mannan oligosaccharides). Significant changes resulted from DFM

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treatments regarding α-and β-galactosidase microbial activities. Bacterial glycolytic enzymes are responsible for the fermentation of undigested carbohydrates, animal performance and health. Adding a DFM in broiler diets may result in a significant reduction of β-glucurinidase activity in the intestine and faeces, and β-glucosidase in the intestine (Mountzouris *et al*., 2007). The importance of glycolytic enzymes can be summarised as follows:

- α-galactosidase aids the hydrolysis of dietary α-galactosides such as rafinose, stachyose, and other oligosaccharide components of feedstuffs such as soybean meal.
- β-galactosidase aids the hydrolysis of β-galactosides as in the case of some prebiotics and lactose.
- α-glucosidase adds to starch fermentation.
- β-galactosidase adds to the hydrolysis of glucose monomers from non-starch polysaccharides (NSP) (e.g., cellulose, β-glucans).

However, it is possible that β-glucosidase could also be involved in the formation of toxic glycons, depending on the nature of plant glycosides, and β-glucuronidase activity is perceived as harmful for health because it is capable of releasing carcinogens from hepatically derived glucuronic acid conjugates, and is a critical factor in the enterohepatic circulation of drugs and other foreign compounds (Mountzouris *et al*., 2007). *Bifidobacterium* and *Lactobacilli* mainly produce bacterial α- and β-galactosidase enzymes. In studies conducted by Mountzouris *et al*. (2007), increased activity of α- and β-galactosidase was seen in the birds intestine when DFM cultures were provided in both the feed and water, or only in the feed, which can be ascribed to the increased levels of *Bifidobacterium* spp. and *Lactobacillus* spp. compared to a diet containing antibiotics.

2.9.2 Other alternatives to AGPs that works synergistically with DFM cultures

2.9.2.1 Phytogenic compounds

Essential oils (EO) have the potential to enhance broiler gut health and digestive functions. Examples of EO are rosemary and sage extracts, oregano essential oils, thymol and cinnamaldehyde and anise oil. These can also help to protect gut microflora with *Lactobacilli* and *Bifidobacteria* at 42 days old and control against *Clostridium perfringens* and *Necrotic enteritis* (Mountzouris *et al*., 2011). Phytogenic feed additives included in the diet help to maintain a good digestive function irrespective of the inclusion level (Mountzouris *et al*., 2011).

Baurhoo *et al*. (2007) also reported an increased population of beneficial bacteria (*Lactobacilli* and *Bifidobacteria*) in the caeca of broilers with the dietary inclusion of mannan

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oligosaccharides, which also reduced *E. coli* load in broiler litter. The chemical structure of oligosaccharides is such that they can only be utilised by, and stimulate the growth of, a limited number of bacteria. These bacteria include *Bifidobacteria* and *Lactobacilli*, which are considered to be the only microorganisms that utilise oligosaccharides and beneficially affect host health (Qiang *et al*., 2009).

2.9.2.2 Organic acids

Organic acids have been used in poultry production to control *E. coli*, *Campylobacter* and *Salmonella*. Fumaric and sorbic acids have been added to broiler food, while some acids were provided in water (Pirgozliev *et al*., 2008). Among all the candidates to replace AGPs in animal feed, organic acids appear to have had the most widespread acceptance by 2005 (Dibner & Richards, 2005). They have a beneficial effect on the broilers' gut health, reduce endogenous losses and improve availability of dietary energy (Pirgozliev *et al*., 2008). Organic acids decrease the pH of the drinking water (to prevent bacteria colonising the drinking system) and also reduce the buffering capacity of the feed, having a major effect on the crop and proventriculus (Huyghebaert *et al*., 2011). The chickens' crop and caeca had more lactic acid bacteria than *Coliforms*, and lactic acid bacteria can be beneficial to the host by inhibiting growth of pathogenic gram-negative flora like *E. coli* and *Salmonella* (Pirgozliev *et al*., 2008).

In contrast, lactic acid inclusion in water and feed showed little benefit in combatting *Campylobacter* bacteria (Wagenaar *et al*., 2005). Sodium butyrate can be supplemented at lower levels than organic acids; it stimulates growth of the duodenal mucosa, enhancing the growth of *Lactobacilli* in the jejunum (Hu *et al*., 2007; Gao *et al.,* 2008).

2.10 Models to study the impact of gut microflora

DNA profiling techniques can be used to determine the changes of microflora composition in the intestine of chickens in response to different environmental conditions or feeding practices (Chambers & Gong, 2011). A multiplex polymerase chain reaction (POSITIVE CONTROLR) protocol was developed for characterization of the pathogenic *E.coli* (APEC) capable of causing poultry colibacillosis (Skyberg *et al*., 2003). The POSITIVE CONTROLR protocol targets four genes: *iss*, the increased serum survival gene, *tsh*, which encodes a temperature sensitive hemaglutinin, *iucC*, a gene encoding a protein involved in aerobactin production, and *cvi*, the colicin V immunity gene (Skyberg *et al*., 2003). These genes were chosen because of their association with avian *E. coli* virulence and their possible linkage to the same plasmid (Skyberg *et*

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al., 2003). Intestinal microbial populations have been characterised using classical plating techniques (Patterson & Burkholder, 2003).

2.11 Gut microflora quantification

After chickens have been treated with a food product or DFM, the ileum and caecum can be excised and immediately frozen in liquid nitrogen and stored at −80ºC for DNA extraction. The birds' crops can also be cut in half and one half treated as above, with liquid nitrogen, while the other half is sent immediately to a laboratory for bacterial culture analysis (Hammons *et al*., 2010). Different techniques to quantify gut microflora are as follows: quantitative polymerase chain reaction (Q-POSITIVE CONTROLR) analysis, POSITIVE CONTROLR-based DNA profiling techniques, DNA microarray, flow cytometry, insertion sequencing, and particularly next generation DNA sequencing and bioinformatics analyses (Gong *et al*., 2012). The gut microflora analysis is done by polymerase chain reaction-denaturing gradient gel electrophoresis (POSITIVE CONTROLR-DGGE) (Hammons *et al*., 2010).

2.12 Conclusion

DFM cultures that are included in broiler rations are preferred over antibiotics, because they do not have harmful effects on consumers. Microorganisms that are capable of changing the gastrointestinal environment, which is stimulated by bacterial cultures, will favour the birds' health status and improve their feed efficiency. DFM cultures improve the feed conversion efficiency by alterations in intestinal flora, and subsequent growth enhancement of non-pathogenic facultative anaerobic and gram positive bacteria. These form lactic acid and hydrogen peroxide, thereby suppressing growth of intestinal pathogens and enhancing digestion of nutrients. Their main advantages are growth improvement, low feed conversion ratio, and enhanced survival by altering gastrointestinal flora through suppressing growth of pathogenic bacteria (Onderci *et al*., 2008).

