Performance and gut microbiome diversity of broilers supplemented with a single strain *Bacillus subtilis* probiotic and a multi-complex carbohydrase

by

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DECLARATION

I, **Rudie Hermann van der Westhuizen,** declare that the thesis, which I hereby submit for the degree MSc (Agric) Animal Science: Animal Nutrition at the University of Pretoria, is my own work and not previously been submitted by me for a degree at this or any other tertiary institution.

Rudie Hermann van der Westhuizen

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ABSTRACT

The use of antimicrobial growth promoters in broiler feed used to be and still is common practice in several parts of the world. However, due to the risk of microbial resistance building up, several countries have banned the use of antimicrobial growth promoters (AGP) and in other countries this movement away from AGP is driven by consumer demands. Unfortunately, as seen in Europe, the removal of AGP from broiler feed is not a simple process, thus several alternative options have since been investigated. The purpose of the study was to evaluate the benefits, if any, of adding a single strain *Bacillus subtilis*, probiotic product, either alone or in combination, with a multi complex carbohydrase (NSPase) to the diet of broilers. The study was conducted in a fully environmentally controlled, semi-commercial broiler house. The house contained 96 pens in total, all set up in one line from the front to the back of the house. The pens were surrounded by commercially produced broilers throughout the duration of the trial, to simulate commercial conditions as far as possible. Two thousand three hundred and fifty Ross 308 male broiler birds were feather-sexed and randomly distributed throughout the pens, 24 broilers per pen at a stocking density of 22.22 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 probiotic and / or NSPase to create six treatments as follows: Negative Control: Basal diet (without AGP, probiotic and NSPase), Positive Control: Basal diet with AGP, Treatment 1: Basal diet with NSPase and AGP, Treatment 2: Basal diet with NSPase, Treatment 3: Basal diet with probiotic, Treatment 4: Basal diet with probiotic and NSPase. Broiler performance was measured periodically, coinciding with feeding phases, in terms of body weight (BW), feed intake (FI) and feed conversion ratio (FCR) from day-old to 35 days-of-age. Two birds per pen were culled on day 25. One bird was used for macroscopic gut health scoring and one to collect caecal samples for quantification of the microbiome.

Broilers that received AGP treatments had a significantly higher BW at 28 days compared to broilers that received probiotic (without NSPase) and NSPase (without AGP) diets. Broilers that received the probiotic (without NSPase) diet had a significantly lower FCR in the final week of the study when compared to both treatments containing AGP. At the end of the study there were no significant differences between any of the treatments for the performance parameters measured including mortalities. Treatment 3 had significantly higher percentage abundance of the phylum *Tenericutes* when compared to the positive control. When compared to the positive control, Treatment 3 also had significantly higher percentage abundance in both the *Bacillales* and *RF39* orders. There was a significant difference in the average percentage abundance between Treatment 3 and Positive Control in the *Bacillaceae*_Unknown, *Clostridium*, *Lachnospiraceae*_Unknown, *Anaerostipes*, *Campylobacter* and *RF39*_Unknown genera. Treatment 3 had significantly higher percentage abundance in *Bacillaceae*_Unknown, *Anaerostipes* and *RF39*_Unknown genera, whereas Positive Control had significantly higher percentage abundance of *Clostridium*, *Lachnospiraceae*_Unknown and *Campylobacter*.

It could not be concluded whether the probiotic, alone or in combination with a NSPase, can be successfully used to replace AGP in commercial broiler production.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AGP	:	Antibiotic growth promoter
BW	:	Body weight
BWG	:	Body weight gain
°C	:	Degrees Celsius
CFCR	:	Cumulative feed conversion ratio
CFU	:	Colony forming unit
EFSA	:	European Food Safety Authority
EU	:	European union
FCR	:	Feed conversion ratio
FI	:	Feed intake
FTU	:	Phytase unit
g	:	Gram
GLM	:	General linear model
g/t	:	Gram per ton
kg	:	Kilogram
LAB	:	Lactic acid producing bacteria
m^2	:	Square meter
MJ	:	Mega joules
ME	:	Metabolisable energy
NE	:	Necrotic enteritis
NSP	:	Non-starch polysaccharides
NC	:	Negative control
PC	:	Positive control
TRT	:	Treatment
QPS	:	Qualified presumption of safety
US	:	United states
VFD	:	Veterinary feed directive
WHO	:	World health organisation

LIST OF TABLES AND FIGURES

CHAPTER 1
Introduction

With the growing population, poultry is becoming increasingly important in terms of its contribution to food security and nutrition. With its short production cycles and the ability to convert a wide range of agri-food by-products and wastes into meat and eggs edible by humans, it is an affordable source of energy, protein and essential nutrients to humans (Mottet and Tempio, 2017). In developing countries, poultry is the fastest growing agricultural sub-sector and is expected to continue to grow as demand for meat and eggs is driven by growing populations, rising incomes and urbanisation (Mottet and Tempio, 2017). However, poultry represents a threat to human health, especially as a vector of diseases and because of its role in antimicrobial resistance (Mottet and Tempio, 2017).

Since Moore *et al.* (1946) showed that the application of antimicrobial growth promoters (AGP) at sub-therapeutic levels in broiler feed resulted in added weight gain, the poultry industry has been utilising these products in large amounts. Unfortunately, this led to resistant microbes surviving and evolving and scientists reporting resistance in birds to antibiotics regularly used to treat humans as early as the 1950s (Kemmett, 2015). Sweden started with a total ban of AGP-use in 1986 when further evidence of drug-resistant bacteria and drug residues in the body of the birds, an imbalance of normal microbiota and the transfer of antibiotic-resistant genes from animal to human microbiota were discovered (Kemmett, 2015). In 2006, Europe officially banned the use of AGP in broiler feed, and ever since then multiple other countries have followed suite (Kocher and Choct, 2008). Several countries started switching over to alternative management systems and feed supplements to replace in-feed AGP, due to pressure from the consumer rather than official legislation by authorities. One of the major problems with the removal of AGP was the increase in the prevalence of necrotic enteritis, intestinal mucosa damage mainly caused by *Eimeria* infection or a change in the normal intestinal microbiota (Kocher and Choct, 2008). A normal balanced intestinal microbiome is important for the maintenance of a healthy gut to ensure optimum digestion of feed and absorption of nutrients and any major change in diet can predispose birds to rapid proliferation of *Clostridium perfringens* or any other pathogenic bacteria (Kocher and Choct, 2008). When AGP are removed from broiler feed and productivity is not threatened by disease, the most

important factor influencing performance is the capability of the animal to convert feed into carcass as efficiently as possible (Rinttilä and Apajalahti, 2013).

Several suggestions for the mode of action of AGP have been investigated and it is likely that a combination of actions contribute to the success obtained by using these products in broiler feed. Engberg *et al*. (2000) concluded that the supplementation of broiler feed with salinomycin and/or zinc bacitracin resulted in significantly lower counts of *Clostridium perfringens* as well as *Lactobacillus salivarius*, which was one of the dominant lactic acid bacterium located in broiler gastro-intestinal tract (GIT) content. High numbers of these lactobacilli may induce broiler growth depression by competing in nutrient uptake or impairing lipid digestion due to bile acid deconjugation. Crisol-Martínez *et al.* 2017 showed that zinc bacitracin changed the composition and increased the diversity of caecal microbiota by reducing dominant species. They also proved that Avilamycin (Elanco Animal Health) only produced minor reductions in the abundance of two microbial taxa, whereas zinc bacitracin produced large shifts in a number of taxa, of which *Lactobacillus* was the primary one. They concluded that the difference in the ability of these two AGP to manipulate the composition of caecal bacteria led to the difference in feed conversion ratio (FCR). Thus, by harnessing specific species of bacteria, it may be possible to develop high-producing strategies in poultry that rely on the use of probiotics and less on in-feed antibiotics.

Since culture-independent molecular techniques have been developed more knowledge about the intestinal system of broiler chickens has come to light, thus leading to increasing evidence suggesting a connection between the metabolisable energy (ME) of the diet and the microbiota in the hindgut of the host (Rinttilä and Apajalahti, 2013). Specialised bacteria in the gut can directly convert some dietary components into high-energy metabolites (Rinttilä and Apajalahti, 2013). Although there is increasing amounts of research being done on the composition of microbial populations present in the broiler caeca, there is still no consensus regarding the type of microbiota related to an ideal animal performance (Rinttilä and Apajalahti, 2013).

Parker (1974) first described probiotics as micro-organisms or substances that aids in the balance of the intestinal microbiome and Fuller (1989) acknowledged that this contribution was beneficial to the host animal. Feeding a probiotic to manipulate the composition of the microbiome is a very popular method to increase production after removal of AGP. Probiotics

are considered to have several modes of actions including, competitive exclusion of pathogenic bacteria, producing metabolites beneficial to the host animal, promoting the proliferation of *Bacteroidetes* in the caeca, which subsequently increase butyric acid production, and the reduction of inflammation in the GIT (Choct *et al.*, 1996; Smits *et al.*, 1997; Torok *et al.*, 2008).

Poultry diets contain high levels of non-starch polysaccharides (NSP). These NSP can reduce the passage rate of feed through the gut and depress bird performance as well as negatively influence and contribute to undesirable shifts in the gut microbiota (Kemmett, 2015; Woest, 2019), leading to disruptions in the dynamic balance of the mucus layer, epithelial cells and immune cells in the intestine which negatively affects feed conversion ratio (FCR) and bird health (Kemmett, 2015). Undigested protein has been suggested as a factor leading to the proliferation of *Clostridium perfringens*, coccidiosis, and associated necrotic enteritis outbreaks in chickens (Kemmett, 2015). Adding exogenous enzymes to the diet can help to improve the digestibility of the overall diet by breaking down the hemicellulose in the plant cell walls allowing the birds to have access to the "encaged" nutrients and therefore to increase the amount of available nutrients in the diet. By breaking down NSP in the diet, the enzyme creates oligosaccharides of different sizes which serves as available substrate for microbiota in the gut. Different raw materials and enzymes will result in different oligosaccharides produced, subsequently promoting the proliferation of certain populations of microbiota (Woest, 2019).

The aim of this trial was to evaluate the effect of a probiotic product (Alterion^{TM}) comprising of a single strain *Bacillus subtilis*, on broiler performance and gut microbiome diversity. A further aim was to determine if Alterion^{TM} can replace antibiotics in broiler feeds either alone or in combination with a multi complex carbohydrase enzyme (Rovabio Advance P^{TM}).
The first null hypothesis (H_0) of this study was that broilers fed a diet with probiotic (Alterion^{TM}) will not perform better than broilers fed a diet that does not contain any probiotic. The alternative hypothesis (H_1) was that broilers fed a diet with probiotic (Alterion^{TM}) will perform better than broilers fed a diet that does not contain any probiotic.
The second null hypothesis was that broilers fed a diet with probiotic (Alterion^{TM}) will perform worse than broilers fed a diet that contains an AGP (virginiamycin). The alternative hypothesis was that broilers fed a diet with probiotic (Alterion^{TM}) will perform the same than broilers fed diets containing an AGP (virginiamycin).
The third null hypothesis was that broilers fed a diet including a combination of a probiotic (Alterion^{TM}) and multi complex carbohydrase enzyme (Rovabio Advance P^{TM}) will perform

the same as broilers fed a diet with only probiotic (Alterion^{TM}), or only multi complex carbohydrase enzyme (Rovabio Advance P^{TM}). The alternative hypothesis was that broilers fed a diet with a combination of a probiotic (Alterion^{TM}) and multi complex carbohydrase (Rovabio Advance P^{TM}) will perform better than broilers fed a diet containing either a probiotic (Alterion^{TM}), or a multi complex carbohydrase, and therefore acting in a synergistic way when combined in feed.

The fourth hypothesis was that broilers fed a diet with a probiotic (Alterion^{TM}) will have a similar caecal microbiome composition than broilers fed a diet containing an AGP (virginiamycin). The alternative hypothesis was that broilers fed a diet with a probiotic (Alterion^{TM}) will have a different caecal microbiome composition when compared to the caecal microbiome composition of broilers receiving a diet with an AGP (virginiamycin).

CHAPTER 2
Literature Review

2.1 Introduction

The aim of this section is to review the current literature on the status of broiler production around the world in terms of antimicrobial growth promotor use compared to the use of alternative solutions, focusing on advantages, disadvantages, and modes of action. This review also investigates the current literature on the broiler gut microbiome with special focus on factors that influence the composition of the microbiome, together with the different methods available to determine the composition. The final part of this review discusses the use of probiotics in combination with multi complex carbohydrase enzymes to successfully replace AGP.

2.2 Antimicrobial growth promoters

Antimicrobials are used all over the world both in animals and humans for the prevention and treatment of infectious diseases (O'Neill *et al.*, 2014; Cuong *et al.*, 2018) . In addition, some countries use antimicrobials in animal production as growth promotors (Pagel and Gautier, 2012; Cuong *et al.*, 2018). The growth promoting effect of antimicrobial products used in poultry feed was discovered by feeding fermentation offal from chlortetracycline production of *Streptomyces aureofaciens* (Jukes and Williams, 1953; Butaye *et al.*, 2003). With the increase in intensive animal production, several antimicrobials have since been added to the list as growth promoters (Butaye *et al.*, 2003). These products improved animal performance in terms of feed conversion and growth, while reducing morbidity and mortality due to clinical and sub-clinical diseases (Butaye *et al.*, 2003). On average the estimated growth improvement in animals are between 4 and 8%, while feed utilisation is improved by 2 to 5% (Ewing and Cole, 1994; Butaye *et al.*, 2003). In a review, Rosen (1995) concluded that antibiotics will improve growth and FCR by 2-3% at 72% of the time (Kocher and Choct, 2019). Apart from improving FCR and performance in animals, it also reduces intestinal wall thickness, selectively modify the gut microbiome, reduce bacterial fermentation and suppress bacterial catabolism (Kocher and Choct, 2019). All of these actions subsequently result in improved health status and an increased nutrient availability to the animal which contributes to the improved performance (Kocher and Choct, 2019). Besides improving performance of animals,

increased nutrient utilisation is also of immense value in areas where there are limited resources and further more reduces the levels of nutrients excreted into the environment (Kocher and Choct, 2019). Improved overall health and welfare of animals receiving AGP and the ability of these substances to protect animals against subclinical infections caused by organisms such as *Eimeria* and *E. coli*, are probably the major reasons why it is still being used (Kocher and Choct, 2019). Microbial resistance to these AGP is the main reason for banning its use in several countries. However, there is considerable ongoing debate about whether a ban on an AGP in feed that are not used in human medicines, is justified with veterinarians questioning the existence of a link between in-feed AGP and resistance patterns in humans (Kocher and Choct, 2019).

In 2014, the European Centre for Disease Control, European Food Safety Agency and European Medicines Agency joined together and estimated in a surveillance report that across 28 European Union (EU) member states, 8927 tonnes of antimicrobial active ingredients were used for animals, compared to 3821 tonnes used for medical purposes (Prevention *et al.*, 2017; Cuong *et al.*, 2018). In the United States (US) 70% of the total antimicrobial consumption came from the animal production sector (Cuong *et al.*, 2018). However, the use of AGP has declined in recent years (Casewell *et al.*, 2003) since it has been banned in the European Union (Cogliani *et al.*, 2011) and limited in the United States by the Veterinary Feed Directive (VFD, 2015; Askelson *et al.*, 2018). However, several countries argue that continuous use of AGP is essential for the economic viability of intensive meat production and the fight against undernutrition in developing countries (Collignon *et al.*, 2005). Some even suggested that starvation would result without the use of AGP (Collignon *et al.*, 2005; Hancock, 2018). The World Health Organisation (WHO) projected a global increase in meat production from 218 million tonnes in 1999 to 376 million tonnes in 2030, with relatively larger increases in developing countries (WHO, 2004; Cuong *et al.*, 2018). The demand for animal protein and increase in intensive farming systems in middle- and low-income countries, has increased the forecast of antimicrobial use from 2010 to 2030 by 67% (Van Boeckel *et al.*, 2015; Cuong *et al.*, 2018). This coincides with the increase in intensive meat production (specifically poultry) in developing countries, where it is more difficult to efficiently regulate the use of antibiotics. To make matters worse is that important human antibiotics such as fluoroquinolones, are often used in developing countries in large quantities at subtherapeutic levels in animal feed (Collignon *et al.*, 2005). Bacteria with antibiotic-resistant genes can enter the human food chain by several ways including, but not limited to, the consumption of meat or other animal products

and through farm runoff water, with the slaughtering house being considered as the most likely place of meat contamination (Collignon *et al.*, 2005). There is considerable debate around the exact mode of action of AGP and Collignon *et al*. (2005) even questioned the actual increase in production, if any, that results from in-feed antibiotics. They mentioned that there are no double-blind, placebo-controlled studies of the continuous use of in-feed antibiotics for growth promotion. When looking at information supplied by pharmaceutical companies for their own and competitors' products, very poor to modest (0.4-2%) improvements in weight gain, when compared to a negative control, were observed (Collignon *et al.*, 2005).