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CHAPTER 3

3. MATERIALS AND METHODS

3.1 Housing and birds

Experimental procedures were approved by the Animal Ethics Committee of the University of Pretoria (ECO6-13). The purpose of the study was to evaluate the benefits, if any, of adding a probiotic product containing *Bacillus subtillus* to the diet of broilers. The trial was conducted at the test facilities at Daybreak Farms, Sundra from 25 February 2014 to 1 April 2014. The trial house was an open-sided house with curtains and a coal boiler (HEATCO) attached to a heat sock as heat source. The house contained 64 pens in total, divided into two rows containing 32 pens each over the length of the house. The pens at both ends of each row were not included in this study. The pens were three meters long and one meter wide. Pens were separated from one another with a wire frame approximately 65cm high, allowing birds to interact socially within each pen and between neighbouring pens (through the wire). Each pen was fitted with two tube feeders and six nipple drinkers on a nipple drinker line. There were two drinker lines running through the pens with three to four nipples on each line per pen. The height of the feeders and drinker lines was adjusted according to bird growth.

The 60 pens were covered with used chicken litter (4cm deep) from another Daybreak Farm. The litter used was from a flock that had the worst performance compared to all the houses on the farm. The purpose of using old litter was to challenge the birds. Minimum and maximum temperatures and the Heat-Co reading were recorded on a daily basis and are given in Appendix B. Temperature and humidity loggers were installed in the house at the beginning of the trial to record any temperature changes throughout the trial. The house was pre-heated to 35°C two days before the chicks were placed in the house, to ensure that the temperature at bird level (35°C) and floor temperature (32°C – 35°C) were acceptable. The feed was placed inside the house one day before the chicks arrived to ensure that the feed also reached room temperature of 35°C. The temperature was gradually decreased from 35.5°C on day two to 23°C at 24 days of age and then kept constant till 35 days of age (Appendix B). An exact amount of feed was weighed off on a scale (Micro PF 1 Platform Scale) and placed in a bin in front of a pen, each containing its own number which corresponded to the pen number. During a phase change, the feed inside these bins was discarded, the bins were cleaned thoroughly and the process was repeated. All the feed was kept in the store room next to the trial house in the same building.

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A total of 3600 day-old Ross 308 broiler chicks (1800 males and 1800 females) with an average weight of 42.18 grams (males 42.32 grams and females 42.05 grams) were randomly distributed among 60 identical pens with concrete floors. The parent flock from Midway chicks received a diet containing an antibiotic growth promoter (Zinc bacitracin), Enviva® Pro (Du Pont) and exogenous enzymes (XAP, AXTRA PHY), but not a coccidiostat. Sixty birds were placed in each pen, at a stocking density of 20/m^2. Chicks were feather sexed when they arrived at the trial house and males and females were placed separately.

On day of placement, extra feed was provided on paper sheets and also one fountain drinker was made available so that chicks would have easy access to the feed and water. The paper sheets were removed after five days. Birds were given *ad libitum* access to feed and water throughout the trial. The feeders were shaken every morning and evening, as well as mid-day if necessary, to make sure the feed was freely available to the chicks. A standard commercial light programme was followed. During days 1 to 6, birds were given 23 hours of daylight; days 7 to 15, birds were given 14 hours of daylight; during days 16 to 22, birds were given 16 hours of daylight; days 23 to 29 birds were given 18 hours of daylight and during days 29 to 35 birds were given 20 hours of daylight (Appendix A).

The house was divided into 5 blocks, with 12 pens per block. Each of the six treatments was repeated twice per block, with one of those pens containing male birds and the other female birds. All pens and housing conditions were inspected three times daily for general health of the birds and constant feed and water supply as well as temperature and ventilation. Any variation in a bird's appearance, excreta appearance, bird activity or behaviour would have been noticed. Birds that appeared to be suffering from pain or distress, or judged unlikely to survive were culled by cervical dislocation. Birds that died from unknown causes, pain or distress were subjected to a necropsy to ascertain the cause of death.

All bird mortalities were recorded on the date of death and the carcasses were removed from the pens and incinerated after full post mortem.

A commercial vaccination programme was used during the trial. At 10 days of age, broilers were vaccinated against Gumboro and Newcastle. On day 16, broilers were re-vaccinated against Gumboro and on day 22 against VH Newcastle. On days of vaccination, the water line was cut off, and the birds were left without water for approximately one hour. All vaccinations were then placed in the drinker lines to allow sufficient intake by the birds.

3.2 Diets and treatments

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A feeding programme consisting of five phases was employed. Birds were fed according to days on feed, namely, Pre-Starter, Starter, Grower, Finisher and Post-Finisher for 10, 7, 10, 3 and 5 days, respectively. At the beginning of each phase, feed from each pen was weighed into an empty bin. Feed from the bin was then added to feeders in the pen as necessary. At the end of each week and phase, feed that was left in the feeders was weighed back and discarded. This amount was added to the weight of feed left in the bin to calculate feed intake.

Six treatments were employed:

- Negative Control : Basal diet (without AGP)
- Positive Control : Basal diet (with AGP)
- DFM at 500g/ton (without AGP)
- DFM at 250g/ton (without AGP)
- DFM at 500g/ton (with AGP)
- DFM at 250g/ton (with AGP)

The basal diet was a typical South African maize-soya based diet. It was formulated to meet or exceed the nutrient requirements of Ross 308 broilers. The antibiotic growth promoter (AGP) used in three of the dietary treatments was zinc bacitracin (zinc bacitracin 15%, antibiotic performance promoter, Virbac, South Africa) at 500g/ton feed. The Direct fed microbial (DFM) included in the treatment diets was a probiotic feed additive product (Enviva Pro^{tm} 201 GT, Danisco Animal Nutrition, Marlborough, UK), which is a blend of three *Bacillus subtilis* strains. The inclusion level was either at the recommended level of 500g/ton feed or at a lower level of 250g/ton feed. The ingredients and calculated nutrient levels are shown in Table 3.1.

All phases except for the post-finisher contained the ionophoric coccidiostat, salinomycin (Salinomycin sodium 12%, Animate, South Africa) at 500g/ton feed. FB3 compound (in feed antimicrobial, Colistine Sulphate 2.4 BOU, V-Tech) was also included in the starter and grower rations of the basal feed at 500g/ton feed. AXTRA XAP (Combination, xylanase, amylase, protease, Danisco Animal Nutrition, Marlborough, UK) was included in all the dietary treatments at 500g/ton feed. Phyzyme AXTRA 10000 LIQ (Phyzyme AXTRA 10000 LIQ DB, Danisco Animal Nutrition, Marlborough, UK) was also included in all the dietary treatments at 100g/ton feed.