2.3 Microbiota in the gastrointestinal tract of broiler chickens

At the moment of hatching, the microbiota in the GIT of the broiler chicken starts to develop from microbes that originate from the environment as well as the surface of the egg shell. These microbiota present on the surface of the egg shell originates from the gastrointestinal tract (GIT) of the breeder hen and the environment in which it was laid. The first inoculum may last for the entire production period of a broiler chicken as it affects the direction of immune system development and composition of intestinal microbiota. The densities of bacteria in the chicken GIT have been shown to increase rapidly after hatching. Apajalahti *et al.* (2004) showed that the maximum density of 10^9 cells/g of ileal digesta and 10^{11} cells/g of caecal digesta was reached less than one week, after which relative stability in the microbiota was obtained for the following 30 days. Facultative aerobes such as *Enterobacteriaceae*, *Lactobacillus*, and *Streptococcus*, are the initial colonisers as the intestinal chick environment shows positive oxidation or reduction potential at hatching (Barnes *et al.*, 1972; Lu *et al.*, 2003; Wise and Siragusa, 2007; Gong *et al.*, 2008; Rinttilä and Apajalahti, 2013).

With its wide biodiversity and number of cells that can reach up to 10^{14}, the microbiota within the gastrointestinal tract (GIT) of mammals and birds can be considered as a metabolically active organ (Macfarlane and Macfarlane, 2004; Bäckhed *et al.*, 2005; Gaggìa *et al.*, 2010). According to a study done by Lu *et al.* (2003) *lactobacilli* species were predominant in the small intestine, while the caecal microbiome were mainly composed of anaerobes and fewer numbers of facultative bacteria. Under normal circumstances the commensal bacteria is responsible and important for the maintenance of health and nutritional functions by protecting the intestinal structure and maintaining homeostasis (Lu *et al.*, 2003). The microbiota in the GIT protects the intestine of the animal against infections, while also affecting the immune

system through developmental and regulatory signals with the host (Gaggìa *et al.*, 2010). Not all microbial species in the chicken GIT have been identified and the effect on the host of some of those that have been identified is not entirely clear. Bacterial species of the genre *Lactobacillus* and *Bifidobacterium* have, however, been shown to provide protection against enteric infections (Gaggìa *et al.*, 2010). O'Hara & Shanahan (2007) found that it may be possible to treat various intestinal disorders and maintain host well-being by promoting a positive gut microbiota, through the addition of live probiotics. According to Ley *et al.* (2008) several surveys of the bacterial communities in the GIT of 60 species of mammals based on 16S rRNA-analysis showed that diet, host phylogeny and gut morphology influence the microbial ecology of the GIT (Gaggìa *et al.*, 2010). Age and health status will also affect the microbial composition present in the GIT (Mueller *et al.*, 2006; Abt and Artis, 2009). Difference in diet will also exert differences in microbial species that are present in the GIT, with large numbers of bacterial phyla present in herbivores and the lowest number in carnivores (Ley *et al.*, 2008; Gaggìa *et al.*, 2010). The major microbial groups in monogastric animals (pig, chicken and rabbits) are *Bacteroides*, *Clostridium*, *Bifidobacterium*, *Eubacterium*, *Lactobacillus*, *Enterobacteriaceae*, *Streptococcus*, *Fusobacterium*, *Peptostreptococcus* and *Propionibacterium* (Gaggìa *et al.*, 2010).

The GIT microbiota can further be subdivided as mucosal microbiota and luminal microbiota (Jeurissen *et al.*, 2002). The luminal microbiota composition is influenced by several factors including the presence of antimicrobial substances, feed passage rate and available nutrients (Shang *et al.*, 2018). Several host factors affect the mucosal-attached microbiota, which include mucus production rate, expression of specific adhesion sites on the enterocyte membrane and secretion of secretory immunoglobulins (Shang *et al.*, 2018). The mucosal-associated microbiota and luminal microbiota are also influenced by each other and therefore, it is important to note that diet can influence and change both communities of microbiota to ultimately influence gut health (Jeurissen *et al.*, 2002; Shang *et al.*, 2018).

2.3.1 Studying microbes in the chicken GIT

Various approaches have been used over the years to study the microbiota present in the chicken GIT. The original preferred method involved culturing of microorganisms, however this was later confirmed to be bias and inaccurate as most bacteria are unable to be cultured due to unknown growth requirements (Barnes *et al.*, 1972; Salanitro *et al.*, 1974; Mohd Shaufi

et al., 2015). Earlier reports have shown that only as little as 60% of the microbiota in the caeca of the gut could be cultured. In the early 2000's, more advanced techniques were introduced, including molecular fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE) and terminal-restriction fragment length polymorphism (T-RFLP) (Gong *et al.*, 2002a, 2002b; Zhu *et al.*, 2002; Diaz-Sanchez *et al.*, 2013; Wei *et al.*, 2013). To study the succession of chicken gut microbiota, Lu *et al*. (2003) utilised Sanger sequencing technology. Unfortunately, due to their low coverage, through-put and semi-quantitative features these techniques were still unable to represent the gut microbiota accurately, but were still more robust than the culture-dependent methods (Zoetendal *et al.*, 2004; Diaz-Sanchez *et al.*, 2013; Stanley *et al.*, 2014; Mohd Shaufi *et al.*, 2015). These methods were also time consuming, expensive and incapable of reflecting the true diversity of a diverse gut microbiota (Zoetendal *et al.*, 2004; Wei *et al.*, 2013). Advances in molecular biotechnology has provided new methods to study the composition, diversity, predicted function and interaction of gut microbiota in different sections of the GIT (Shang *et al.*, 2018). There are currently a broad range of molecular techniques available, each with different strengths and weaknesses, summarised in Table 2.1.

Table 2.1 Summary of the 16S rRNA-based molecular approaches for studying microbial ecology (adapted from Shang *et al.*, 2018)

Technique	Sample Capacity	Applications	Limitations	Advantages
Sequencing analysis targeted amplicons				
16S rDNA sequencing	Limited with Sanger sequencing. Non-limited with next generation sequencing	16S rRNA gene sequence, wide range identification of genus/species/strain, as database rich	Bias in DNA extraction and Primers, PCR amplification and numbers of clones, costly, high labour required	Each clone represents single molecule of rDNA. Allows precise identification of a relatively small number of OTUs
Real-time PCR (RT-PCR)	Limited	Specific gene expression in targeted groups, high in sensitivity	Bias in DNA extraction and RT-PCR, costly	
Profiling approaches				
Fingerprinting DGGE[1], TGGE[2], TTGE[3], T-RFLP[4], and SSCP[5]	Good	Amplify common 16S rDNA sequences, diversity profiles within the targeted group, rapid, comparative	Bias in DNA extraction, primers, inter and intra laboratory reproducibility remains a major challenge. Provides relatively coarse taxonomic resolution, data usually is qualitative or semi-quantitative	Amplicons may be used to form sequencing
Gene quantification				
FISH[6]	Limited	Enumeration of the bacterial population	High level of labour required at species level	Sensitivity has been improved by means of fluorescent probes
DNA microarray technology				
Diversity arrays	High	Diversity profiles, different gene expression levels	High level of labour required, costly	
DNA microarrays	High	Transcriptional fingerprint, comparative	Bias in nucleic acids extraction and their labeling, costly	

[1] Denaturing gradient gel electrophoresis
[2] Temperature gradient gel electrophoresis
[3] Temporal temperature gel electrophoresis
[4] Terminal restriction fragment length polymorphism
[5] Single strand conformation polymorphism
[6] Fluorescence in situ hybridisation

The first report utilising high-throughput sequencing of 16S rRNA genes for investigating the difference in populations of microbial communities and their interaction in the chicken GIT was published in 2013 and ever since this method had been extensively used in other research fields, to such an extent that it has become the current method of choice for these type of studies (Diaz-Sanchez *et al.*, 2013; Shang *et al.*, 2018). The 16S rRNA molecule is a small subunit of the ribosome that possesses regions of sequence similarity that are highly conserved across all bacteria (Shang *et al.*, 2018). These genes are amplified through polymerase chain reaction (PCR) by using broad-range primers, which target conserved regions of the 16S rRNA gene on microbial DNA extracted from either faecal or digesta samples (Apajalahti *et al.*, 2004; Shang *et al.*, 2018). Sequencing of the amplified products (amplicons) provides the ability to determine the composition of the bacterial community as well as the relative abundance of each species in the original sample. Sequencing can generally be done up to the genus or species level (Weisburg *et al.*, 1991; Flint *et al.*, 2006; Shang *et al.*, 2018).

High-throughput next-generation sequencing (HT-NGS) is the latest direction in which molecular technology is moving towards, due to its ability to provide large scale analysis with extraordinary coverage and depths at a relatively low cost (Pettersson *et al.*, 2009; Park *et al.*, 2013). Illumina HiSeq and MiSeq instruments are the two NGS platforms that are the most often used process in recent chicken GIT microbiome and metagenomic research (Shang *et al.*, 2018). Although these two platforms offer many advantages, they unfortunately suffer limitations such as short read assembly and high costs (Kumar and Pitta, 2015).

Single molecule real-time (SMRT) and nanopore sequencing form part of the third-generation sequencing platforms which require less time for DNA preparation and are also less expensive (Kumar and Pitta, 2015). Continuous developments and improvements in these platforms will result in new understanding of the chicken microbiome (Shang *et al.*, 2018).

2.3.2 Functions of microbes in the gut

The complex microbiome in the GIT compartments of chickens are composed of densely populated communities (protozoa, archaea, bacteria, fungi and virus) that are dominated by bacteria (Shang *et al.*, 2018). Over the years several benefits of commensal intestinal bacteria have been identified. Commensal bacteria contribute to the development of specific organs, tissues and the immune systems, as well as providing several nutritional compounds and aiding

in the digestion of less digestible compounds found in broiler diets (Dibner and Richards, 2005; Gaggìa *et al.*, 2010). Several researchers noted that the commensal bacteria of the normal intestinal microbiome play a significant role in what is called, colonisation resistance, against bacterial pathogens in the GIT of broilers. Colonisation resistance consist of two theories; competitive exclusion and immune-modulation of the developing chick (Gabriel *et al.*, 2006; Revolledo *et al.*, 2006; Brisbin *et al.*, 2007; Lee *et al.*, 2010; Chambers and Gong, 2011). Microbiota also influenced the development of the GIT; subsequently having an influence on functions such as nutrient digestion, digestive enzymes, gut mucosal proliferation, vitamin synthesis and utilisation as well as utilisation of fermentation and endogenous products (Lan *et al.*, 2005; Gabriel *et al.*, 2006).

Due to the extensive influences of the intestinal microbiota, the manipulation thereof has become a strategy of interest for the prevention of intestinal infection and promotion of broiler health and performance (Chambers and Gong, 2011). The possible impact of the microbiota on the control of pathogens has been evaluated by Nardi *et al.* (1990), where survival rate to 7 days was 100% in conventional mice and 0% in germ-free mice following challenge with 1.7 x 10^2 CFU *Salmonella enteritidis*. Several other researchers had similar results with conventional mice or guinea pigs always having improved resistance and survival rates when inoculated with a variation of pathogens like *Clostridium botulinum* and *Shigella felxneri* (Maier *et al.*, 1972; Moberg and Sugiyama, 1979). By attaching to the epithelial walls of the enterocytes the microbiota present in the gut can form a protective barrier and thus prevent the colonisation of pathogenic bacteria (Yegani and Korver, 2008; Shang *et al.*, 2018). Metabolic by-products of microbes such as vitamins, short-chain fatty acids (SCFA), organic acids, and antimicrobial compounds, can serve as nutrients for the host or aid in the inhibition of pathogenic bacteria (Apajalahti, 2005; Dibner and Richards, 2005; Yegani and Korver, 2008; Shang *et al.*, 2018).

A normal gut microbial community do not only have benefits, but also costs to the host (Gaskins *et al.*, 2002; Dibner and Richards, 2005; Shang *et al.*, 2018). Commensal microbiota provide several benefits of which the most important ones include immune stimulation and programming, competitive exclusion of pathogens and non-indigenous microbes, and contributions to host nutrition (Dibner and Richards, 2005; Shang *et al.*, 2018). Microbiota present in the GIT modulates the development of the gut through short-chain fatty acids (SCFA) produced as a by-product of fermentation (Chambers and Gong, 2011). Lactic acid

and SCFA are the main products produced by microbial fermentation in the hindgut. The major SCFA found in digesta from the caeca include butyrate, acetate and propionate (Lan *et al.*, 2005). These SCFA are available to the host as nutrients and aid in increasing intestinal tissue weight by accelerating gut epithelial cell proliferation (Chambers and Gong, 2011). Foodborne pathogens, such as *Salmonella* spp. can be eliminated by the bacteriostatic properties of SCFA produced in the hindgut of broilers (Ricke, 2003; Shang *et al.*, 2018). It has also been determined that SCFA production lowers the pH of the content in the colon, which prevents the conversion of bile to secondary bile products (Christl *et al.*, 1997; Shang *et al.*, 2018). According to Shang *et al.*, (2018), the microbiota in the gut contribute to the metabolism of host nitrogenous compounds through the conversion of uric acid into ammonia by caecal bacteria, which can be subsequently absorbed by the bird and used to produce amino acids such as glutamine. Microbiota themselves can be considered as a source of protein or amino acids as some of the nitrogen from the diet is used and incorporated into bacterial cellular protein (Metges, 2000). NSP are found in most broiler diets and serves as the main source of carbon and energy for commensal bacteria in the lower GIT, due to the inability of the hosts' intestinal enzymes to successfully degrade these complex carbohydrates in the small intestine (Rinttilä and Apajalahti, 2013). Undigested dietary protein and other metabolic secretions by the GIT and pancreas also contribute to the nutrient supply of intestinal bacteria (Rinttilä and Apajalahti, 2013). Large quantities of host-derived substances like mucins and sloughed epithelial cells are degraded by intestinal microbiota, subsequently producing microbial metabolites which can either be beneficial or have a negative effect on the health status of the animal (Rinttilä and Apajalahti, 2013). The concentration and type of metabolites that are formed depends on the substrate available as well as the microbial species involved in the fermentation process (Rinttilä and Apajalahti, 2013). The number of goblet cells producing acidic mucin as well as the composition of the mucin produced are all influenced by the composition of the developing microbiota (Forder *et al.*, 2007; Chambers and Gong, 2011). Delayed feeding after hatch will negatively influence mucosa development in the chick (Smirnov *et al.*, 2006). According to Lan *et al.* (2005), the composition and interaction between the diet and microbiota will have an influence on the intestinal development, mucosal architecture, and the mucus composition of the lower digestive tract.

Although the commensal microbiota is mostly considered as beneficial, it also incurs a cost to the host (Shang *et al.*, 2018). Specifically, in the upper part of the GIT (gizzard and small intestine), microbes compete with the host for protein and energy (Shang *et al.*, 2018).

Microbes catabolise bile acids and produce toxic metabolites in both the proximal and distal gut, which may decrease fat digestibility and depress growth of the birds, respectively (Gaskins *et al.*, 2002). The presence of microbiota in the gut results in increased amounts of mucin secreted by the gut mucus layer as well as increased epithelial cell turnover rate, thus allowing the GIT to remain lubricated while also preventing microorganisms from damaging and entering intestinal epithelial cells of the host (Shang *et al.*, 2018). The bacterial composition in the gut is also partly regulated by the intestinal immune system which secretes IgA that specifically binds to bacterial epitopes (Mitchell *et al.*, 2006; Suzuki and Nakajima, 2014). These processes are generally considered as beneficial; however, they will have an influence on the growth performance since they require higher levels of protein and energy from the host (Shang *et al.*, 2018).