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Table 3.1. Composition of the basal diet

	Pre-Starter	Starter	Grower	Finisher	Post-Finisher
Ingredients (%)					
Maize (yellow)	59.21	63.72	66.92	72.02	72.02
Soybean oilcake (46 %)	23.5	16.6	13.62	19.2	19.56
Sunflower oilcake	4	4.3	2.28	2.76	3.1
White gluten (60 %)	2.35	3.72	2.8	2.26	2.2
Full fat soya	5	7.5	8.12	10	10
DL - threonine	0.04	0	0	0	0
DL - methionine	0.11	0.04	0.02	0	0
Methionine hydroxy analogue	0.1	0.1	0.1	0.09	0.09
Synthetic lysine (lysine HCl)	0.37	0.34	0.29	0.26	0.26
Salt	0.43	0.43	0.41	0.38	0.38
Soya oil	1.25	0	0	0	0
Mono-di-calcium phosphate	1.5	1.27	0.84	0.64	0.64
Limestone	1.8	1.62	1.32	1.16	1.16
Sodium carbonate	0.097	0.05	0	0	0
Hominy chop	0	0	3	0	0
Premix	0.19	0.23	0.226	0.22	0.116
Salinomycin	0.05	0.05	0.05	0.05	0
FB3 Compound[1]	0	0.05	0.05	0	0
Phyzyme AXTRA 10000 LQT[2]	0.01	0.01	0.01	0.01	0.01
AXTRA XAP[3]	0.05	0.05	0.05	0.05	0.05
Calculated analysis (%)					
Dry matter	89.35	89.34	89.18	89.18	89.18
Crude protein	21	20	18	17	17
AME for chicks[4]	11.8	11.89	12.3	12.5	12.5
Crude fibre	3.47	3.49	3.15	3.2	3.26
Fat	4.62	3.94	4.32	4.49	4.49
Lysine[5]	1.2	1.1	0.95	0.88	0.88
Methionine	0.5	0.5	0.4	0.39	0.39
Total sulphur amino acids	0.8	0.8	0.7	0.64	0.64
Threonine	0.7	0.64	0.59	0.56	0.55
Tryptophan	0.2	0.2	0.2	0.15	0.15
Arginine	1.2	1.13	1	0.95	0.95
Isoleucine	0.8	0.8	0.7	0.63	0.63
Valine	0.9	0.9	0.77	0.73	0.73
Glycine and serine	1.6	1.5	1.33	1.25	1.26
Calcium[6]	1.03	0.9	0.75	0.65	0.66
Potassium	0.87	0.78	0.73	0.67	0.67
Chloride	0.29	0.29	0.29	0.27	0.28
Total phosphorous	0.71	0.63	0.51	0.46	0.46
Retainable phosphorous[7]	0.47	0.42	0.34	0.29	0.29
Sodium	0.22	0.19	0.17	0.16	0.16

[1]FB3 compound, in feed antimicrobial – Colistine Sulphate (Vtech)

[2]Phyzyme AXTRA 10000LIQ DB, Danisco Animal Nutrition, Marlborough, UK. This enzyme was included at a rate of 100g/ton finished feed

[3]AXTRA XAP, Danisco Animal Nutrition, Marlborough, UK. This enzyme was included at a rate of 500g/ton finished feeds

[4] OE for broiler chicks (CVB)

[5] Amino acid available for broiler chicks (CVB) for all amino acids

[6] 0.08% Ca made available by supplementation of phytase enzyme

[7] 0.08% P made available by supplementation of phytase enzyme

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3.3 Feed samples

Samples were collected from all the raw materials at the feed mill before the trial feeds were formulated and mixed. Chemical composition of raw materials were determined using a NIR scanner (Perten instruments, DA7200, Sweden) to obtain the protein, moisture, fat, Ca, P and ash content of each raw material. Feed samples were taken of every treatment from each phase just after exiting the mixer, and also after pelleting. Samples were stored in a freezer (-8 degrees Celsius).

The raw materials were weighed individually using an electronic scale (Eastrand, Micro PF) and manually added to the mixer (A.C. Trading). Micro packs were weighed off by hand at Pennville Premix (Pretoria, South Africa). These micro packs contained lysine HCl, DL methionine, threonine, limestone, mono-dicalciumphosphate, salt, Methionine Hydroxy Analogue, AXTRA XAP, broiler premix, Phyzyme AXTRA 10000TPT, salinomycin 12% and Kembind® (Kemin® Product specialist). The treatment packs, containing sodium carbonate, zinc bacitracin (15%), FB 3 Compound and Enviva Pro 201 GT, were prepared for each specific treatment and specific phase in that treatment. Macro packs were produced, containing the micro pack and the treatment pack mixed together. Every treatment in each phase had its own macro pack that was added to the mixer together with the other raw materials. Extra moisture (8.2L) was added to the mixer to aid with the mixing and pelleting processes. Kembind was used as a binding agent in the feed at a total of 0.82 litres per treatment. Each bag was marked with a different colour label. After mixing and pelleting, the feed was bagged in a standard 50kg feed bag containing an inner plastic sealer to prevent contamination and nutrient losses.

3.4 Avian Pathogenic *E. coli* isolation (APEC) and *E. coli* and *Lactobacillus* Enumeration

At placement (day 0), 20 live chicks were randomly selected and transported to Deltamune Laboratories (Centurion). The chicks were culled via cervical dislocation and the GIT from the proximal duodenum to the anus was removed. The two ends were tied off using a cotton string and the 20 samples were placed together in one sealable bag and stored at 4°C for 24 hours. All 20 samples were pooled together and blended (Stomacher 400) to obtain a sample of 31.45g. The sample was then diluted 1:10 by adding 300ml peptone growth medium. Using a Finn pipette ® F2 (Thermo scientific) fixed at 1000µl, the mixture was further diluted and plated on two different agar plates using a Spiral machine (Interscience). The first plate was a MRS Agar plate (containing Campygen to decrease the oxygen levels) to grow *Lactobacillus* cultures, and the second one a Brilliance EC Agar plate to grow *E. coli* cultures. After plating the mixture on the agar plates, the MRS plate was incubated for three days at 30°C and the Brilliance plate for 24 hours at 37°C. After

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incubation, the *E. coli* colonies were purple in colour. The agar plates were scanned (Scan 500®, interscience) and FTA cards (Whatman® FTA® card technology, FTA Classic cards, GE Healthcare Life Sciences) were prepared. To prepare the FTA cards, purple *E. coli* colonies were swabbed using a sterile swab stick and then spread on the space provided on the FTA card. The cards were shipped to Waukeshea in America for APEC analysis. The FTA cards were scanned using a POSITIVE CONTROLR to obtain the number of APEC genes present in the sample of *E. coli*. The more APEC genes present in the *E. coli* sample, the more likely it is that the tested *E. coli* is pathogenic (Amerah *et al*. 2013).

At 22 days of age, one bird per pen was randomly selected. The body weight of the selected bird was within 50g of the average weight of the birds in that pen. The birds were marked according to the pen number and individual body weights were recorded. Birds were culled via cervical dislocation and the GIT from the proximal duodenum to the anus removed and spread on a clean dissection surface. Relevant sample information was recorded on the sample collection bag (i.e. bird number, pen, date). Three portions of the GIT were collected: the ascending portion of the duodenal loop; a 10 to 15 cm segment of the jejunum ending at the Meckl's Diverticulum; and the ileum, approximately 10 cm long. The remaining portions of the GIT were discarded. The contents from all three sections were squeezed out and the sections of the GIT were rinsed with 5ml of sterile peptone buffer until the contents were clear. Each section was cut longitudinally to expose the mucosal surface. The scissors used to cut the three sections of the GIT were sterilized with an alcohol solution and flame dried every time before handling the next sample number to prevent cross-contamination. Gloves were used as a standard procedure to protect the dissectors from any unknown diseases. Each sample (containing the three segments of the GIT) was weighed (OHAUS®, Pioneer™) to obtain the total weight of the three sections, and any abnormal pathology of the GIT was recorded. All three sections were combined into a sterile whirl-pak bag. After weighing the sections, 9ml of sterile peptone solution per gram of weight obtained was added to the whirl-pak bag and masticated at five strokes per second for 60 seconds. FTA cards were prepared as previously described.

3.5 DNA isolation and Multiplex PCR

APEC isolates had to possess two or more of the five virulent genes (iss, iucC, tsh, cvaC, and irp2) screened by multiplex PCR to be accepted as an APEC culture.

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3.6 Statistical design

The experimental procedures, with respect to placement, feeding, counting, recording of mortality, weighing of feed allocations and returns, and weighing of birds, were carried out according to the Standard Operating Procedures: Daybreak Trial Facility No. 601-0 (Placement of Broiler Chicks); Daybreak Trial Facility No. 602-0 (Trial Routine Procedures); and Daybreak Trial Facility No. 604 (Trial Weighing Days). A randomised block design was used in this trial. There were 10 pens (replications) for each treatment in the house. The house was divided into five blocks, with 12 pens per block (2 – 13, 14 – 25, 26 – 31, as well as 34 – 39, 40 – 51 and 52 – 63). Each of the six treatments was repeated twice per block (two replications per treatment per block), and one of those pens had male birds and the other female birds. There were only two fixed factors in the trial, namely, the specific treatment and sex. Each treatment had 10 replications throughout the trial.

3.7 Statistical Methods

The Generalised Line Treatment Model (GLM) function in Minitab Statistical Software (version17) was used in preference to the balanced ANOVA, so that *post hoc* multiple comparison tests could be run on the treatment means where the GLM found significant differences in performance between treatments. The *post hoc* multiple comparison test used was the Bonferroni test, which is appropriate for small numbers of comparisons and is stricter than Tukey's test. The confidence level was set at 95%. Block effects were accounted for by including "block" as a random factor in the model (two levels). The variables to be analysed were body weight, weekly body weight gains, phase feed intake, cumulative feed intake, weekly food conversion ratio, cumulative feed conversion ratio, performance efficiency factor, weekly mortality and cumulative mortality. These can be calculated, respectively, from the following measurements: bird counts, initial body weight, successive body weights, feed weighed in and feed weighed out (weighed on day 7, 14, 21, 28 and 35), and mortality records.

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CHAPTER 4

4. RESULTS

4.1 Body weight of broilers from 0 to 35 days of age

The influence of treatments on the body weight (BW) of broilers is summarised in Table 4.1. Body weight at 7 days of age was not significantly different between males and females ($P > 0.05$), but differed significantly ($P < 0.05$) at 14, 21, 28, and 35 days of age.

Table 4.1: The effect of DFM and zinc bacitracin on body weight of broilers from 0 to 35 days of age

Main Effect	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
Sex						
Male	42.32	166.83	415.93^{a}	821.63^{a}	1380.49^{a}	1980.03^{a}
Female	42.05	165.74	404.41^{b}	771.51^{b}	1260.78^{b}	1769.51^{b}
Treatment						
Negative Control[1]	42.17	162.82^{cd}	404.62^{bc}	787.54^{c}	1287.7^{c}	1847.9^{c}
Positive Control[2]	42.00	166.83^{bc}	412.56^{ab}	805.25^{ab}	1346.6^{a}	1905.4^{a}
DFM (500g/ton)[3]	42.10	166.64^{bc}	410.95^{bc}	797.64^{abc}	1322.3^{ab}	1873.6^{bc}
DFM (250g/ton)[4]	42.12	161.73^{d}	402.18^{c}	784.51^{c}	1303.7^{bc}	1863.9^{bc}
DFM (500g/ton) + AGP[5]	42.35	167.73^{b}	409.50^{bc}	792.49^{bc}	1327.9^{ab}	1873.8^{bc}
DFM (250g/ton) + AGP[6]	42.37	171.96^{a}	421.18^{a}	811.99^{a}	1335.6^{a}	1884^{ab}
Pooled SEM		1.5374	3.5272	7.2349	8.9654	11.3594
Probability						
Sex		0.3654	<0.0001	<0.0001	<0.0001	<0.0001
Treatment		0.0002	0.0029	0.0127	0.0003	0.0073
Treatment*Sex		0.1068	0.3407	0.0727	0.487	0.9284

$^{a-d}$Means within a column with different superscripts differ significantly ($P < 0.05$)

[1]Negative Control, Standard commercial maize, soya diet

[2]Positive Control, Standard commercial maize soya diet + Antibiotic Growth Promoter (Zinc bacitracin, Virbac) at 500g/ton

[3]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 500g/ ton (150000 CFU)

[4]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 250g/ ton (75000 CFU)

[5]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 500g /ton (150000 CFU) with AGP (zinc bacitracin, Virbac)

[6]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 250g/ ton (75000 CFU) with AGP (zinc bacitracin, Virbac)

The broilers from the Positive Control were significantly heavier ($P < 0.05$) than those of the Negative Control from day 21 onwards. The addition of DFM at either 250 or 500g/ton did not improve the growth of broilers compared to the Negative Control ($P > 0.05$). Supplementation of AGP with the DFM had the same effect on BW than feeding AGP alone (Positive Control),

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although the DFM 500g/ton + AGP broilers had a significantly lower BW on day 35 (P < 0.05) than the Positive Control. The only significant advantage of supplementing DFMs was noted on day 7 where the DFM 250g/ton + AGP group performed better than all other treatments in respect of BW (P < 0.05).

4.2 Cumulative feed intake of broilers from 0 to 35 days of age

Cumulative feed intake (CFI) at 7 days of age (Table 4.2) was not significantly different between males and females (P > 0.05), but the males had a higher feed intake (P < 0.05) from day 14 to 35 (P < 0.05).

Table 4.2. The effect of DFM and zinc bacitracin on cumulative feed intake of broilers from 0 to 35 days

Main Effect	Days 0-7	Days 0-14	Days 0-21	Days 0-28	Day 0-35
Sex					
Male	142.79	470.33^{a}	1100.00^{a}	2086.90^{a}	3286.00^{a}
Female	143.03	458.74^{b}	1036.80^{b}	1906.30^{b}	2934.60^{b}
Treatment					
Negative Control[1]	141.34	462.08^{ab}	1065.90^{b}	1988.30^{bc}	3094.50^{bc}
Positive Control[2]	144.06	467.94^{ab}	1074.60^{ab}	2003.80^{ab}	3114.60^{ab}
DFM (500g/ton)[3]	142.82	461.70^{ab}	1064.90^{bc}	1994.20^{b}	3102.70^{bc}
DFM (250g/ton)[4]	140.90	456.67^{b}	1044.40^{c}	1957.00^{c}	3051.40^{c}
DFM (500g/ton) + AGP[5]	144.43	468.18^{ab}	1072.80^{ab}	2004.60^{ab}	3133.30^{ab}
DFM (250g/ton) + AGP[6]	143.91	470.63^{a}	1087.40^{a}	2031.80^{a}	3165.20^{a}
Pooled SEM	1.8072	4.7778	8.5745	12.904	19.2404
Probability					
Sex	0.8714	0.0012	<0.0001	<0.0001	<0.0001
Treatment	0.6404	0.1695	0.0081	0.0075	0.0041
Treatment*Sex	0.5552	0.53	0.2348	0.6608	0.993