According to Carter *et al.* (2009), the immunity of the chicken depends on the innate immune response and the acquired immune response which includes antibody- and cell-mediated immune responses (Chambers and Gong, 2011). The innate immunity is the first line of defence and acts in a rapid, non-specific and short-lived manner (Chambers and Gong, 2011). The acquired immune response is considered as more specific, requiring more time to be activated and will last much longer (Chambers and Gong, 2011). T-lymphocytes form part of the acquired cell mediated immunity and their function is controlled by cytokines and chemical signals (Chambers and Gong, 2011). In order to avoid inflammation of the GIT and to maintain intestinal homeostasis, there needs to be a delicate balance between immune regulation and activation (Brisbin *et al.*, 2007). The mucosal membrane can be protected by the commensal bacteria within the intestinal lumen through immune modulation and non-inflammatory processes; however, these mechanisms are not yet fully understood (Chambers and Gong, 2011). To ensure a proper immune response and avoid over stimulation of the immune system that could lead to inflammation of the GIT, the host must be able to distinguish between commensal bacteria and pathogenic bacteria (Chambers and Gong, 2011). The epithelial monolayer, mucus layer, the intestinal immune cells, and the lamina propria all for part of the immune system which development can be stimulated by the commensal microbiota (Shakouri *et al.*, 2009; Oakley *et al.*, 2014; Shang *et al.*, 2018). These tissues form a barrier between the microbes and host as well as combat the proliferation of undesirable gut microorganisms (Shang *et al.*, 2018). According to Brisbin *et al.* (2007), the immune response can be modulated by the commensal bacteria by means of increasing or decreasing the amount of mediators secreted by immune system cells associated with the intestine and by stimulating T helper and

regulatory cells. Ultimately, the microbiota plays a significant role in the immune response of the chicken and when the microbial communities are disrupted the health of the chickens can be negatively affected (Chambers and Gong, 2011).

2.3.3 Factors influencing the composition of gut microbiome

Dysbiosis is the term used to describe an imbalanced gut microbiome and can be defined as qualitative and/or quantitative imbalance of normal microbiota in the small intestine, which may lead to several reactions in the GIT, including a reduction in the intestinal barrier function and poor nutrient digestibility (Shang *et al.*, 2018). Poor nutrient digestibility may consequently lead to an increase in the risk of bacterial translocation and inflammatory responses (Teirlynck *et al.*, 2011). Dysbacteriosis can be the result of both infectious and non-infectious stressors (Shang *et al.*, 2018). Coccidiosis, toxic metabolites produced by harmful microorganisms such as *Clostridium perfringens,* or bacterial and viral challenges are all considered as infectious factors, whereas; nutritional imbalances, environmental stressors, dietary changes, poor management, mycotoxins, enzymatic dysfunction or host genetics, compose most of the non-infectious factors (Teirlynck *et al.*, 2011).

The composition of microbiota in the GIT is influenced by several factors including the diet, age, location in the GIT and level of stress experienced by the animal (Chambers and Gong, 2011; Shang *et al.*, 2018). The age of the bird is one of the most important factors that influence GIT bacterial composition, cell density and metabolic function (Shang *et al.*, 2018). At day-old the broiler chick already has a community of microorganisms within their intestinal tract (Ballou *et al.*, 2016). The acquisition of microorganisms can already happen in the pre-hatching phase through two main pathways, directly from the oviduct of the hen or from the environment through the pores in the eggshell (Cason *et al.*, 1994; Gantois *et al.*, 2009; Roto *et al.*, 2016). In commercial broiler production of today, strict hygiene practices together with fumigation of eggs when they are collected from breeder farms, reduce the overall bacterial load in the hatching environment, as well as limiting the spread of pathogens. These practices consequently expose newly hatched chicks to a diverse range of bacteria from environmental origin rather than from the breeder birds (Donaldson *et al.*, 2017). According to a recent study by Donaldson *et al.* (2017), contaminating broiler eggs with caecal microbiota from donor birds reduced bird-to-bird variation in microbiota composition but failed to transfer FCR performance from donor birds to recipients. According to Lu *et al.* (2003) the GIT of broilers

at 3 days-of-age contained *L. delbrueckii, C. perfringens*, and *Campylobacter coli*, whereas from 7 to 21 days-of-age, *Streptococcus, L. acidophilus* and *Enterococcus* were more common. The GIT contained *L. crispatus* at 28 and 49 days of age, but the overall composition was significantly different from other ages (Lu *et al.*, 2003). In other work, HT-NGS technology has shown that successional changes in gut microbial communities involved increasing concentration of *Clostridium* with the age of the bird, whereas lactobacilli concentrations remained relatively low throughout the growth cycle (Shang *et al.*, 2018). Variability in results may be ascribed to method used as coverage and accuracy of conventional microbiological and molecular methods are limited when compared to high-throughput NGS platforms, which offer higher coverage and depth in determining microbial communities (Shang *et al.*, 2018).

The GIT of the chicken is made up of the crop, proventriculus, gizzard, duodenum, jejunum, ileum, caeca, large intestine, and cloaca (Yeoman *et al.*, 2012). Each of these sections has different metabolic functions that influence the composition of the microbiota in that specific section, therefore it is important to consider sampling location and study design (Shang *et al.*, 2018). Lactobacilli is the dominating bacteria in the crop, with a total bacteria concentration of 10^8 to 10^9 cfu/g (Gong *et al.*, 2007; Rehman *et al.*, 2007). In a study done by Choi *et al.* (2014), the difference in time between feeding and sampling resulted in large variations in microbial composition among individual broilers fed on similar diets. The gizzard has a similar concentration of bacteria as the crop, although bacterial fermentation activities are low due to the low pH (Shang *et al.*, 2018). The main bacteria found in the gizzard are lactobacilli, enterococci, lactose-negative enterobacteria and coliform bacteria (Rehman *et al.*, 2007). Due to short passage time and a dilution of digesta by secreted bile, the duodenum has the lowest bacterial density among the small intestine segments (Shapiro and Sarles, 1949). Clostridia, streptococci, enterobacteria and lactobacilli are the main bacterial communities present in the duodenum (Waite and Taylor, 2015). Of all the small intestine segments, the ileum microbiota has been studied the most (Shang *et al.*, 2018). By examining 16S rRNA gene sequences Lu et al., (2003) found *Lactobacillus* (70%) as the major group of bacteria in the ileum, followed by members of the family *Clostridiaceae* (11%), *Streptococcus* (6.5%) and *Enterococcus* (6.5%). The caecum is the section of the GIT that has the most diverse, rich and stable microbial community including some anaerobes (Salanitro *et al.*, 1974; Videnska *et al.*, 2013; Shang *et al.*, 2018). According to Oakley *et al.* (2014), the caecal microbial communities change significantly from day of hatch to six weeks of age in commercial broilers (Oakley and Kogut, 2016). They also found significant differences when comparing caecal samples with faecal

samples from the same individual (Oakley and Kogut, 2016; Shang *et al.*, 2018). The richness and diversity in the caecum increased significantly during the six week period, with rapid shifts in the taxonomic composition from Proteobacteria, Bacteroides and Firmicutes, to mainly Firmicutes at three weeks-of-age (Oakley *et al.*, 2014; Oakley and Kogut, 2016). In another study done by Kumar *et al.* (2018), Firmicutes were the most abundant phylum in both the caeca and ileum at all ages from day 0 to day 41, however on day 42, Bacteroidetes were the most abundant in the caeca (Shang *et al.*, 2018). Differences in the nucleic acid extraction protocols, primers, sequencing approach, environmental factors, dietary treatment or composition, breed, and geographical conditions are all reasons for the difference in bacterial compositions (Shang *et al.*, 2018).

In the GIT of humans and animals, the mucosal layer, together with the intestinal epithelium, serves as the first sensory line of defence between the internal milieu and the luminal environment (Guarner and Malagelada, 2003). Various mucosal secretions including trefoil peptides, surfactant phospholipids and mucin glycoproteins interact and contribute to the formation of the mucus layer (Guarner and Malagelada, 2003). Surface enterocytes, M cells and intestinal dendritic cells are immunosensory cells involved in the active sampling of resident bacteria, pathogens and antigens (Gaggìa *et al.*, 2010). The resident bacteria may exert a dual function, the maintenance of the homeostasis of the immune response and the stimulation of mucosal mechanisms of defence (Gaggìa *et al.*, 2010).

The year to year increase in broiler growth rate is mainly due to genetic selection over the past decade which resulted in a very high capacity for protein deposition together with high feed intake (Havenstein *et al.*, 2003; Qaisrani *et al.*, 2014). To harness the full genetic potential of modern-day broilers, diets need to be formulated with high content of digestible protein and energy (Qaisrani *et al.*, 2014). In the absence of sufficient fermentable carbohydrates in the hindgut, undigested amino acids may be used as an energy source by microbes and subsequently result in the proliferation of certain microbial species (Reid and Hillman, 1999; Qaisrani *et al.*, 2014). Undigested protein can be used as energy source by microbes through a series of steps, starting with proteolysis then followed by deamination and decarboxylation of amino acids which then delivers a substrate that can be used as an energy source (Jeaurond *et al.*, 2008; Qaisrani *et al.*, 2014). Sulphur containing compounds, indoles, phenols, ammonia and amines are all compounds that can be produced by microbiota from undigested nitrogen in the caeca (Qaisrani *et al.*, 2014). Due to the production of volatile fatty acids, fermentation of

carbohydrates are considered beneficial, however, protein fermentation are mostly considered as detrimental to health and performance due to the production of toxic compounds such as amines and ammonia (Macfarlane *et al.*, 1988; Qaisrani *et al.*, 2014). Unfortunately, under practical circumstances, diets may still contain significant amounts of undigestible protein, thus the level and source of broiler dietary protein can influence the composition of gut microbiota as well as overall gut health of the bird (Kaldhusdal and Skjerve, 1996; Drew *et al.*, 2004; Qaisrani *et al.*, 2014). According to a study done by Drew *et al*. (2004), the level and source of dietary protein had significant effects on intestinal populations of *Clostridium perfringens* in broiler chickens. Higher levels of methionine and glycerine present in fish meal, when compared to soy protein concentrate, may be one of the contributing factors for the increased number of *Clostridium perfringens* in the caeca of broilers receiving fish meal as main protein source (Nakamura *et al.*, 1968; Muhammed *et al.*, 1975; Drew *et al.*, 2004). Under normal circumstances the number of *Clostridium perfringens* in the intestine is low (~ 10^4 cfu/g of digesta) but under certain conditions *Clostridium perfringens* may multiply, increasing numbers to 10^7 to 10^9 cfu/g of digesta, which can ultimately cause clinical disease such as necrotic enteritis (NE) (Drew *et al.*, 2004). Although *Clostridium perfringens* is recognised as the etiological agent of NE, other co-factors such as coccidiosis, infection, environment, climate, management of hygiene and diets, are usually necessary to actually cause an outbreak of the disease (Elwinger *et al.*, 1992; Kaldhusdal and Skjerve, 1996; Kaldhusdal, 2000; Drew *et al.*, 2004).

2.4 Probiotics

Probiotics is a term first introduced by Lilly and Stillwell (1965) to describe the growth promoting effects by micro-organisms, derived from the Greek word meaning “pro-life” (Jin *et al.*, 2007). Probiotics are described as viable microbial species that are purposefully ingested, at specific quantities, to alter the gastrointestinal microbiome in such a way that it promotes health benefits to the host (Gogineni *et al.*, 2013). According to Ozen & Dinleyici (2015), the history of probiotics can be traced back to the ancient times of approximately 10 000 years ago, where microorganisms, originating from fermentation products such as beer, bread, cheese and wine, were frequently used for nutritional and therapeutic purposes.

Parker (1974) first described probiotics as microorganisms or substances that aids in the balance of intestinal microbiome (Jin *et al.*, 2007). Crawford (1979) later defined probiotics as

'a culture of specific living microorganisms', which establishes itself in the animal it is fed to, where it subsequently aids in the establishment of the populations of beneficial organisms (Jin *et al.*, 2007).

2.4.1 Mode of action of probiotics

The main mechanisms promoting the beneficial effects of probiotics are still not clearly understood, but according to several researchers they are likely to be multifactorial (Bermudez-Brito *et al.*, 2012). Specific modes of action that are already identified, some more researched than others, includes the following: the secretion of antimicrobial substances, promoting and strengthening of the integrity of the gut epithelial barrier, modulating the immune system, as well as maintaining a balance of beneficial microbiota (Bermudez-Brito *et al.*, 2012). Not all mechanisms are applicable to all probiotics, but they can act through several ways against pathogens.

2.4.1.1 Maintaining a balance of beneficial microbiota

One of the characteristics of a healthy animal is having a well-functioning alimentary tract that allows the animal to efficiently digest feed and absorb nutrients (Jin *et al.*, 2007). Having a balanced microbiome with dominant populations of beneficial microorganisms is one of the key elements to achieve a well-functioning GIT. The microbial balance is disturbed when the animal is exposed to stressful conditions such as high temperatures and humidity, change of feed, poor ventilation and transportation (Jin *et al.*, 2007). When fed continuously, probiotics has been found to maintain the beneficial intestinal microbiota in two ways; by antagonistic activity toward pathogenic bacteria and by competitive exclusion (Jin *et al.*, 2007).

Antagonistic Activity: Chateau *et al*. (1993) isolated 103 *Lactobacillus* species from two commercial probiotic products and tested their ability to inhibit pathogens *in vitro*. They found that lactic acid bacteria were able to inhibit the growth of poultry pathogens like *Salmonella* and *E.coli* (Chateau *et al.*, 1993). Several other researchers also confirmed the ability of various species of *Lactobacillus* bacteria to inhibit the proliferation of various strains of *Salmonella* and *E. coli*. Lactic acid bacteria are able to produce these antagonistic activities towards pathogenic bacteria due to their ability to produce bactericidal substances. Bacteriocins, organic acids and hydrogen peroxide are a few of these bactericidal substances produced by

Lactobacilli. Bacteriocins were defined by Tagg *et al.* (1976) as compounds produced by bacteria that have a biologically active protein moiety and a bactericidal action (Jin *et al.*, 2007). One bacteriocin-like substance produced by intestinal lactobacillus species, in humans and several laboratory animals, is called lactocidin. Vincent *et al.* (1959) described lactocidin as active between pH 5.0 and 7.8, non-dialysable and insensitive to catalyse activity. They found that crude lactocidin displayed inhibitory activity against several bacterial species including *Proteus* spp., *E. coli* spp., *Staphylococcus* and *Salmonella* spp. Due to its broad spectrum of antimicrobial activities, *L. acidophilus* could play an important role in controlling undesirable microorganisms in the intestinal tract of animals and humans (Vincent *et al.*, 1959; Jin *et al.*, 2007).

Some metabolic end-products of lactic acid bacteria are associated with antagonistic activities. Organic acids such as acetic and lactic acid together with hydrogen peroxide are produced as metabolic by-products from the metabolism of *Lactobacillus* and are capable of antagonistic activities *in vitro* (Wheater *et al.*, 1952; Tramer, 1966; Dahiya and Speck, 1968; Price and Lee, 1970; Sorrells and Speck, 1970; Jin *et al.*, 2007). Acetic and lactic acids inhibit the growth of multiple bacterial species including pathogenic gram-negative organisms (Jin *et al.*, 2007). Sorrells and Speck (1970) and Adams and Hall (1988b) found that the activity of these acids depends on the pH of their surroundings (Jin *et al.*, 2007). The bactericidal activity of the acids are increased at lower pH due to the increased volume of acids in their undissociated forms (Jin *et al.*, 2007). Hydrogen peroxide produced by *Lactobacillus* spp. also induced antagonistic effects on other bacterial species (Wheater *et al.*, 1952; Price and Lee, 1970). According to Juven *et al.* (1988), certain strains of *Lactobacillus* and *Pediococcus* isolated from meat, produced high levels of hydrogen peroxide *in vitro* to initiate the oxidation of biomolecules (Jin *et al.*, 2007). Vincent *et al.* (1959) demonstrated, however that catalase had no effect on the inhibitory action produced by *L. acidophilus*, thus hydrogen peroxide was not involved. However, Gilliland and Speck (1977) found that when active catalase was added to co-cultured bacterial strains, the inhibitory action produced by *L. acidophilus* was only reduced, and not eliminated, thus suggesting that hydrogen peroxide do play a role in the antagonistic properties of *L. acidophilus.* The antibacterial action produced by *L. acidophilus* can probably be ascribed to a combination of different factors which include hydrogen peroxide, acids and bacteriocins.

Competitive Exclusion: Oral inoculation of day-old broiler chicks with a 1:10 dilution of intestinal contents from healthy adult broilers one day before being orally challenged with

Salmonella infantis, reduced the infection rate from 100% in control birds to 23% in the treated birds (Nurmi and Rantala, 1973). Many studies have been done during the past two decades on the efficacy of competitive exclusion for the control of pathogenic bacteria like *Salmonella*, *E.coli* and *Campylobacter* (Jin *et al.*, 2007). Lactic acid bacteria are the most popular bacteria used in competitive exclusion studies but the results are contradictory. Several researchers found that different *Lactobacillus* strains were able to significantly reduce coliform counts in different parts of the GIT (Watkins & Miller, 1983; Jin *et al.*, 1996). However, Adler and DaMassa (1980) found that treating day-old chicks with a single dose of *Lactobacillus* did not change the lactobacilli-coliform balance in the caecum (Jin *et al.*, 2007).

The exact mechanisms of probiotics to exclude the proliferation of pathogenic bacteria has yet to be described, although several mechanisms have been suggested including competition for adhesion sites, competition for nutrients and production of bactericidal substances (Jin *et al.*, 2007). Chick intestinal microbiota reached maximum colonisation at 48-72 hours after treatment with selected faecal microbiota, forming a mat of microbiota through interconnecting fibres (Soerjadi *et al.*, 1982; Jin *et al.*, 2007). This dense mat of microbiota may be responsible for early protection of the chick against colonisation of S*almonellae* in the caeca (Soerjadi *et al.*, 1982; Jin *et al.*, 2007). This, together with studies done by Muralidhara *et al.* (1977) and Conway *et al.* (1987), support the theory that direct competition for attachment sites is probably the main mechanism of competitive exclusion.

Competition for available nutrients is another theory suggested as a means of controlling intestinal bacterial populations, but it is unlikely to be an effective mechanism for competitive exclusion (Jin *et al.*, 2007). According to a study by Rolfe (1991), nutrients from the diet of the host and manipulation of ingredients through digestion processes of the host and several other environmental factors may enhance the growth of specific microbial populations and subsequently result in the exclusion of others (Jin *et al.*, 2007). Fructo-oligosaccharides are substrates of digestion and are not digested by host intestinal enzymes, thus serving as nutrients for *Lactobacilli* and *Bifidobacteria* which can utilise the compound as energy source, ultimately causing an increase in the size of their population (Mulder, 1991; Jin *et al.*, 2007). Diet composition and available substrates can ultimately influence microbial composition and subsequently gut health can be optimised or compromised.

2.4.1.2 Enhancing epithelial barrier functions

The intestinal lining of epithelial cells is in permanent contact with the luminal content and the ever changing, dynamic population of microbiota. In order for the organism to remain protected from the environment, it is important to maintain epithelial integrity by means of a proper intestinal barrier (Bermudez-Brito *et al.*, 2012). A proper intestinal barrier consist of four defence mechanisms which include the mucous layer, secretory IgA, antimicrobial peptides and the epithelial junction adhesion complex (Bermudez-Brito *et al.*, 2012). Intestinal disorders may occur when inflammatory responses are induced by food and bacterial antigens reaching the submucosa after disruption of the barrier function. Even though the mechanism by which probiotics enhance intestinal barrier function are not fully understood, several researchers have found that probiotic bacteria are involved in the maintenance of the barrier functions. Another possible mechanism to enhance intestinal barrier function is by increased expression of the genes involved in tight junction signalling (Anderson *et al.*, 2010).

2.4.1.3 Immuno-modulation

The development of the immune response can be influenced by the manipulation of gut microbiota through administration of probiotics (Anderson *et al.*, 1999). Although, the exact mechanism by which probiotics influence the immune system is not fully understood, some studies have shown that probiotics stimulate different levels of immune system cells to produce cytokines, which subsequently play a role in the regulation and induction of the immune system (Maassen *et al.*, 2000; Christensen *et al.*, 2002; Lammers *et al.*, 2003; Haghighi *et al.*, 2005). When human peripheral blood mononuclear cells were stimulated *in vitro* with *Lactobacillus rhamnosus* it resulted in the production of interleukin (IL)-4, IL-6, IL-10, tumor necrosis factors alpha, and gamma interferon (Schultz *et al.*, 2003; Haghighi *et al.*, 2005). More studies confirmed lactobacilli induced increased levels of Th2 cytokines, such as IL-4 and IL-10 (Christensen *et al.*, 2002; Lammers *et al.*, 2003; Rakoff-Nahoum *et al.*, 2004; Haghighi *et al.*, 2005). Increased production of Th2 cytokines subsequently leads to the increased development of B cells as well as the switching of immunoglobulin isotype required for the production of antibodies (Haghighi *et al.*, 2005). Increased production of the mucosal IgA response is dependent on other cytokines, such as transforming growth factor (Lebman and Edmiston, 1999; Haghighi *et al.*, 2005). Various species and strains of lactobacilli are able to induce the

production of transforming growth factor β, although the efficacy of this varies among strains (Blum *et al.*, 2002; Haghighi *et al.*, 2005). Lactobacilli can modulate the systemic antibody response to antigens in broilers (Huang *et al.*, 2004; Koenen *et al.*, 2004; Haghighi *et al.*, 2005). Enhanced immunity to *Eimeria acervulina* may be brought about by the secretion of cytokines and changes in lymphoid cells in the GIT of broilers, due to the administration of probiotics (Dalloul *et al.*, 2003a, 2003b; Haghighi *et al.*, 2005). However, more research is required on the induction of a systemic antibody response to soluble and cellular antigens as well as on the antibody response in the GIT (Haghighi *et al.*, 2005).

2.4.2 Main probiotic species

Lactobacillus

The genus of *Lactobacillus* compromises of more than 100 different species and is considered as a wide and heterogeneous taxonomic unit, belonging to the group of lactic acid-producing bacteria (LAB) (Gaggìa *et al.*, 2010). They usually constitute a significant portion of the normal gut microbiota of humans and animals, although the occurrence and number are host dependent. According to Hammes and Hertel (2006), several of the species in this genus are purposely introduced in various sectors of the food chain, being involved in a range of food and feed fermentation processes, while also being used as probiotics in humans and animals. *Lactobacillus casei* and *Lactobacillus rhamnosus* might sometimes be involved in human diseases, as stated by several reports (Vesterlund *et al.*, 2007), however no report can be found on safety concerns related to lactobacilli in animals (Gaggìa *et al.*, 2010). A list of species has been proposed for Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) (Authority, 2008; Gaggìa *et al.*, 2010).

Enterococcus

The *Enterococcus* genus also belongs to the LAB group. They are naturally found in food products and are normal human and animal commensals. They are often used as starter cultures in food products, such as cheese, as probiotic cultures for humans and animals as well as silage additives (Foulquié Moreno *et al.*, 2006; Gaggìa *et al.*, 2010). *E. faecium* seems to be the most prevalent in animals, while *E. faecium* and *E. faecalis* are the most common in the human GIT (Fisher and Phillips, 2009; Gaggìa *et al.*, 2010).

The *Enterococcus* genus is sometimes associated with human infections and is of medical relevance due to its increased incidence as a cause of disease in hospital acquired infections, as well as antibiotic resistance towards available antibiotic therapies (Gaggìa *et al.*, 2010). According to Leavis *et al.* (2006), several virulence factors have been described and the number of enterococci that shows resistance towards vancomycin is also increasing. Species such as *E. durans* and *E. hirae* have been associated with infections in chickens, whereas strains belonging to *E. faecium* have been used rather safely in industrial and agricultural applications (Chadfield *et al.*, 2005; Abe *et al.*, 2006; Gaggìa *et al.*, 2010). The use of enterococci as probiotics, therefore, remain a controversial issue. Even though the probiotic benefits of some strains are well established, the fact that multiple antibiotic resistant strains together with the emergence and increased association of enterococci with human diseases, have raised concerns regarding their use as probiotics (Gaggìa *et al.*, 2010). According to Kayser (2003) and Foulquie Moreno *et al.* (2006), there is a concern of antimicrobial resistance genes or genes encoding virulence factors that could be transferred to other bacteria in the GIT. None of the *Enterococcus* genus members have been proposed for QPS status due to safety concerns (Authority, 2008).

Bacillus

The *Bacillus* species are Gram-positive, spore-forming microorganisms usually associated with water, air and soil. They are usually found in the intestinal tract due to involuntary ingestion of contaminated feed and are thus normally considered as allochthonous microbes (Gaggìa *et al.*, 2010). According to Sanders *et al.* (2003), several *Bacillus* species used as animal feed supplements, probiotics, plant protection products or seed coating agents are also known as agents of food poisoning. The safety of some species could be assured by the QPS approach, due to knowledge gained from their use as animal feed supplements (Authority, 2008). The fact that most *Bacillus* species potentially possess toxigenic traits means the absence of toxigenic activity needs to be verified before it can qualify as possible probiotic (Gaggìa *et al.*, 2010). *Bacillus subtilis* is a specific strain of *Bacillus* that are commonly used as an animal feed additive. Its spore forming abilities enable it to withstand feed production processes and make it resistant to acidic and alkalic environments, ensuring that it reaches the target organs in the GIT of the chicken (Gaggìa *et al.*, 2010). *Bacillus subtilis* is an aerobic bacteria, consuming large amounts of oxygen while reproducing in the GIT, thus enabling the proliferation of favourable anaerobic bacteria such as *Lactobacillus*, *Bifidobacterium* and

yeasts, while strongly resisting the growth of the majority aerobic pathogenic bacteria (Wang *et al.*, 2006; Gao *et al.*, 2017).

Saccharomyces

Saccharomyces spp. is also part of the residual microbial population of the intestinal microbiome and falls into a genus of budding yeast (Gaggìa *et al.*, 2010). *Saccharomyces cerevisiae* can be found in plants, fruit and soil and is in essence widespread in nature. With its specific fermentation process, it is widely used in food and beverage processes as well as in some health foods (Gaggìa *et al.*, 2010). *Saccharomyces boulardii* is considered as a biotype of *S. cerevisiae*, and is used as a probiotic in ruminants and swine feed (van der Aa Kühle and Jespersen, 2003; Gaggìa *et al.*, 2010).

2.5 Non-starch polysaccharides

According to Bailey (1973), cellulose, non-cellulosic polymers and pectic polysaccharides forms the three main groups of NSP. Non-cellulosic polymers include xyloglucan, mannans, mixed-linked β-glucans and arabinoxylans, whereas the group of pectic polysaccharides are formed by polygalacturonic acids substituted with arabinogalactan, arabinan and galactan (Sinha *et al.*, 2011; Choct, 2015). A group of NSP that is not as abundant as cellulose, hemicellulose or pectins includes the fructans, glucomannans and galactomannans (Sinha *et al.*, 2011).

Maize and soybean meal are generally considered as highly digestible feed ingredients and contributes around 65% of apparent metabolizable energy and 80% of crude protein respectively to broiler diets in South Africa (Cowieson, 2005). Unfortunately, according to several researchers, 15-25% of feed consumed by broiler chickens will escape digestion mainly because of insufficient activity of specific enzymes and the presence of anti-nutritional factors (Zanella *et al.*, 1999; Bedford and Partridge, 2010; Cowieson, 2010). Non-starch polysaccharides are one of the main anti-nutrient factors that may limit the nutritive value of feed and according to Irish and Balnave (1993) maize and soybean meal-based diets can contain various levels of NSP (Tahir *et al.*, 2008).

Digestion and absorption of other nutrients can be affected, either directly or indirectly by NSP (Sinha *et al.*, 2011; De Vries *et al.*, 2012; Woest, 2019). Direct effect involves encapsulation of starch, fat and protein, by the NSP coat, inhibiting digestive enzymes to access those nutrients. Indirect effect is caused by the presence of NSP which results in the increase of the viscosity of the digesta in the lumen of the GIT, subsequently resulting in reduced motility of digestive enzymes and impaired absorption (Căpriţă *et al.*, 2010; Woest, 2019). Intestinal microbiota composition and quantity may also be affected by the presence of NSP in raw materials and forms part of the anti-nutritive effects of NSP (Simon, 1998; Apajalahti, 2001; Woest, 2019).

2.5.1 Non-starch polysaccharide effect on gut microbiome

The GIT of the animal is not only considered as the largest immunological organ, but also as the most important organ when it comes to nutrient digestion, absorption as well as protection against pathogens (Choct, 2009; Woest, 2019). The microbiota present in the GIT of the animal has an influence on several biological processes including digestion, development of the intestines, absorption of nutrients as well as alterations of the immune system, thus ultimately influencing the health status and growth performance of the animal (Yang *et al.*, 2009; Matin *et al.*, 2012; Woest, 2019). Pathogenic and beneficial microbiota is present during all times, but a favourable balance should be maintained to obtain optimum production results. The balance between pathogenic and beneficial organisms can be disrupted by several factors including age, pH, diet, passage rate, mucosal secretions as well as any disorder affecting the immune system (Matin *et al.*, 2012; Woest, 2019). The extent to which these factors can influence the microbial balance depends on the circumstances under which the host finds itself as well as the composition and activity of the gut microbiome (Yang *et al.*, 2009; Bedford and Cowieson, 2012; Woest, 2019).

High levels of soluble NSP in the diet will result in a viscous intestinal environment which will decrease the rate at which feed moves through the intestine, and lower the rate of nutrient digestion (Salih *et al.*, 1991; Bederska-Łojewska *et al.*, 2017; Woest, 2019). Almost 90% of bacteria in the GIT are present in the large intestine but a reduced passage rate will allow the opportunity for some of these bacteria to migrate into the small intestine, subsequently increasing the number of anaerobic microbes in the upper parts of the GIT (Smits and Annison, 1996; McNab and Boorman, 2002; Apajalahti *et al.*, 2004; Jozefiak *et al.*, 2007; Parker *et al.*,

2007). Thus, high levels of soluble NSP can lead to the proliferation of pathogenic bacteria in the small intestine, which may reduce the availability of nutrients to the host due to the bacteria's ability to utilise nutrients such as starch and protein (Salih *et al.*, 1991; Bedford, 1995; Bedford and Cowieson, 2012). Rivière *et al.* (2014) found that high amounts of undegraded arabinoxylan that reached the colon stimulated the development of residing bacteria such as *Bifidobacterium, Lactobacillus, Clostridium, Bacteriodes* and *Eubacterium*.

2.5.2 Effect of carbohydrase enzyme on gut microbiota

The microbiota in the lumen of the GIT plays an influential role in the absorption and digestion of nutrients and may also be affected by carbohydrase enzymes in the diet (Woest, 2019). When feeding animals a diet high in NSP content, adding carbohydrase enzymes may improve gut health by reducing digesta viscosity, subsequently resulting in reduced shedding of microorganisms and a decrease in the proliferation of pathogenic bacteria (Vahjen *et al.*, 1998; Castillo and Gatlin III, 2015; Woest, 2019). Exogenous enzymes have a multifactorial effect in the lumen of the GIT, by partitioning nutrients to the host as well as producing nutrients for specific populations of bacteria by means of their mode of action (Bedford and Cowieson, 2012; Woest, 2019). According to a study done by Adeola & Cowieson (2011), beneficial bacteria populations may be promoted by the action of exogenous carbohydrase enzymes, subsequently leading to improved gut and overall health of the animal. The breakdown of plant cell wall carbohydrates by non-starch polysaccharide degrading enzymes, result in the production of smaller polymers and oligomers, which subsequently act as substrate for bacterial fermentation. Alteration of the population profiles of gut-associated microbiota by exogenous enzymes, can positively alter volatile fatty acid production (Bedford & Apajalahti, 2001; Woest, 2019).

2.6 Conclusion

When the European Union started with the ban on AGP in broiler feed, there was a significant increase in the incidence of reported NE cases from 4% to 12.5% of diseases in France in the years 1995 to 1999. Similar figures were reported in other European countries as well, thus it is important to understand the effect of AGP on bird performance and the possible consequences of removing them from broiler diets, to efficiently put together a successful strategy to replace them. Looking at the mode of action of exogenous carbohydrase enzymes

and probiotics, it might be a possible solution to promote optimum gut health and aid in obtaining required bird performance in the absence of AGP, either each on their own or in combination.

CHAPTER 3
Materials and Methods

3.1 Facilities and birds

Experimental procedures were approved by the Faculty of Natural and Agricultural Sciences Research Ethics committee of the University of Pretoria (NAS178/2020). The trial was conducted at the Wincanton trial facility of Sovereign Foods in Uitenhage, South Africa. A total of 2304 male Ross 308 day-old chicks were obtained from Sovereign Foods Hatchery in Uitenhage, South Africa. The birds were housed in an environmentally controlled, semi-commercial broiler house. The house had a solid concrete floor that was evenly covered with pine shavings. The house contained 96 pens of 1.04m x 1.04m each, giving a floor space of 1.08 m^2 per pen. The pens were arranged in a single row over the length of the house and were surrounded by a further 58 000 Ross 308 broilers not restricted to pens to simulate commercial conditions as much as possible. The 2304 broilers used in the experiment were randomly allocated to the pens, with 24 birds per pen, at a stocking density of 22.22 birds/m^2.

3.2 Hygiene and biosecurity

The biosecurity protocol of Sovereign Foods was strictly adhered to at all times. The broiler house was cleaned and washed with Pharmabac and then disinfected with Paragon Plus (Immuno-Vet Services, Kya Sand, Randburg, South Africa) before placing the birds. Foot baths (Vet Fluid-O, Immuno-vet services) were placed at the entrance of the broiler house and all workers used only gumboots allocated to this specific house. All farm visits, truck deliveries and pests were monitored to promote maximum biosecurity. All people working with the chickens were required to shower before entering and exiting the farm. Mortalities were collected, weighed and recorded accordingly on a daily basis. Dead and culled birds were removed from the broiler house for post-mortem examination and incineration.