[a-d]Means within a column with different superscripts differ significantly (P < 0.05)

[1]Negative Control, Standard commercial maize, soya diet

[2]Positive Control, Standard commercial maize soya diet + Antibiotic Growth Promoter (Zinc bacitracin, Virbac) at 500g/ton

[3]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 500g/ ton (150000 CFU)

[4]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 250g/ ton (75000 CFU)

[5]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 500g /ton (150000 CFU) with AGP (zinc bacitracin, Virbac)

[6]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 250g/ ton (75000 CFU) with AGP (zinc bacitracin, Virbac)

Feed intake did not differ between the Positive Control and Negative Control groups. Adding DFM alone to the diets revealed no significant difference (P > 0.05) on feed intake

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compared to Negative Control. In general, the addition of AGP in combination with DFM to the diets had no apparent effect on feed intake by the birds. At the end of the trial at day 35, the DFM 250g/ton group's feed intake was significantly lower than all groups that received AGPs in their feed ($P < 0.05$).

4.3 Cumulative feed conversion ratio of broilers from 0 to 35 days of age

The data revealed no significant difference ($P > 0.05$) between males and females in terms of FCR throughout the trial, for all treatments fed (Table 4.3).

Table 4.3 The effect of DFM and zinc bacitracin on feed conversion ratio of broilers from 0 to 35 days

Main Effect	**Day 0-7**	**Day 0-14**	**Day 0-21**	**Day 0-28**	**Day 0-35**
Sex					
Male	1.1481	1.2593	1.4117	1.5598	1.6969
Female	1.1575	1.2663	1.4217	1.5647	1.6981
Treatment					
Negative Control[1]	1.1728^{a}	1.2759^{a}	1.4334^{a}	1.5974^{a}	1.7113^{ab}
Positive Control[2]	1.1541^{a}	1.2628^{abc}	1.4056^{b}	1.5363^{d}	1.6744^{c}
DFM (500g/ton)[3]	1.1467^{ab}	1.2515^{bc}	1.4094^{b}	1.5575^{bc}	1.6937^{bc}
DFM (250g/ton)[4]	1.1786^{a}	1.2687^{ab}	1.4072^{b}	1.5515^{cd}	1.6754^{c}
DFM (500g/ton) + AGP[5]	1.1526^{a}	1.2753^{ab}	1.4313^{a}	1.5595^{bc}	1.7110^{ab}
DFM (250g/ton) + AGP[6]	1.1119^{b}	1.2427^{c}	1.4132^{ab}	1.5712^{b}	1.7194^{a}
Pooled SEM	0.01284	0.00907	0.00718	0.00614	0.00818
Probability					
Sex	0.3739	0.315	0.0911	0.3365	0.86
Treatment	0.011	0.043	0.0192	<0.0001	0.0003
Treatment*Sex	0.7738	0.8393	0.8078	0.166	0.6036

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

[1]Negative Control, Standard commercial maize, soya diet

[2]Positive Control, Standard commercial maize soya diet + Antibiotic Growth Promoter (Zinc bacitracin, Virbac) at 500g/ton

[3]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 500g/ ton (150000 CFU)

[4]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 250g/ ton (75000 CFU)

[5]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 500g /ton (150000 CFU) with AGP (zinc bacitracin, Virbac)

[6]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 250g/ ton (75000 CFU) with AGP (zinc bacitracin, Virbac)

From day 21 onwards, broilers that received AGP (Positive Control) in their diets had a lower FCR ($P < 0.05$) than the Negative Control (without AGP). Addition of DFM to the feed,

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improved the FCR to the same level as that of the Positive Control birds (P > 0.05). This improvement in FCR was, however, not noted for birds that have received the combination of AGP and DFM.

4.4 Cumulative mortality for broilers from 0 to 35 days of age

The cumulative mortalities from 0 to 35 days of age are summarised in Table 4.4. In this study, sex had no effect on mortalities up to 21 days of age. During the last two weeks, however, male birds showed a higher mortality rate than female birds (P < 0.05). The mortalities were not associated with any treatment (P > 0.05).

Table 4.4. The effect of DFM and zinc bacitracin on cumulative mortality for broilers from 0 to 35 days

Main Effect	Days 0-7	Days 0-14	Days 0-21	Days 0-28	Days 0-35
Sex					
Male	0.0089	0.01391	0.02059	0.0428[a]	0.05789[a]
Female	0.0083	0.00945	0.01222	0.0300[b]	0.03393[b]
Treatment					
Negative Control[1]	0.005[ab]	0.0101	0.0134	0.0335	0.0436
Positive Control[2]	0.010[ab]	0.0150	0.0183	0.0400	0.0517
DFM (500g/ton)[3]	0.003[b]	0.0083	0.0117	0.0283	0.0317
DFM (250g/ ton)[4]	0.010[ab]	0.0117	0.0167	0.0367	0.0500
DFM (500g/ ton) + AGP[5]	0.008[ab]	0.0167	0.0217	0.0384	0.0467
DFM (250g/ ton) + AGP[6]	0.0037	0.0083	0.0167	0.0417	0.0517
Pooled SEM	0.8512	0.0045	0.0057	0.0065	0.01
Probability					
Sex	0.2916	0.2271	0.0778	0.0194	0.0003
Treatment	0.9214	0.692	0.8533	0.7282	0.3947
Treatment*Sex	0.9214	0.932	0.9452	0.8032	0.3658

[a-d]Means within a column with different superscripts differ significantly (P < 0.05)

[1]Negative Control, Standard commercial maize, soya diet

[2]Positive Control, Standard commercial maize soya diet + Antibiotic Growth Promoter (Zinc bacitracin, Virbac) at 500g/ton

[3]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 500g/ ton (150000 CFU)

[4]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 250g/ ton (75000 CFU)

[5]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 500g /ton (150000 CFU) with AGP (zinc bacitracin, Virbac)

[6]Basal diet + Enviva Pro™ 201 GT (Du Pont[tm]) at 250g/ ton (75000 CFU) with AGP (zinc bacitracin, Virbac)

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4.5 APEC Counts of the GIT of broilers at 21 days of age, both pooled and individual samples

The number of Avian Pathogenic *E. coli* colonies (APEC) and non-pathogenic *E. coli* colonies are shown in Figure 4.1.