3.3 General management

Birds were placed, managed and cared for according to the standard operating procedures of Sovereign Foods. Each pen was provided with one tube feeder and shared one line of nipple drinkers with five nipples in each pen. The height of the feeder and drinker line were adjusted according to bird growth. The standard heating and lighting programs of Sovereign Foods were

followed, and which is shown in Table 3.1 and Table 3.2, respectively. The birds had *ad libitum* access to feed and water throughout the trial. Tube feeders were refilled when necessary and shaken twice a day to ensure consistent feed availability throughout the trial. Environmental conditions were monitored and controlled throughout the duration of the trial. The chicks were vaccinated on day 0, 7, 12 and 17 following the schedule indicated in Table 3.3.

Table 3.1 Temperature profile for trial

Days	Target temperature °C
0 - 6	35
7 - 13	31
14 - 20	27
21 - 27	25
28 - 35	23

Table 3.2 Lighting program for the duration of the trial

Days	Hours light on	Hours light off
0 - 1	24	0
2 - 7	23	1
8 - 21	18	6
22 - 31	20	4
32 - 33	22	2
34 - 35	23	1

Table 3.3 Vaccination program

Age	Disease	Application method
Day 0	Infectious Bronchitis	Course Spray (Hatchery)
Day 0	Newcastle Disease	Course Spray (Hatchery)
Day 0	Infectious Bursal Disease (Gumboro)	Course Spray (Hatchery)
Day 12	Infectious Bursal Disease (Gumboro)	Course Spray (On-farm)
Day 12	Newcastle Disease	Course Spray (On-farm)
Day 17	Infectious Bursal Disease (Gumboro)	Course Spray (On-farm)
Day 17	Newcastle Disease	Course Spray (On-farm)

3.4 Experimental design and diets

In order to evaluate the efficacy and extent to which the single strain *Bacillus subtilis* probiotic, either on its own or in combination with a multi-enzyme product, can alter the performance and composition of the caecal microbiome, a study was done using a randomised complete block design.

The description of the treatment groups and experimental diets can be seen in Table 3.4. All diets were formulated based on the standard commercial nutrient specification and raw materials used by Sovereign Foods. The negative control group (NC) received the basal diet which contained no AGP, probiotic or NSP-enzyme. The positive control group (PC) received the same basal diet, with the only difference being the inclusion of an AGP (Stafac; Phibro Animal Health) at 40 g/ton. The Rovabio Advance enzyme (Adisseo, France) was added at 50 g/ton to the basal diet for Treatments 2 and 4 as well as to diet of Treatment 1 (also contained AGP). The probiotic Alterion 2% (Adisseo, France) was added at 500 g/ton to the basal diet for Treatment 3 and Treatment 4.

Table 3.4 The experimental groups and diets used in the trial

Experimental groups	Experimental diet description
NC = Negative Control	Basal diet without feed additives
PC = Positive Control	Basal diet + AGP
Treatment 1 (PCE)	Basal diet + AGP + NSP-enzyme
Treatment 2 (NCE)	Basal diet + NSP-enzyme (without AGP)
Treatment 3 (NCP)	Basal diet + Probiotic (without AGP)
Treatment 4 (NCEP)	Basal diet + NSP-enzyme + Probiotic (without AGP)

AGP: Antimicrobial growth promoter (Stafac at 40 g/ton)
NSP-enzyme: Non-starch polysaccharide degrading enzyme (Rovabio Advance (Adisseo, France) at 50 g/ton)
Probiotic: Alterion 2% (Adisseo, France) at 500 g/ton

Three feeding phases were used over the five week study period. The first phase was a starter diet, which was fed from day 0 to day 10, followed by a grower diet, from day 10 to day 21. The third phase (finisher) diet was fed from day 21 to day 28. The fourth and final phase (post-finisher) diet was fed from day 28 to day 35. The starter diet was in crumble form, while the grower, finisher and post-finisher diets were in pellet form. Each of the six treatments included in the study was repeated once within a block, with a total of 16 replicates per treatment.

3.5 Feed formulas

Least cost feed formulation software (Format International, UK) was used to formulate the broiler diets for the starter, grower, finisher and post-finisher phases. The ME content, crude protein, and digestible amino acids levels of a typical South African maize and soybean meal-based diet were used and formulated according to the nutrient specifications of Ross 308. The starter and grower diets were formulated to be 0.5 MJ AME lower in energy and 0.1% higher in digestible amino acid levels, than those of typical standard commercial diets, in an attempt to stimulate hindgut protein fermentation and thereby inducing a microbial challenge in the caeca of the broilers. For the same purpose the finisher and post finisher diets were formulated to be 0.3 MJ AME lower in energy and 0.01% higher in digestible amino acid levels. All the diets were formulated to contain expected levels of 1000 FTU/kg of a phytase enzyme (Axtra Phy 10000 TPT, Du Pont Delaware, United States) at an inclusion level of 100 mg/kg. Dietary Treatments 1, 2 and 4 were formulated to contain a minimum level of 1250 visco units/kg of xylanase at an inclusion level of 50 mg/kg of Rovabio Advance (Adisseo, France). Dietary Treatments 3 and 4 were formulated to contain a minimum level of 1.10^8 CFU *Bacillus subtilis*/kg feed at an inclusion level of 500 mg/kg Alterion 2% (Adisseo, France).

The experimental diets were mixed by SimpleGrow (Pty) Ltd (Pretoria, South Africa). The feed ingredients to be used for the diets were procured and stored separately. Representative samples of the feed ingredients were collected and analysed, with NIR, prior to feed formulation in order to formulate the diets based on accurate nutrient concentrations. Metabolisable energy and digestible amino acids were calculated based on standard procedures (CVB, 2007). All additives (AGP, NSP-enzyme, probiotic) were added to the basal diets without assigning it any nutrient values during formulation. Tables 3.5 to 3.8 show the raw material composition and calculated nutrient specifications of the starter, grower, finisher and post finisher basal diets.

Table 3.5 Raw material composition of the starter diets on an as-fed basis (%)

Ingredient	PC	NC	PCE	NCE	NCP	NCEP
Yellow maize	55.50	55.50	55.50	55.50	55.50	55.50
Soybean oilcake (46.5%)	32.45	32.45	32.45	32.45	32.45	32.45
Sunflower oilcake (36%)	4.00	4.00	4.00	4.00	4.00	4.00
Full fat soya	3.50	3.50	3.50	3.50	3.50	3.50
Limestone	1.66	1.66	1.66	1.66	1.66	1.66
Mono dicalcium phosphate	1.27	1.27	1.27	1.27	1.27	1.27
Sodium bicarbonate	0.36	0.36	0.36	0.36	0.36	0.36
Salt	0.12	0.12	0.12	0.12	0.12	0.12
Lysine HCL (78%)	0.28	0.28	0.28	0.28	0.28	0.28
Methionine DL (99%)	0.30	0.30	0.30	0.30	0.30	0.30
Threonine L (98%)	0.14	0.14	0.14	0.14	0.14	0.14
Vitamin and mineral premix	0.15	0.15	0.15	0.15	0.15	0.15
Choline chloride (60%)	0.20	0.20	0.20	0.20	0.20	0.20
Coccidiostat Coyden 25%	0.05	0.05	0.05	0.05	0.05	0.05
Phytase enzyme (Axtra Phy 10000)	0.01	0.01	0.01	0.01	0.01	0.01
Antimicrobial growth promotor (Stafac)	0.004	0.00	0.004	0.00	0.00	0.00
NSP enzyme (Rovabio Advance)	0.00	0.00	0.005	0.005	0.00	0.005
Probiotic (Alterion 2%)	0.00	0.00	0.00	0.00	0.05	0.05

Vitamin and mineral premix composition of the starter feed in a 1.5kg unit, with contribution per kg of complete feed: vitamin A: 12 000IU; vitamin D3: 4000 IU; vitamin E: 60 IU; vitamin K3: 4mg; vitamin B1: 4mg; vitamin B2: 9mg; vitamin B3: 60mg; vitamin B5: 15mg; vitamin B6: 5mg; vitamin B9: 2mg; vitamin B12: 0.025mg; vitamin H: 0.2mg; antioxidant: 200mg; Mn: 100mg; Fe: 70mg; Zn: 60mg; Cu: 20mg; Se: 0.3mg; I: 1.25mg. Selenium is supplied in the form of sodium selenite, and iodine in the form of calcium iodate. Copper, manganese, iron and zinc are supplied in the form of sulphates. NC=Negative Control, PC=Positive Control, PCE=Treatment 1, NCE=Treatment 2, NCP=Treatment 3, NCEP=Treatment 4.

Table 3.6 Raw material composition of the grower diets on an as-fed basis (%)

Ingredient	PC	NC	PCE	NCE	NCP	NCEP
Yellow maize	59.95	59.95	59.95	59.95	59.95	59.95
Soybean oilcake (46.5%)	26.75	26.75	26.75	26.75	26.75	26.75
Sunflower oilcake (36%)	5.00	5.00	5.00	5.00	5.00	5.00
Full fat soya	4.35	4.35	4.35	4.35	4.35	4.35
Limestone	1.62	1.62	1.62	1.62	1.62	1.62
Mono dicalcium phosphate	0.71	0.71	0.71	0.71	0.71	0.71
Sodium bicarbonate	0.37	0.37	0.37	0.37	0.37	0.37
Salt	0.11	0.11	0.11	0.11	0.11	0.11
Lysine HCL (78%)	0.29	0.29	0.29	0.29	0.29	0.29
Methionine DL (99%)	0.26	0.26	0.26	0.26	0.26	0.26
Threonine L (98%)	0.13	0.13	0.13	0.13	0.13	0.13
Vitamin and mineral premix	0.15	0.15	0.15	0.15	0.15	0.15
Choline chloride (60%)	0.20	0.20	0.20	0.20	0.20	0.20
Coccidiostat Coyden 25%	0.05	0.05	0.05	0.05	0.05	0.05
Phytase enzyme (Axtra Phy 10000)	0.01	0.01	0.01	0.01	0.01	0.01
Antimicrobial growth promotor (Stafac)	0.004	0.00	0.004	0.00	0.00	0.00
NSP enzyme (Rovabio Advance)	0.00	0.00	0.005	0.005	0.00	0.005
Probiotic (Alterion 2%)	0.00	0.00	0.00	0.00	0.05	0.05

Vitamin and mineral premix composition of the starter feed in a 1.5kg unit, with contribution per kg of complete feed: vitamin A: 12 000IU; vitamin D3: 4000 IU; vitamin E: 60 IU; vitamin K3: 4mg; vitamin B1: 4mg; vitamin B2: 9mg; vitamin B3: 60mg; vitamin B5: 15mg; vitamin B6: 5mg; vitamin B9: 2mg; vitamin B12: 0.025mg; vitamin H: 0.2mg; antioxidant: 200mg; Mn: 100mg; Fe: 70mg; Zn: 60mg; Cu: 20mg; Se: 0.3mg; I: 1.25mg. Selenium is supplied in the form of sodium selenite, and iodine in the form of calcium iodate. Copper, manganese, iron and zinc are supplied in the form of sulphates. NC=Negative Control, PC=Positive Control, PCE=Treatment 1, NCE=Treatment 2, NCP=Treatment 3, NCEP=Treatment 4.

Table 3.7 Raw material composition of the finisher diets on an as-fed basis (%)

Ingredient	PC	NC	PCE	NCE	NCP	NCEP
Yellow maize	68.50	68.50	68.50	68.50	68.50	68.50
Soybean oilcake (46.5%)	16.10	16.10	16.10	16.10	16.10	16.10
Sunflower oilcake (36%)	5.00	5.00	5.00	5.00	5.00	5.00
Full fat soya	6.85	6.85	6.85	6.85	6.85	6.85
Limestone	1.46	1.46	1.46	1.46	1.46	1.46
Mono dicalcium phosphate	0.57	0.57	0.57	0.57	0.57	0.57
Sodium bicarbonate	0.39	0.39	0.39	0.39	0.39	0.39
Salt	0.10	0.10	0.10	0.10	0.10	0.10
Lysine HCL (78%)	0.31	0.31	0.31	0.31	0.31	0.31
Methionine DL (99%)	0.20	0.20	0.20	0.20	0.20	0.20
Threonine L (98%)	0.11	0.11	0.11	0.11	0.11	0.11
Vitamin and mineral premix	0.15	0.15	0.15	0.15	0.15	0.15
Choline chloride (60%)	0.20	0.20	0.20	0.20	0.20	0.20
Coccidiostat Coyden 25%	0.05	0.05	0.05	0.05	0.05	0.05
Phytase enzyme (Axtra Phy 10000)	0.01	0.01	0.01	0.01	0.01	0.01
Antimicrobial growth promotor (Stafac)	0.004	0.00	0.004	0.00	0.00	0.00
NSP enzyme (Rovabio Advance)	0.00	0.00	0.005	0.005	0.00	0.005
Probiotic (Alterion 2%)	0.00	0.00	0.00	0.00	0.05	0.05

Vitamin and mineral premix composition of the starter feed in a 1.5kg unit, with contribution per kg of complete feed: vitamin A: 12 000IU; vitamin D3: 4000 IU; vitamin E: 60 IU; vitamin K3: 4mg; vitamin B1: 4mg; vitamin B2: 9mg; vitamin B3: 60mg; vitamin B5: 15mg; vitamin B6: 5mg; vitamin B9: 2mg; vitamin B12: 0.025mg; vitamin H: 0.2mg; antioxidant: 200mg; Mn: 100mg; Fe: 70mg; Zn: 60mg; Cu: 20mg; Se: 0.3mg; I: 1.25mg. Selenium is supplied in the form of sodium selenite, and iodine in the form of calcium iodate. Copper, manganese, iron and zinc are supplied in the form of sulphates. NC=Negative Control, PC=Positive Control, PCE=Treatment 1, NCE=Treatment 2, NCP=Treatment 3, NCEP=Treatment 4.

Table 3.8 Raw material composition of the post finisher diets on an as-fed basis (%)

Ingredient	PC	NC	PCE	NCE	NCP	NCEP
Yellow maize	68.50	68.50	68.50	68.50	68.50	68.50
Soybean oilcake (46.5%)	16.10	16.10	16.10	16.10	16.10	16.10
Sunflower oilcake (36%)	5.00	5.00	5.00	5.00	5.00	5.00
Full fat soya	6.85	6.85	6.85	6.85	6.85	6.85
Limestone	1.46	1.46	1.46	1.46	1.46	1.46
Mono dicalcium phosphate	0.57	0.57	0.57	0.57	0.57	0.57
Sodium bicarbonate	0.39	0.39	0.39	0.39	0.39	0.39
Salt	0.10	0.10	0.10	0.10	0.10	0.10
Lysine HCL (78%)	0.31	0.31	0.31	0.31	0.31	0.31
Methionine DL (99%)	0.20	0.20	0.20	0.20	0.20	0.20
Threonine L (98%)	0.11	0.11	0.11	0.11	0.11	0.11
Vitamin and mineral premix	0.15	0.15	0.15	0.15	0.15	0.15
Choline chloride (60%)	0.20	0.20	0.20	0.20	0.20	0.20
Coccidiostat Coyden 25%	0.00	0.00	0.00	0.00	0.00	0.00
Phytase enzyme (Axtra Phy 10000)	0.01	0.01	0.01	0.01	0.01	0.01
Antimicrobial growth promotor (Stafac)	0.004	0.00	0.004	0.00	0.00	0.00
NSP enzyme (Rovabio Advance)	0.00	0.00	0.005	0.005	0.00	0.005
Probiotic (Alterion 2%)	0.00	0.00	0.00	0.00	0.05	0.05

Vitamin and mineral premix composition of the starter feed in a 1.5kg unit, with contribution per kg of complete feed: vitamin A: 12 000IU; vitamin D3: 4000 IU; vitamin E: 60 IU; vitamin K3: 4mg; vitamin B1: 4mg; vitamin B2: 9mg; vitamin B3: 60mg; vitamin B5: 15mg; vitamin B6: 5mg; vitamin B9: 2mg; vitamin B12: 0.025mg; vitamin H: 0.2mg; antioxidant: 200mg; Mn: 100mg; Fe: 70mg; Zn: 60mg; Cu: 20mg; Se: 0.3mg; I: 1.25mg. Selenium is supplied in the form of sodium selenite, and iodine in the form of calcium iodate. Copper, manganese, iron and zinc are supplied in the form of sulphates. NC=Negative Control, PC=Positive Control, PCE=Treatment 1, NCE=Treatment 2, NCP=Treatment 3, NCEP=Treatment 4.