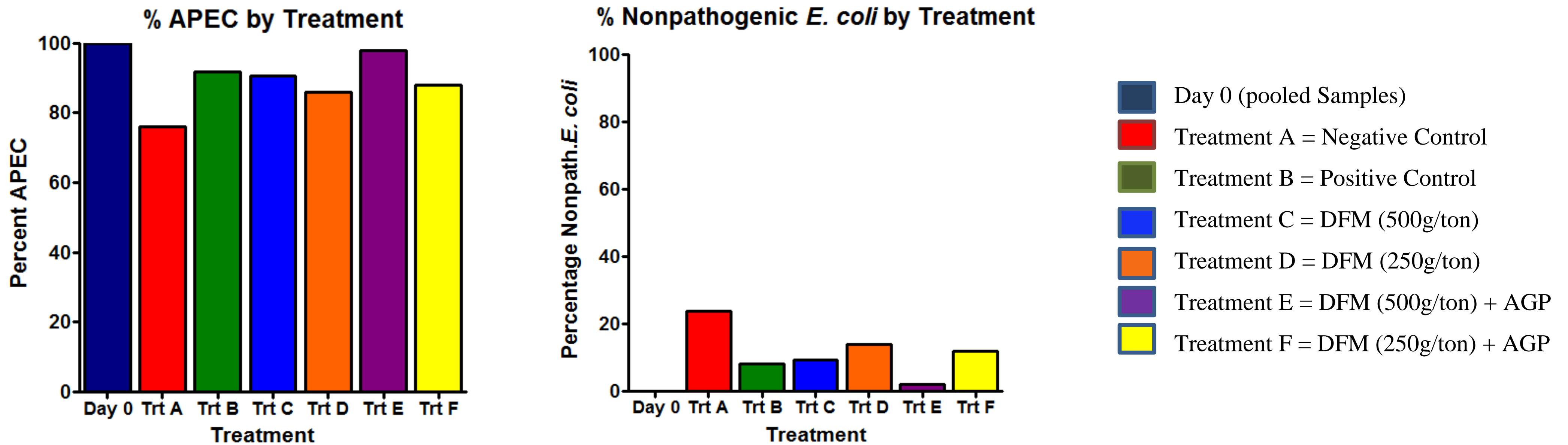

Figure 4.1: Effect of DFM and zinc bacitracin on the percentage of *E. coli* isolates with corresponding number of Avian Pathogenic *E. coli* colonies (APEC) and without the corresponding number of Avian Pathogenic *E. coli* genes.

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The pooled sample taken from 10 random birds at placement (day 0) showed a 100% APEC count. Negative Control broilers showed the lowest APEC count compared to all the other treatments at 22 days of age. Broilers from the DFM (250g/ton) as well as DFM (250g/ ton) + AGP groups had lower APEC counts compared to the DFM (500g/ton) and DFM (500g/ ton) + AGP groups.

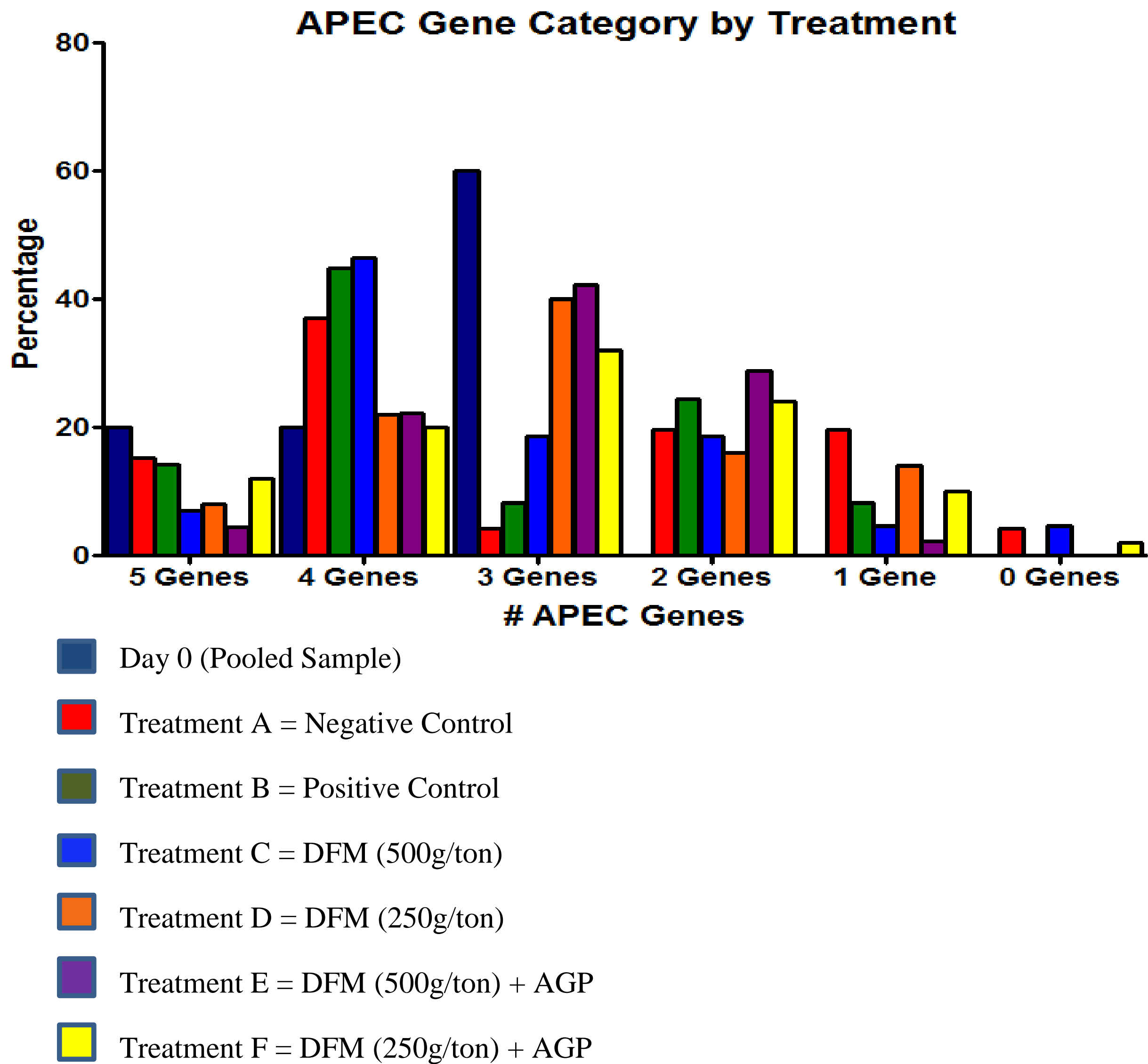

Figure 4.2 The effect of Avian Pathogenic *E. coli* colonies (APEC) on gene category by treatment at 22 days of age

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The data summarised in Figure 4.2 shows that the number of *E. coli* genes tested positive for APEC was too high. The majority tested APEC positive between 3 and 4 genes, compared to the ideal of between 0 and 1 APEC gene. Broilers that received DFM (500g/ton) tested the highest APEC gene percentage at 4 genes, followed by the Positive Control and Negative Control groups, and DFM (500g/ton) + AGP as well as the pooled sample, which tested the highest APEC percentage at 3 genes.

There was no significant difference in average APEC counts between the Positive Control and DFM (250g/ton) + AGP groups (Table 4.5). DFM (500g/ton) + AGP revealed the lowest APEC count compared to all other diets fed ($P < 0.05$), except for Negative Control.

Table 4.5 Average Avian Pathogenic *E. coli* colonies (APEC) counts (log^{10} *cfu*/g) in the gastro intestinal tract by treatment as relates to the Negative Control

Main Effect	Days 22	P-Value
Treatment		
Day 0 (Pooled sample)	5.82	
Negative Control	2.74	
Positive Control	3.77	0.03*
DFM (500g/ ton)	3.14	0.4
DFM (250g/ ton)	3.62	0.06
DFM (500g/ ton) + AGP	3.02	0.63
DFM (250g/ ton) + AGP	3.79	0.03*

*Significant at P<0.05

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CHAPTER 5

5. DISCUSSION

5.1 Effect of sex on broiler performance

In the current study, males were heavier than the females from 14 to 35 days of age. Research supporting this finding highlighted the fact that males grow faster than females, because their intestinal tract and absorption capacity develops more rapidly, and due to a better growth potential, allowing them to grow faster (Miles *et al*., 2006). Males showed a higher feed intake from 14 to 35 days of age compared to females, even though the FCR remained non-significantly different between sexes.

5.2 The effect of zinc bacitracin on broiler performance

Dietary antibiotics are a major factor influencing the digestive microflora (Chambers & Gong, 2011). A common practice for promoting growth and preventing diseases is the use of sub-therapeutic doses of antibiotics in a broiler diet. However, it reduces both the stability of the microflora and also the *Lactobacillus* population in the intestines (Chambers & Gong, 2011). The dietary antibiotic effects on the microflora composition are dose and age dependent. Positive Control revealed a significantly higher body weight from 21 to 35 days compared to Negative Control. Feed intake for Positive Control birds were non-significantly different from Negative Control birds at 35 days, however, FCR were significantly lower from 21 to 35 days compared to Negative Control. This illustrates that AGPs improve the performance of broiler birds.

5.3 The effect of DFM on broiler performance

No dose response was observed between the two DFM doses, with and without AGP, in terms of BW, FI and FCR. The body weight of broilers at 35 days that received the DFM (500g/ton and 250g/ton) without AGP diets, was not significantly different from the Negative Control, and also not significant from the DFM (500g/ton and 250g/ton) + AGP groups. The DFM (500g/ton and 250g/ton) alone diets had no significant effect on feed intake compared to Negative Control, although, DFM 250g/ton diet revealed a significantly lower FI compared to DFM (500g/ton and 250g/ton) + AGP and Positive Control. Broilers from this group (DFM 250g/ton) also showed a significantly lower FCR compared to Negative Control, and DFM (500g/ton and 250g/ton) + AGP. An optimal intake level of DFMs has not yet been established; even though it is generally accepted

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that efficacy for most DFM microorganisms is demonstrated with a daily consumption of 10^8 to 10^9 microorganisms per day in animals (Mountzouris *et al*., 2007). Even though the doses of DFMs used in this study were lower compared to the doses used in the research done by Mountzouris (2007), no significant differences were found in terms of BW compared to all treatments containing DFM.

Other researchers found that supplementing a lower dose of probiotic product limits its efficiency to improve the birds BWG and BW (O'Dea *et al*., 2006). Mechanisms by which DFMs improve feed conversion efficiency include modification of intestinal flora (Kabir, 2009), enhancement of growth of non-pathogenic facultative anaerobic and gram positive bacteria producing lactic acid and hydrogen peroxide (Kabir, 2009), suppression of growth of intestinal pathogenns (Kabir, 2009), and improvement of digestion and utilisation of nutrients (Kabir, 2009).

The major outcomes when using DFMs in feed include improvement in growth, reduction in mortality, and improvement in feed conversion efficiency (Kabir, 2009). In contrast to these findings, some studies showed no influence on feed intake and weight gain but, however, improved FCR compared to diets containing AGP at 35 days (Amerah *et al*., 2013).

The DFM + AGP diets in this study might have disturbed the microfloral population in the gut of broilers, and therefore, a lower FCR was observed for broilers receiving the DFM 250g/ton diet. According to Bai *et al*. (2003), antibiotics may limit microbial enzymatic activity, lower digestion and overall absorption of nutrients, and increase faecal output of nutrients. The bird will respond in such a way that it increases its CFI to maintain an optimal nutrient intake for normal body functions and muscle production (Bai *et al*., 2013). Broilers that received DFM alone diets had no significant difference in terms of mortality compared to all other diets fed. Recent studies showed that a DFM product of *Lactobacillus fermentum* and *Saccharomyces cerevisiae* stimulated the T-cell immune system in the intestine without sacrificing growth performance in broilers during their first 21 days (Bai *et al*., 2013).

5.4 The effect of a combination of DFM with AGP on broiler performance

No dose response was seen between DFM + AGP diets in terms of BW, FI and FCR at 35 days of age. The DFM (500g/ton) + AGP had a significantly lower BW compared to Positive Control at 35 days, although, revealed no significant difference in terms of FI, but a significantly higher FCR at 35 days compared to Positive Control. This may be due to nutrients wasted by the micro-organisms in the GIT at levels of 500g/ton DFM in the diets. The nutrients consumed were most likely not converted into muscle resulting in a high FCR. Factors affecting the intestinal microflora, such as diet (including antibiotics), age and major stresses (Chambers & Gong, 2011)

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could cause a drop in performance. Dietary ingredients are also nutrients for bacterial growth, therefore, the intestinal microflora is a function of the diet itself (Chambers & Gong, 2011). Diet composition and microflora can affect the mucosal architecture as well as the mucus composition of the intestinal tract (Chambers & Gong, 2011).

The birds that received DFM + AGP diets (both 500g/ton and 250g/ton) in this study had a higher FCR compared to Positive Control diets. DFM 250g/ton + AGP was also revealed not significantly different in terms of BW and FI compared to the Positive Control, although, showed a significantly higher FCR at 35 days. Thus, broiler performance from the DFM 250g/ton + AGP group was more efficient in terms of FCR than DFM 500g/ton + AGP diets.

The mode of action of DFMs is in contrast to that of AGPs. AGPs improve broiler performance by decreasing overall microbial load in the digestive tract, reducing competition for nutrients, and decreasing microbial metabolites that depress growth (Stutz *et al*., 1983; Engberg *et al*., 2000; Amerah *et al*., 2013). Therefore, if day-old chicks receive antibiotics in their diets, their gut microflora populations will be destroyed. Research is lacking on the effects of AGP on physical changes to the GIT due to diets containing DFMs, because the focus of studies over the past few years has been on the effect of easily cultured bacterial populations such as *Lactobacillus* and *Clostriduim perfringes* on poultry health (Miles *et al*., 2006). Not all antibiotics control growth and proliferation by the same mechanism, and they differ with regard to their ability to influence certain disease states or improve growth and feed efficiency (Miles *et al*., 2006).

5.5 The potential of a DFM to replace AGPs in the diet of broilers

Positive Control diets had a significantly higher BW compared to DFM (500g/ton and 250g/ton) alone diets. Direct Fed Microbial 250g/ton had a significantly lower FI compared to Positive Control, and FCR was non-significantly different from Positive Control. It would, therefore, appear to us if an inclusion level of 250g/ton of DFM per ton of feed could be used to replace AGPs in broiler diets.