Table 3.9 Calculated nutrient specifications of the four basal diets on an as-fed basis (%)

	Starter	Grower	Finisher	Post Finisher
Dry matter	89.47	89.41	89.31	89.31
AME broiler (MJ/kg)	10.90	11.20	11.80	11.81
Crude protein	22.97	21.27	17.78	17.78
Crude fat	3.13	3.39	4.03	4.03
Crude fat (acid hydrolysis)	3.70	3.95	4.60	4.60
Crude fibre	3.58	3.83	3.88	3.88
Total calcium	1.00	0.88	0.79	0.79
Digestible calcium	0.68	0.64	0.58	0.58
Sodium	0.16	0.16	0.16	0.16
Chloride	0.20	0.20	0.20	0.20
Potassium	1.01	0.92	0.76	0.76
Total lysine	1.42	1.31	1.09	1.09
Total sulphur amino acids	1.02	0.94	0.79	0.79
Total threonine	1.00	0.92	0.76	0.76
Total tryptophan	0.26	0.24	0.19	0.19
Total isoleucine	0.97	0.89	0.72	0.72
Total arginine	1.55	1.42	1.14	1.14
Total leucine	1.90	1.78	1.54	1.54
Digestible lysine	1.25	1.15	0.95	0.95
Digestible methionine	0.61	0.56	0.46	0.46
Digestible cysteine	0.30	0.28	0.24	0.24
Digestible sulphur amino acids	0.91	0.84	0.70	0.70
Digestible threonine	0.86	0.79	0.65	0.65
Digestible tryptophan	0.23	0.21	0.17	0.17
Digestible isoleucine	0.86	0.78	0.63	0.64
Digestible arginine	1.39	1.27	1.03	1.03
Digestible leucine	1.70	1.60	1.39	1.39
Digestible valine	0.93	0.86	0.71	0.72
Digestible histidine	0.54	0.50	0.41	0.41

AME: Apparent metabolisable energy

3.6 Production parameters

3.6.1 Body weight and body weight gain

The body weight of all the chickens were determined at day old, day 10, 21, 28 and 35 on a per pen basis. All birds in a pen were placed in a single crate and weighed simultaneously. Average body weight (g/bird), weekly body weight gain (g/bird/day) and body weight gain (g/bird) for the overall trial period were calculated for each pen.

3.6.2 Feed intake

The amount of feed consumed by the chickens were determined per pen with each transition from the one phase feed to the next (day 10, 21 and 28) as well as at the end of the rearing period on day 35. At the beginning of each phase period, feed from each pen was weighed into an empty bin. Feed from the bin was then added to the feeders in the pen as necessary. If the bin was empty before the end of the period, extra feed was weighed into the bin first and then added to the feeders as necessary. With every change to the next phase, the left-over feed from the previous phase was weighed back and discarded. The average feed intake for all birds were calculated per phase period as well as cumulative from day 0.

3.6.3 Feed conversion ratio

FCR; Feed conversion ratio (unit of feed consumed per unit of live mass gained) were calculated by dividing the amount of feed consumed by the body weight gained. To correct for feed consumed before a bird died, the weight of the dead birds in every pen during the period was added to the live weight of the pen at the end of the period. Feed conversion ratio was calculated per period at days 10, 21, 28 and 35 and cumulative from day 0.

3.7 Sampling and processing

On day 25, two birds per pen (192 birds in total) were selected based on individual weights closest to the mean body weight of birds in the pen. The selected broilers were euthanised via cervical dislocation prior to all sampling. Euthanised birds were dissected under aseptic

conditions, the entire digestive system caudal to the proventriculus removed for scoring of gut health and collection of caecal samples.

3.7 Macroscopic gut health scoring

Macroscopic scoring was done at 25 days-of-age by a registered veterinarian from Adisseo (France). To allow no bias in results, scoring was performed blind, with dedicated persons collecting the birds and other doing the actual scoring in a different location outside the house. Scoring of individual birds followed immediately after removal of the digestive system caudal to the proventriculus. The proposed method by Teirlynck *et al.* (2011) was followed and each bird received a score between 0 and 10 for intestinal dysbacteriosis parameters, where 0 represented a normal GIT and 10 represented severe dysbacteriosis. Ten parameters were assessed. Both sections cranial and caudal to the Meckel's diverticulum were scored individually for the following parameters: presence of ballooning; reduction in overall thickness of the gut wall; significant redness of the serosa or mucosa; and abdominal gut content (mucous, water or gas) as well as undigested feed particles caudal to the ileocaecal junction as proposed by Teirlynck *et al.* (2011). First, ballooning was assessed with the gut intact, thereafter 10 cm incisions (situated approximately 10 cm caudal and cranial from Meckel's diverticulum) were made to expose the serosa for scoring. Thickness of the gut wall was evaluated by judging the flaccidity of the gut edges three seconds after opening of the intestinal segment by longitudinal incision. Caudal to the ileocaecal junction, a 10 cm incision was made to expose the digesta for scoring. An average score per pen was calculated from the scores of both birds and used as a single data point.

3.8 Microbial analysis

Birds from specific treatments (Negative Control, Positive Control, Treatment 2, Treatment 3, Treatment 4) were selected for digesta sampling at 25 days-of-age. These treatments were selected as it was of most interest for this study and limited funds prevented analyses of all treatments. Caecal content samples were collected from two broilers from all pens relating to previously mentioned treatments. Both caeca were removed and emptied into 5 mL Eppendorf tubes, labelled and stored in dry ice. Samples were frozen at -80°C until analysis on microbial populations could commence. Ten Eppendorf tubes from each treatment were analysed for

microbiome diversity. The populations of total bacteria were determined by q-PCR performed by Genoscreen (Lille, France). In short, the genomic DNA samples were PCR amplified using a universal primer pair (341F and 785R – targeting V3 and V4 of the 16S rRNA gene). Resulting amplicons were gel purified, end repaired and illumina specific adapter sequences were ligated to each amplicon. Following fluorometric quantification, the samples were individually indexed, and a further ampure bead based purification step was performed. Amplicons were then sequenced on Illumina's MiSeq platform, using MiSeq v3 (600 cycles) kit. Only reads of sufficient Q scores (>q20) and lengths were analysed. 20MB of data (2x300bp paired-end reads) were produced for each sample. For each sample, a breakdown of the different levels of classification (kingdom, phylum, class, order, family, and genus) of present microbes was supplied and analysed.

3.9 Statistical analysis

3.9.1 Performance data

The linear model used is described by the following equation:

$Y_{ijk} = \mu + T_i + L_j + TL_{ij} + B_k + e_{ijk}$

Where Y = variable studied during the period

μ = overall mean of the population

T = effect of the i^{th} treatment

L = effect of the j^{th} level

TL = effect of the ij^{th} interaction between treatment and level

B = effect of the k^{th} block

e = error associated with each Y

Data were analysed statistically as a randomised complete block design with the GLM model (SAS, 2018) for the average effects over time. The row of 96 pens was divided into 16 blocks with 6 pens per block. Repeated Measures Analysis of Variance with the GLM model was used for repeated period measures. Means and standard errors were calculated and significance of difference ($P < 0.05$) was determined by Fischer's test (Samuels, 1989).

Mortality data and macroscopic gut scoring were analysed with the frequency model of SAS (2018), by means of the standard chi-square tests. The level of statistical significance was $P < 0.05$.

3.9.2 Microbial data

The distribution of the % abundance of microbes was not symmetrical or reasonably Normal with constant variance, so nonparametric tests that do not make any strong assumptions about the actual form of the distributions, were used (Freund *et al.*, 2010). Firstly, Friedman's nonparametric test for analysing a randomised complete block design, (i.e. the data consists of observations on k treatments assessed under n different conditions (blocks)) was used to test for differences between the five treatment group effects per microbe. Then the Mann-Whitney U test was used to compare the negative control treatment (NC) against the combined four other treatments (NCE, NCP, NCEP & PC), and finally, to compare the positive control (PC) against treatment 3 (T3) (Siegel, 1956). Data were analysed using the statistical program GenStat® (VSN International, 2017). The level of statistical significance was $P < 0.05$. A level of $P < 0.1$ was accepted as a tendency towards a significant difference.

CHAPTER 4
Results

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

The influence of treatments on the body weight (BW) of broilers is summarised in Table 4.1. Chick weight did not differ significantly ($P > 0.05$) between the treatments at the start of the trial (day 0) or any other time after that, except for a significant difference ($P < 0.05$) on day 28 between PC, PCE, NCE and NCP, with PC and PCE having a significantly higher BW than NCE and NCP. The body weight of the broilers was above the Ross 308 breed standards (Aviagen, 2014) for all treatments throughout the trial.

Table 4.1 The effect of feed additive on the average periodic body weight (g) of broilers from day 0 to 35

Treatment	Day 0	Day 10	Day 21	Day 28	Day35
NC	45.20	327.0	1124	1753[ab]	2496
PC	44.98	329.9	1098	1785[a]	2516
PCE	44.93	327.9	1121	1785[a]	2518
NCE	45.34	321.2	1103	1736[b]	2475
NCP	45.23	327.9	1087	1736[b]	2501
NCEP	45.14	323.8	1120	1746[ab]	2492
SEM	0.19	3.35	23.58	14.41	23.44

[a,b] Column means with common superscript did not differ significantly for least square means ($P > 0.05$)
NC = Negative Control (Base diet without AGP/Probiotic/NSP-enzyme
PC = Positive Control (Base diet + AGP (Stafac 40 g/t))
PCE = Basal diet + AGP (Stafac 40 g/t) + NSP -enzyme (Rovabio 50 g/t)
NCE = Basal diet + NSP-enzyme (Rovabio 50 g/t)
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
NCEP = Basal diet + NSP-enzyme (Rovabio 50 g/t) + Probiotic (Alterion 2% 500 g/t)
SEM: Standard error of the mean

4.2 Average periodic weight gain of broilers

There were no significant differences ($P > 0.05$) in the periodic weight gain of broilers between any of the treatments during the trial. The periodic weight gain of broilers is summarised in Table 4.2.

Table 4.2 The effect of all treatments on average periodic weight gain (g / bird / period) of broilers from 0 to 35 days

Treatment	Day 0 - 10	Day 10 - 21	Day 21 - 28	Day 28 - 35
NC	281.4	794.7	572.7	732.0
PC	284.5	762.5	637.7	710.7
PCE	282.6	790.4	604.5	706.9
NCE	275.5	782.0	581.4	714.5
NCP	281.9	754.5	589.7	750.0
NCEP	278.2	793.2	569.4	735.5
SEM	3.33	23.05	27.02	19.57

NC = Negative Control (Basal diet without AGP/Probiotic/NSP-enzyme
PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
PCE = Basal diet + AGP (Stafac 40 g/t) + NSP -enzyme (Rovabio 50 g/t)
NCE = Basal diet + NSP-enzyme (Rovabio 50 g/t)
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
NCEP = Basal diet + NSP-enzyme (Rovabio 50 g/t) + Probiotic (Alterion 2% 500 g/t)
SEM: Standard error of the mean

4.3 Feed intake of broilers

The average feed intake of broilers over the different phase periods is summarised in Table 4.3. There were no significant differences ($P > 0.05$) in feed intake between any of the treatments during the trial.

Table 4.3 The effect of each treatment on periodic feed intake (g / bird / period) of broilers from 0 to 35 days corrected for mortalities

Treatment	Day 0 - 10	Day 10 - 21	Day 21 - 28	Day 28 - 35
NC	322.8	1084	961.3	1245
PC	325.4	1096	968.0	1245
PCE	325.4	1098	969.5	1223
NCE	320.9	1087	957.7	1227
NCP	325.3	1094	959.0	1223
NCEP	323.3	1088	969.3	1226
SEM	3.32	8.68	8.98	14.59

NC = Negative Control (Basal diet without AGP/Probiotic/NSP-enzyme
PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
PCE = Basal diet + AGP (Stafac 40 g/t) + NSP -enzyme (Rovabio 50 g/t)
NCE = Basal diet + NSP-enzyme (Rovabio 50 g/t)
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
NCEP = Basal diet + NSP-enzyme (Rovabio 50 g/t) + Probiotic (Alterion 2% 500 g/t)
SEM: Standard error of the mean

4.4 Cumulative feed intake of broilers

The cumulative feed intake of all treatments is shown in Table 4.4. There were no significant differences (P > 0.05) between treatments in terms of cumulative feed intake during the trial.

Table 4.4 The effect of treatment on the average cumulative feed intake (g / bird) of broilers from 0 to 35 days

Treatment	Day 0 - 10	Day 0 - 21	Day 0 - 28	Day 0 - 35
NC	322.8	1409	2441	3701
PC	325.4	1427	2459	3733
PCE	325.4	1426	2471	3732
NCE	320.9	1408	2431	3693
NCP	325.3	1424	2460	3703
NCEP	323.3	1414	2455	3696
SEM	3.32	10.86	19.55	32.98

NC = Negative Control (Basal diet without AGP/Probiotic/NSP-enzyme
PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
PCE = Basal diet + AGP (Stafac 40 g/t) + NSP -enzyme (Rovabio 50 g/t)
NCE = Basal diet + NSP-enzyme (Rovabio 50 g/t)
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
NCEP = Basal diet + NSP-enzyme (Rovabio 50 g/t) + Probiotic (Alterion 2% 500 g/t)
SEM: Standard error of the mean

4.5 Periodic feed conversion ratio of broilers

The periodic feed conversion ratio of the broilers in the different treatments, are shown in Table 4.5. There were no significant differences (P > 0.05) for FCR of the broilers between any of the treatments during the first three periods of the trial, however, broilers from NCP performed significantly better (P < 0.05) in the last 7 days of the trial compared to the PC and PCE.

Table 4.5 The average periodic feed conversion ratio of broilers (g feed intake / g body weight gain) for different treatments from day 0 to 35

Treatment	Day 0 - 10	Day 10 - 21	Day 21 - 28	Day 28 - 35
NC	1.14	1.38	1.51	1.68^{ab}
PC	1.14	1.46	1.40	1.72^{b}
PCE	1.15	1.41	1.45	1.70^{b}
NCE	1.16	1.42	1.51	1.68^{ab}
NCP	1.15	1.47	1.48	1.60^{a}
NCEP	1.16	1.38	1.52	1.65^{ab}
SEM	0.01	0.05	0.05	0.03

^{a,b} Column means with common superscript did not differ significantly for least square means (P > 0.05)
NC = Negative Control (Basal diet without AGP/Probiotic/NSP-enzyme
PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
PCE = Basal diet + AGP (Stafac 40 g/t) + NSP -enzyme (Rovabio 50 g/t)
NCE = Basal diet + NSP-enzyme (Rovabio 50 g/t)
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
NCEP = Basal diet + NSP-enzyme (Rovabio 50 g/t) + Probiotic (Alterion 2% 500 g/t)
SEM: Standard error of the mean

4.6 Cumulative feed conversion ratio

The cumulative feed conversion ratio (CFCR) of the broilers in the different treatments is shown in Table 4.6. The broilers from all treatments performed better than Ross 308 breed standards (Objectives, 2014) in terms of CFCR during the 35-days rearing period. There were no significant differences (P > 0.05) amongst the treatments over the 10, 21 and 35-day periods. There were, however, a significant difference between the PC, PCE and NCP for the 28 day period, with improved performance of the PC and PCE when compared to NCP.

Table 4.6 The average cumulative feed conversion ratio of broilers (g feed intake / g body weight gain) for different treatments from day 0 to 35

Treatment	Day 0 - 10	Day 0 - 21	Day 0 - 28	Day 0 - 35
NC	1.143	1.261	1.393^{ab}	1.483
PC	1.141	1.313	1.378^{a}	1.485
PCE	1.149	1.284	1.385^{a}	1.483
NCE	1.165	1.288	1.401^{ab}	1.494
NCP	1.149	1.321	1.418^{b}	1.482
NCEP	1.157	1.270	1.406^{ab}	1.483
SEM	0.010	0.029	0.010	0.012

^{a,b} Column means with common superscript did not differ significantly for least square means (P > 0.05)
NC = Negative Control (Basal diet without AGP/Probiotic/NSP-enzyme
PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
PCE = Basal diet + AGP (Stafac 40 g/t) + NSP -enzyme (Rovabio 50 g/t)
NCE = Basal diet + NSP-enzyme (Rovabio 50 g/t)
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
NCEP = Basal diet + NSP-enzyme (Rovabio 50 g/t) + Probiotic (Alterion 2% 500 g/t)
SEM: Standard error of the mean

4.7 Mortality

The chi-square frequency analysis of the total mortalities of the broilers for the different treatments is shown in Table 4.7. The mortality of the broilers were not significantly affected by treatment (P > 0.05).

Table 4.7 Chi-square frequency analysis of total mortalities of the broilers for the different treatments from day 0 to 35

	Mortality (%)				
Treatment	Day 10	Day 21	Day 28	Day 35	Total
NC	1.04	0.52	4.69	0.52	6.77
PC	0.78	1.56	4.17	1.04	7.55
PCE	0.78	0.78	4.95	1.3	7.81
NCE	0.78	0.01	4.43	1.3	6.52
NCP	1.56	1.3	4.95	0.78	8.59
NCEP	1.04	0.78	4.69	0.52	7.03

NC = Negative Control (Basal diet without AGP/Probiotic/NSP-enzyme
PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
PCE = Basal diet + AGP (Stafac 40 g/t) + NSP -enzyme (Rovabio 50 g/t)
NCE = Basal diet + NSP-enzyme (Rovabio 50 g/t)
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
NCEP = Basal diet + NSP-enzyme (Rovabio 50 g/t) + Probiotic (Alterion 2% 500 g/t)

4.8 Macroscopic gut health score

The chi-square frequency analysis of the macroscopic gut health score of the broilers for the different treatments, are shown in Table 4.8. Feed additive had no significant effect (P > 0.05) on the macroscopic gut health score of the broilers at 25 days-of-age.

Table 4.8 The effect of feed additive on macroscopic gut health score of broilers at 25 days-of-age

	Macroscopic score (Number of birds observed)				
Treatment	0	1	2	3	4
NC	4	3	3	4	2
PC	5	6	2	2	1
PCE	8	6	1	0	1
NCE	4	5	4	2	1
NCP	2	7	3	3	1
NCEP	3	3	7	1	2

NC = Negative Control (Basal diet without AGP/Probiotic/NSP-enzyme
PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
PCE = Basal diet + AGP (Stafac 40 g/t) + NSP -enzyme (Rovabio 50 g/t)
NCE = Basal diet + NSP-enzyme (Rovabio 50 g/t)
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
NCEP = Basal diet + NSP-enzyme (Rovabio 50 g/t) + Probiotic (Alterion 2% 500 g/t)

4.9 Microbiome and microbial diversity

Table 4.9 shows the main effect of feed additive on the caecal microbiome at a phylum level of male broilers at 25 days-of-age. There was no significant difference in percentage abundance between any of the treatments ($P > 0.05$) when looking at the Friedman's nonparametric test, however there was a tendency towards a significant effect ($P < 0.10$) on the percentage abundance of *Tenericutes*, with NCEP having the highest average and NCE the lowest.

Table 4.9 The effect of feed additive on the percentage abundance of the caecal microbiome at a phylum level of broilers at 25 days of age (Friedman's non-parametric test)

Phylum	NC	NCE	NCP	NCEP	PC	P-Value
Other	0.02	0.03	0.60	0.06	0.00	0.66
Actinobacteria	15.11	7.09	10.47	2.52	19.21	0.35
Bacteroidetes	31.07	23.90	40.55	26.49	23.34	0.14
Firmicutes	46.98	60.43	45.34	67.24	51.53	0.32
Lentisphaerae	0.04	0.00	0.01	0.01	0.00	0.63
Proteobacteria	6.33	8.36	2.62	3.15	5.67	0.53
Tenericutes	**0.40**	**0.17**	**0.37**	**0.52**	**0.21**	**0.08**[1]
Verrucomicrobia	0.05	0.01	0.02	0.00	0.03	0.87

NC = Negative Control (Basal diet without AGP/Probiotic/NSP-enzyme)
PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
NCE = Basal diet + NSP-enzyme (Rovabio 50 g/t)
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
NCEP = Basal diet + NSP-enzyme (Rovabio 50 g/t) + Probiotic (Alterion 2% 500 g/t)
[1] Tendency towards significant effect at $P < 0.1$

Table 4.10 and Figure 4.1 shows the main effect of feed additive on the caecal microbiome at a phylum level of male broilers at 25 days-of-age when comparing NCP (Probiotic) against the PC (AGP). There was a significant difference ($P < 0.05$) in percentage abundance of the *Tenericutes* phyla between NCP and the PC according to the Mann-Whitney U test, with NCP having a significantly higher average percentage abundance. There was also a tendency towards a significant effect ($P < 0.10$) on the percentage abundance of the *Bacteroidetes* and *Proteobacteria* phyla, with NCP having the highest average of *Bacteroidetes* and the PC the highest average of *Proteobacteria*. There were no significant differences ($P > 0.05$) between the two treatments when comparing the other phyla analysed.

Table 4.10 The effect of feed additive on the percentage abundance of the caecal microbiome at a phylum level of broilers at 25 days-of-age when comparing the positive control treatment (PC) to Treatment 3 (NCP) with the Mann-Whitney U test

Phylum	NCP	s.d.	PC	s.d.	P-Value
Other	0.60	1.88	0	0.01	0.48
Actinobacteria	10.47	18.73	19.21	20.38	0.19
Bacteroidetes	**40.55^{A}**	**19.21**	**23.34^{B}**	**21.91**	**0.06**
Firmicutes	45.34	12.32	51.53	20.09	0.39
Lentisphaerae	0.01	0.03	0	0	0.47
Proteobacteria	**2.62^{B}**	**2.23**	**5.67^{A}**	**3.88**	**0.06**
Tenericutes	**0.37^{a}**	**0.57**	**0.21^{b}**	**0.67**	**0.02**
Verrucomicrobia	0.02	0.06	0.03	0.06	0.72

PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
s.d. = Standard deviation
^{a,b} Row means with different superscript differed significantly (P > 0.05)
$^{A, B}$ Row means with different superscript showed a tendency towards a significant difference (P > 0.1)

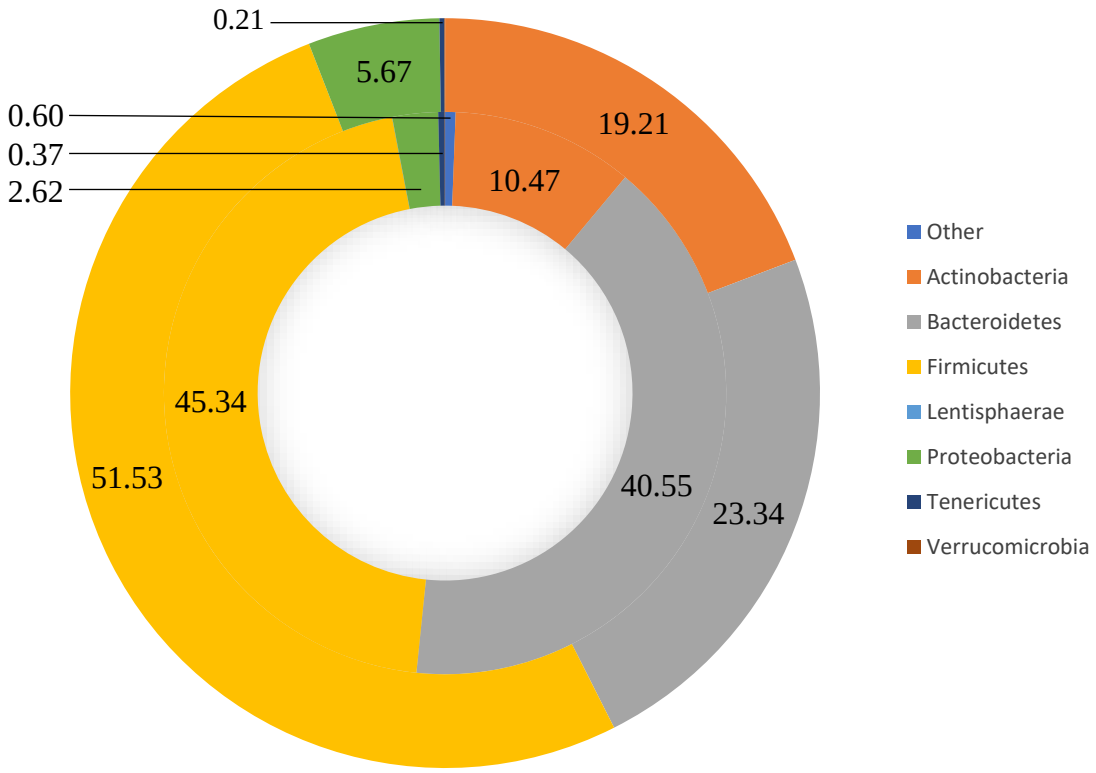

Figure 4.1 Composition of the caecal microbiome at a phylum level of male broilers at 25d of age. The inner and outer rings indicate NCP and PC, respectively

Table 4.11 shows the main effect of feed additive on the caecal microbiome at an order level of male broilers at 25 days-of-age. There were significant differences ($P < 0.05$) in percentage abundance between all treatments in the *Bacillales* and *RF39* order levels considering the Friedman's nonparametric test, with NCEP having the highest average percentage abundance and the PC the lowest in both orders. There were no significant differences ($P > 0.05$) between the treatments for any of the other orders analysed.

Table 4.11 The effect of feed additive on the percentage abundance of the caecal microbiome at an order level of broilers at 25 days of age (Friedman's non parametric test)

Order	NC	NCE	NCP	NCEP	PC	P-Value
Other	0.02	0.03	0.60	0.06	0	0.66
Bifidobacteriales	15.09	6.97	10.47	2.52	19.21	0.35
Coriobacteriales	0.02	0.12	0	0	0	0.26
Bacteroidetes_Other	0.09	0.12	0.12	0.36	1.31	0.39
Bacteroidales	30.98	23.78	40.44	26.13	22.03	0.14
Firmicutes_Other	0	0	0.15	0	0	0.41
Bacillales	**0.02**	**0.03**	**0.08**	**0.08**	**0**	**0.05**[1]
Lactobacillales	0.57	0.49	0.10	0.05	0.12	0.98
Clostridiales	46.40	59.90	45.00	67.09	51.40	0.30
Erysipelotrichales	0	0	0.01	0.01	0.01	0.86
Victivallales	0.04	0	0.01	0.01	0	0.63
Proteobacteria_Other	0	0	0	0	0	0.65
Rickettsiales	0	0	0.01	0	0	0.20
Sphingomonadales	0	0	0.01	0	0	0.26
Burkholderiales	1.10	0.90	0.41	0.70	0.77	0.58
Desulfovibrionales	0	0	0	0.40	0.05	0.48
Campylobacterales	1.88	5.67	0.68	1.58	2.49	0.48
Aeromonadales	0	0	0.03	0	0.08	0.48
Enterobacteriales	3.34	1.78	1.48	0.47	2.29	0.25
Anaeroplasmatales	0.01	0	0.03	0.04	0.19	0.87
RF39	**0.39**	**0.17**	**0.34**	**0.49**	**0.02**	**0.02**[1]
Verrucomicrobiales	0.05	0.01	0.02	0	0.03	0.87

NC = Negative Control (Basal diet without AGP/Probiotic/NSP-enzyme
PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
NCE = Basal diet + NSP-enzyme (Rovabio 50 g/t)
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
NCEP = Basal diet+ NSP-enzyme (Rovabio 50 g/t) + Probiotic (Alterion 2% 500 g/t)
[1] Significant effect at $P < 0.05$

Table 4.12 and Figure 4.2 shows the main effect of feed additive on the caecal microbiome at an order level of male broilers at 25 days-of-age when comparing NCP (Probiotic) against the PC (AGP). According to the Mann-Whitney U test there was a significant difference ($P < 0.05$) in percentage abundance of the *Bacillales* and *RF39* orders between NCP and the PC, with NCP having a significantly higher average percentage abundance in both orders. There were no evidence of *Bacillales* in the PC treatment. There was also a tendency towards a significant effect ($P < 0.10$) on the percentage abundance of the *Bacteroidales* and *Campylobacterales* orders, with NCP having the highest average of *Bacteroidales* and the PC the highest average of *Campylobacterales*. There were no significant differences ($P > 0.05$) between the two treatments for any of the other orders analysed. NCP, however, showed a greater abundance of bacterial orders present in the caeca than the PC.

Table 4.12 The effect of feed additive on the percentage abundance of the caecal microbiome at an order level of broilers at 25 days-of-age when comparing the positive control treatment (PC) to treatment 3 (NCP) with the Mann-Whitney U test

Order	NCP	s.d.	PC	s.d.	P-Value
Other	0.601	1.876	0.004	0.007	0.480
Bifidobacteriales	10.470	18.730	19.210	20.380	0.190
Coriobacteriales	0	0	0	0	1.000
Bacteroidetes_Other	0.116	0.189	1.310	3.807	0.889
Bacteroidales	**40.440^{A}**	**19.160**	**22.030^{B}**	**22.230**	**0.052**
Firmicutes_Other	0.149	0.471	0	0	1.000
Bacillales	**0.080^{a}**	**0.116**	**0**	**0**	**0.011**
Lactobacillales	0.104	0.140	0.117	0.192	0.719
Clostridiales	45.000	12.430	51.400	20.100	0.353
Erysipelotrichales	0.006	0.018	0.007	0.023	1.000
Victivallales	0.011	0.029	0	0	0.474
Proteobacteria_Other	0.001	0.003	0	0	1.000
Rickettsiales	0.006	0.013	0.002	0.005	0.721
Sphingomonadales	0.006	0.017	0.003	0.007	1.000
Burkholderiales	0.407	0.824	0.768	0.803	0.105
Desulfovibrionales	0	0	0.047	0.147	1.000
Campylobacterales	**0.684^{B}**	**0.685**	**2.485^{A}**	**2.802**	**0.089**
Aeromonadales	0.031	0.099	0.076	0.210	0.737
Enterobacteriales	1.484	1.867	2.293	3.147	0.435
Anaeroplasmatales	0.028	0.089	0.188	0.594	1.000
RF39	**0.341^{a}**	**0.569**	**0.025^{b}**	**0.079**	**0.013**
Verrucomicrobiales	0.022	0.064	0.030	0.058	0.721

PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
s.d. = Standard deviation
^{a,b} Row means with different superscript differed significantly ($P > 0.05$)
$^{A, B}$ Row means with different superscript showed a tendency towards a significant difference ($P > 0.1$)

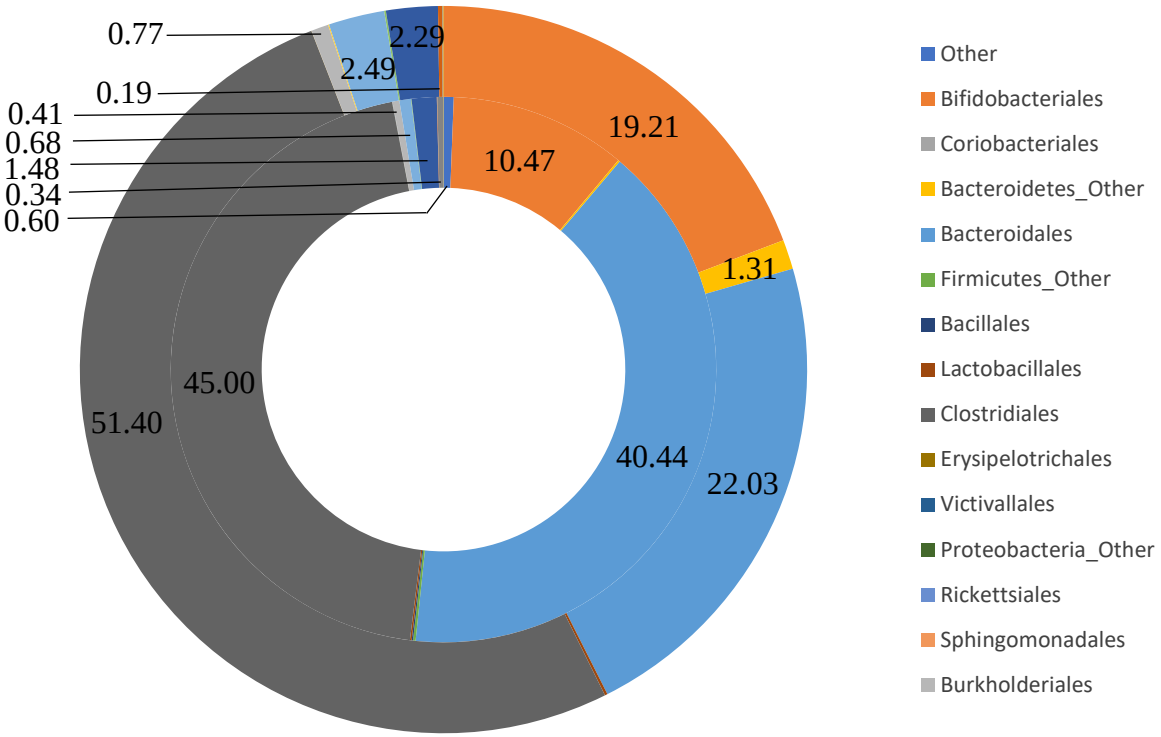

Figure 4.2 Composition of the caecal microbiome at an order level of male broilers at 25 days-of-age. The inner and outer rings indicate NCP and PC, respectively

The main effect of feed additive on the caecal microbiome at a genus level of male broilers at 25 days-of-age is shown in Table 4.13. Feed additives showed a tendency towards a significant effect ($P < 0.10$) between treatments in five different genera. The PC had a tendency ($P < 0.10$) toward a higher percentage abundance in the *Clostridium*, *Lachnospiraceae*_Unknown and *Mogibacteriaceae*_Unknown genera, whereas NCP and NCEP had a tendency ($P < 0.10$) towards a higher percentage abundance in *Dehalobacteriaceae*_Unknown and *Ruminococcaceae*_Unknown respectively. There was a significant difference ($P < 0.05$) in percentage abundance amongst treatments in the *Bacillaceae*_Unknown, *Clostridiales*_Unknown, *Dehalobacterium*, *Anaerostipes*, *Coprococcus* and *RF39*_Unknown genera, with NCEP having the highest average percentage abundance in the genera *Bacillaceae*_Unknown, *Clostridiales*_Unknown, *Dehalobacterium* and *RF39*_Unknown, NCP the highest *Anaerostipes* and the PC the highest *Coprococcus* average percentage abundance.