5.6 The effect of DFM alone or a combination of DFM + AGP on the Avian Pathogenic *E. coli* colony levels in the gut of broilers

The average number of APEC genes in the GIT of chicks was influenced significantly via the use of lower doses of DFMs in the diet. Amerah *et al*. (2013) indicated that the number of mucosa-associated APEC genes was influenced significantly by dietary treatments. Lee *et al*. (2010) also reported that strains of *B. subtilis* exert an inhibitory effect on avian pathogenic

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Escherichia coli or *Clostridium perfringens*. In the current study, the pooled sample showed a 100% APEC count, which can be related to the hatchery, parent flock diet and environment. APEC can be transmitted from the parent through the uterus (yolk sac) to the foetus, which could explain the high percentage of APEC in the pooled sample. Only the highly resistant APEC genes are transmitted from the parents to the foetus. Negative Control showed significantly lower APEC counts, which may have resulted from the used litter system used in the trial. Day-old chicks are exposed to pathogens that stimulate the immune system and help to establish a healthy microflora population in the GIT. Research also demonstrated that some *Bacillus* spp. were capable of directly inhibiting APEC as well as other microbes in the gut (Amerah *et al*, 2013; Lee *et al*, 2010). A higher dose of DFMs (both DFM alone and DFM + AGP diets) revealed lower APEC counts in the gut.

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CHAPTER 6

6. CONCLUSION

Male birds were more efficient in terms of growth without any difference in FCR, therefore, required less feed to grow heavier compared to female birds. No dose response was observed between the two levels of DFM (500g/ton and 250g/ton) in terms of BW, FI, and FCR at 35 days of age. A lower dose of DFM (250g/ton) in the diets of broilers revealed a significantly lower FCR compared to diets without DFMs (Negative Control), and DFM (500g/ton and 250g/ton) + AGP, and no significant difference in FCR compared to Positive Control at 35 days of age. However, when combining a DFM with AGP in the diet, the FCR of broilers increased due to a lower weight obtained with a higher feed intake at 35 days, showing an antagonistic effect between a DFM and AGP in feed. The DFM (500g/ton) without AGP feed revealed a non-significant difference in FI and FCR compared to Positive Control, with a lower BW at 35 days. More nutrients seemed to be lost with the higher dose of DFMs (500g/ton). Therefore the lower dose of DFM (250g/ton) without AGPs will be the recommended dosage in broiler diets.

The DFM (500g/ton) + AGP had a significantly lower body weight, no difference in FI, and a higher FCR in broilers compared to Positive Control at 35 days. However, a lower dose of DFM (250g/ton) + AGP did not influence the BW, and FI of broilers compared to only AGPs in the diet (Positive Control), but increased the FCR significantly. Therefore, it will be more beneficial to include a lower dose of DFMs when combined with AGPs in the diet.

Apart from the Negative Control, most treatments fed throughout this trial showed a significantly lower average APEC count compared to the Positive Control, although DFM (250g/ton) with AGP showed no significant difference compared to the Positive Control. This study revealed that a lower dose of DFMs had no difference in terms of FCR compared to AGPs alone. Furthermore when a DFM was combined with an AGP, the FCR increased significantly. It is therefore recommended to use a lower dose of DFMs (250g/ton) that can be used as an alternative to AGPs in broiler diets.

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CHAPTER 7

7. RECOMMENDATIONS

The DFM (500g/ton and 250g/ton) with AGP combination diet revealed a lowering effect on FCR at 35 days of age. I showed that there may be an antagonistic effect between a DFM and AGP in broiler diets. Highly recommended to use a combination diet (DFM with AGP) as done in the study.

Challenging birds with used chicken litter may have had a synergistic effect on the broilers that received the Negative Control feed through inoculation of the day old chicks. It may be recommended to use clean shavings at placement as done commercially in South Africa to avoid external effects on the study.

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APPENDIX A

Table 1: Lighting program followed during the experimental period

Day	Controller set point		Day Light	Darkness
	Lights on	Lights off		
1	00:00	23:00	23:00	01:00
2	00:00	23:00	23:00	01:00
3	00:00	23:00	23:00	01:00
4	00:00	23:00	23:00	01:00
5	00:00	23:00	23:00	01:00
6	00:00	23:00	23:00	01:00
7	05:00	19:00	14:00	10:00
8	05:00	19:00	14:00	10:00
9	05:00	19:00	14:00	10:00
10	05:00	19:00	14:00	10:00
11	05:00	19:00	14:00	10:00
12	05:00	19:00	14:00	10:00
13	05:00	19:00	14:00	10:00
14	05:00	19:00	14:00	10:00
15	05:00	19:00	14:00	10:00
16	04:00	20:00	16:00	08:00
17	04:00	20:00	16:00	08:00
18	04:00	20:00	16:00	08:00
19	04:00	20:00	16:00	08:00
20	04:00	20:00	16:00	08:00
21	04:00	20:00	16:00	08:00
22	04:00	20:00	16:00	08:00
23	03:00	21:00	18:00	06:00
24	03:00	21:00	18:00	06:00
25	03:00	21:00	18:00	06:00
26	03:00	21:00	18:00	06:00
27	03:00	21:00	18:00	06:00
28	03:00	21:00	18:00	06:00
29	02:00	22:00	20:00	04:00
30	02:00	22:00	20:00	04:00
31	02:00	22:00	20:00	04:00
32	02:00	22:00	20:00	04:00
33	02:00	22:00	20:00	04:00
34	02:00	22:00	20:00	04:00
35	02:00	22:00	20:00	04:00

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APPENDIX B

Table 2: Temperature profile of the houses during the experimental period

Day	Temperature (°C, 50 % rH)		
	Lower Temp	Target Temp	Upper Temp
-1	34	**35.5**	37
-2	34	**35.5**	37
0	34	**35.5**	37
1	34	**35.5**	37
2	34	**35.5**	37
3	33	**34.5**	36
4	33	**34.5**	36
5	33	**34.5**	36
6	32	**33.5**	35
7	32	**33.5**	35
8	32	**33.5**	35
9	28.2	**29.7**	31.2
10	28.2	**29.7**	31.2
11	28.2	**29.7**	31.2
12	25.7	**27.2**	28.7
13	25.7	**27.2**	28.7
14	25.7	**27.2**	28.7
15	24.7	**26.2**	27.7
16	24.7	**26.2**	27.7
17	24.7	**26.2**	27.7
18	23.5	**25**	26.5
19	23.5	**25**	26.5
20	23.5	**25**	26.5
21	22.5	**24**	25.5
22	22.5	**24**	25.5
23	22.5	**24**	25.5
24	21.5	**23**	24.5
25	21.5	**23**	24.5
26	21.5	**23**	24.5
27	21.5	**23**	24.5
28	21.5	**23**	24.5
29	21.5	**23**	24.5
30	21.5	**23**	24.5
31	21.5	**23**	24.5
32	21.5	**23**	24.5
33	21.5	**23**	24.5
34	21.5	**23**	24.5
35	21.5	**23**	24.5

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