Table 4.13 The effect of feed additive on the percentage abundance of the caecal microbiome at a genus level of broilers at 25 days-of-age (Friedman's non-parametric test)

Genus	NC	NCE	NCP	NCEP	PC	P-Value
Other	0.017	0.034	0.601	0.058	0.004	0.633
Bifidobacteriaceae_Unknown	0	0.005	0.012	0.004	0.006	0.379
Bifidobacterium	15.090	6.960	10.460	2.520	19.210	0.345
Coriobacteriaceae_Unknown	0.023	0.124	0	0	0	0.255
Bacteroidetes_Other	0.094	0.118	0.116	0.364	1.310	0.392
Bacteroides	0.001	0	0	0	0.005	0.255
Rikenellaceae_Other	0.005	0.011	0.010	0.006	0.008	0.464
Rikenellaceae_Unknown	30.790	23.190	40.420	25.730	21.630	0.138
Alistipes	0.097	0.002	0.001	0.021	0.352	0.685
Barnesiellaceae_Unknown	0.089	0.561	0	0.374	0.010	0.133
Barnesiella	0.002	0.014	0	0	0	0.558
Bacillaceae_Unknown	**0.020**	**0.032**	**0.080**	**0.085**	**0**	**0.050[1]**
Enterococcus	0.013	0.049	0.030	0.008	0.033	0.492
Lactobacillus	0.018	0.008	0.017	0.007	0.002	0.245
Streptococcus	0.537	0.436	0.057	0.039	0.082	0.980
Clostridiales_Other	0.387	0.667	0.771	0.657	0.221	0.129
Clostridiales_Unknown	**7.740**	**12.360**	**7.930**	**22.770**	**5.010**	**0.003[1]**
Christensenellaceae_Unknown	0.349	0.388	0.285	0.526	0.320	0.428
Clostridium	**0.512**	**0.319**	**0.193**	**0.431**	**2.130**	**0.073[2]**
Dehalobacteriaceae_Unknown	**0.003**	**0.007**	**0.008**	**0**	**0.001**	**0.075[2]**
Dehalobacterium	**0.005**	**0.016**	**0.018**	**0.043**	**0.010**	**0.019[1]**
Anaerofustis	0.002	0.002	0	0.005	0.001	0.157
Lachnospiraceae_Other	0.722	0.996	0.889	0.692	1.781	0.417
Lachnospiraceae_Unknown	**1.612**	**1.976**	**1.059**	**1.974**	**3.495**	**0.062[2]**
Anaerostipes	**0.023**	**0**	**0.088**	**0.008**	**0**	**0.010[1]**
Blautia	0.427	0.498	0.638	0.333	1.214	0.118
Coprococcus	**2.688**	**2.806**	**5.960**	**2.727**	**11.076**	**0.010[1]**
Defluviitalea	0.763	0.473	0.919	1.262	0.356	0.677
Dorea	0.652	0.530	0.495	0.372	0.762	0.300
Lachnospira	0.006	0.020	0.006	0	0	0.360
Roseburia	0	0.010	0	0.016	0	0.558
Ruminococcus	2.323	5.863	3.628	3.181	4.191	0.176
Ruminococcaceae_Other	0.396	0.501	0.485	0.493	0.408	0.224
Ruminococcaceae_Unknown	**4.150**	**3.852**	**2.470**	**5.598**	**1.498**	**0.054[2]**
Anaerotruncus	1.925	0.267	0.544	0.334	0.470	0.967
Butyricicoccus	3.580	7.336	4.207	4.229	3.802	0.417
Clostridium	0.005	0.019	0.005	0.009	0.010	0.599
Faecalibacterium	0.003	0.004	0.001	0.003	0	0.414
Oscillospira	16.940	19.230	13.050	20.030	13.510	0.345
Ruminococcus	0.684	1.356	0.895	0.925	0.408	0.130
Subdoligranulum	0.001	0.002	0	0	0.002	0.406

Megasphaera	0.004	0.026	0	0.075	0.014	0.736
Phascolarctobacterium	0.059	0.028	0.015	0.044	0.129	0.817
Mogibacteriaceae_Unknown	**0.338**	**0.221**	**0.374**	**0.262**	**0.566**	**0.083[2]**
Erysipelotrichaceae_Other	0.001	0.001	0.006	0.007	0.007	0.856
Victivallaceae_Unknown	0.039	0.005	0.011	0.011	0	0.632
Proteobacteria_Other	0.005	0	0.001	0.002	0	0.652
Sutterella	1.098	0.902	0.407	0.697	0.768	0.579
Desulfovibrio	0	0.002	0	0.399	0.045	0.478
Campylobacter	1.369	5.009	0.229	0.675	1.135	0.417
Helicobacter	0.515	0.659	0.454	0.903	1.351	0.533
Succinatimonas	0	0.003	0.031	0	0.076	0.478
Escherichia	3.334	1.773	1.483	0.472	2.285	0.245
Klebsiella	0	0	0	0	0.007	0.406
Proteus	0	0.007	0	0.002	0	0.558
Shigella	0.005	0.003	0.001	0	0.001	0.736
Anaeroplasma	0.008	0	0.028	0.038	0.188	0.874
RF39_Unknown	**0.387**	**0.173**	**0.341**	**0.487**	**0.025**	**0.016[1]**
Akkermansia	0.051	0.008	0.022	0.005	0.030	0.870

NC = Negative Control (Basal diet without AGP/Probiotic/NSP-enzyme
PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
NCE = Basal diet + NSP-enzyme (Rovabio 50 g/t)
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
NCEP = Basal diet + NSP-enzyme (Rovabio 50 g/t) + Probiotic (Alterion 2% 500 g/t)
[1] Significant effect at $P < 0.05$
[2] Tendency toward a significant effect at $P < 0.1$

Table 4.14 and Figure 4.3 shows the main effect of feed additive on the caecal microbiome at genus level of male broilers at 25 days-of-age when comparing NCP (Probiotic) against the PC (AGP). There was a tendency towards a significant difference ($P < 0.10$) in the percentage abundance of *Rikenellaceae*_Unknown, *Barnesiellaceae*_Unknown, *Clostridiales*_Other, *Dehalobacteriaceae*_Unknown, *Coprococcus* and *Ruminococcaceae*_Other genera, with NCP having the highest average percentage abundance in *Rikenellaceae*_Unknown, *Clostridiales*_Other, *Dehalobacteriaceae*_Unknown and *Ruminococcaceae*_Other genera, whereas the PC had the highest average percentage abundance in *Barnesiellaceae*_Unknown and *Coprococcus* genera.

There was a significant difference ($P < 0.05$) in the average percentage abundance between NCP and PC in the *Bacillaceae*_Unknown, *Clostridium*, *Lachnospiraceae*_Unknown, *Anaerostipes*, *Campylobacter* and *RF39*_Unknown genera. NCP having significantly ($P<0.05$) higher percentage abundance in *Bacillaceae*_Unknown, *Anaerostipes* and *RF39*_Unknown genera, whereas PC had significantly ($P<0.05$) higher percentage abundance of *Clostridium*, *Lachnospiraceae*_Unknown and *Campylobacter*.

Table 4.14 The effect of feed additive on the percentage abundance of the caecal microbiome at a genus level of broilers at 25 days-of-age when comparing the positive control treatment (PC) to Treatment 3 (NCP) with the Mann-Whitney U test

Genus	NCP	s.d.	PC	s.d	P-Value
Other	0.601	1.876	0.004	0.007	0.480
Bifidobacteriaceae_Unknown	0.012	0.028	0.006	0.010	1.000
Bifidobacterium	10.460	18.710	19.210	20.380	0.190
Coriobacteriaceae_Unknown	0	0	0	0	1.000
Bacteroidetes_Other	0.116	0.189	1.310	3.807	0.889
Bacteroides	0	0	0.005	0.011	0.474
Rikenellaceae_Other	0.010	0.016	0.008	0.009	0.761
Rikenellaceae_Unknown	**40.420^{A}**	**19.160**	**21.630^{B}**	**22.150**	**0.052**
Alistipes	0.001	0.004	0.352	1.108	0.737
Barnesiellaceae_Unknown	**0**	**0**	**0.010^{A}**	**0.015**	**0.087**
Barnesiella	0	0	0	0	1.000
Bacillaceae_Unknown	**0.080^{a}**	**0.116**	**0**	**0**	**0.011**
Enterococcus	0.030	0.077	0.033	0.072	0.544
Lactobacillus	0.017	0.031	0.002	0.004	0.206
Streptococcus	0.057	0.070	0.082	0.172	1.000
Clostridiales_Other	**0.771^{A}**	**0.796**	**0.221^{B}**	**0.496**	**0.057**
Clostridiales_Unknown	7.930	6.490	5.010	6.763	0.218
Christensenellaceae_Unknown	0.285	0.233	0.320	0.603	0.494
Clostridium	**0.193^{b}**	**0.294**	**2.130^{a}**	**2.159**	**0.007**
Dehalobacteriaceae_Unknown	**0.008^{A}**	**0.012**	**0.001^{B}**	**0.003**	**0.087**
Dehalobacterium	0.018	0.035	0.010	0.033	0.582
Anaerofustis	0	0	0.001	0.003	1.000
Lachnospiraceae_Other	0.889	0.502	1.781	2.894	0.436
Lachnospiraceae_Unknown	**1.059^{b}**	**0.601**	**3.495^{a}**	**3.097**	**0.011**
Anaerostipes	**0.088^{a}**	**0.136**	**0**	**0**	**0.011**
Blautia	0.638	0.891	1.214	1.362	0.165
Coprococcus	**5.960^{B}**	**9.907**	**11.076^{A}**	**6.830**	**0.063**
Defluviitalea	0.919	1.008	0.356	0.610	0.165
Dorea	0.495	0.563	0.762	0.666	0.165
Lachnospira	0.006	0.015	0	0	0.474
Roseburia	0	0	0	0	1.000
Ruminococcus	3.628	4.303	4.191	3.533	0.796
Ruminococcaceae_Other	**0.485^{A}**	**0.499**	**0.408^{B}**	**1.040**	**0.052**
Ruminococcaceae_Unknown	2.470	1.460	1.498	1.471	0.105
Anaerotruncus	0.544	0.817	0.470	0.722	0.853
Butyricicoccus	4.207	5.240	3.802	5.422	0.481
Clostridium	0.005	0.010	0.010	0.013	0.266
Faecalibacterium	0.001	0.004	0	0	1.000
Oscillospira	13.050	7.267	13.510	10.764	0.796
Ruminococcus	0.895	0.865	0.408	0.399	0.218
Subdoligranulum	0	0	0.002	0.004	0.474
Megasphaera	0	0	0.014	0.038	0.474
Phascolarctobacterium	0.015	0.028	0.129	0.289	0.154

Mogibacteriaceae_Unknown	0.374	0.193	0.566	0.398	0.353
Erysipelotrichaceae_Other	0.006	0.018	0.007	0.023	1.000
Victivallaceae_Unknown	0.011	0.029	0	0	0.474
Proteobacteria_Other	0.001	0.003	0	0	1.000
Sutterella	0.407	0.824	0.768	0.803	0.105
Desulfovibrio	0	0	0.045	0.143	1.000
Campylobacter	**0.229[b]**	**0.165**	**1.135[a]**	**1.722**	**0.011**
Helicobacter	0.454	0.657	1.351	2.359	0.377
Succinatimonas	0.031	0.099	0.076	0.210	0.737
Escherichia	1.483	1.864	2.285	3.143	0.435
Klebsiella	0	0	0.007	0.023	1.000
Proteus	0	0	0	0	1.000
Shigella	0.001	0.004	0.001	0.003	1.000
Anaeroplasma	0.028	0.089	0.188	0.594	1.000
RF39_Unknown	**0.341[a]**	**0.569**	**0.025[b]**	**0.079**	**0.013**
Akkermansia	0.022	0.064	0.030	0.058	0.721

PC = Positive Control (Basal diet + AGP (Stafac 40 g/t))
NCP = Basal diet + Probiotic (Alterion 2% 500 g/t)
s.d. = Standard deviation
[a,b] Row means with different superscript differed significantly (P > 0.05)
[A, B] Row means with different superscript showed a tendency towards a significant difference (P > 0.1)

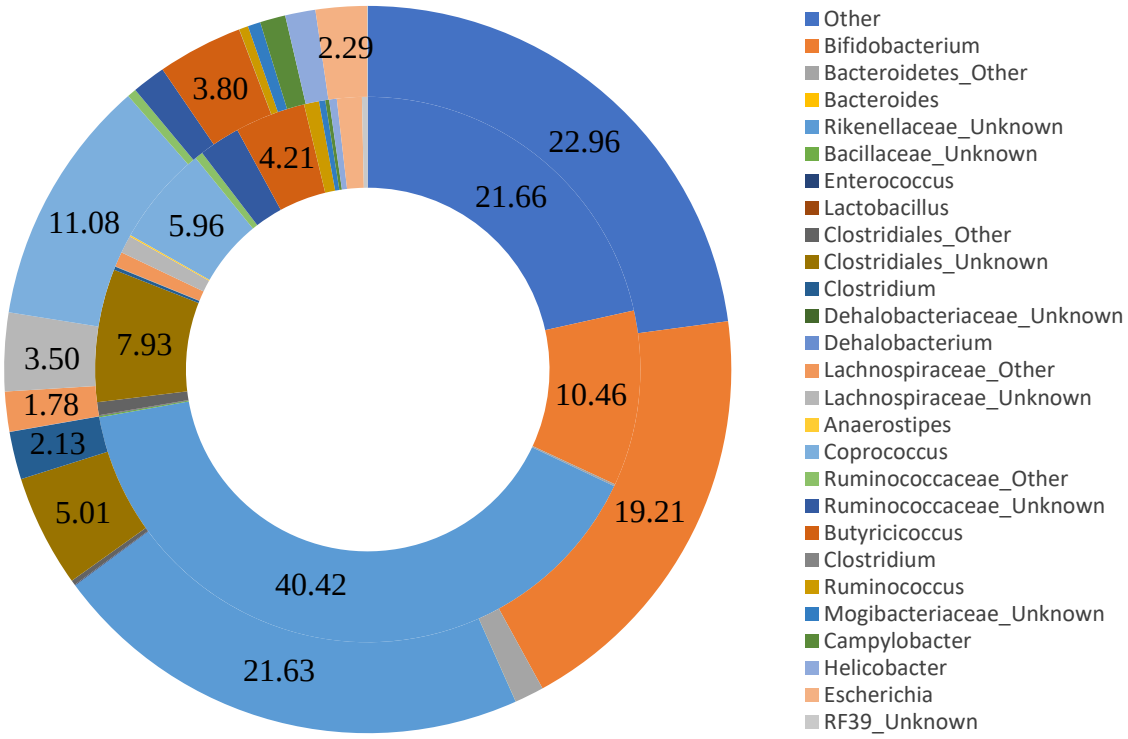

Figure 4.3 Composition of the caecal microbiome at a genus level of male broilers at 25 days-of-age. The inner and outer rings indicate NCP and PC, respectively

CHAPTER 5
Discussion

5.1 Production parameters

In the present study, dietary treatment had no effect on any of the production parameters throughout the experiment. Other similar studies (Kocher *et al.*, 2002; Vieira *et al.*, 2006), also found no improvements in production parameters when enzymes were added to the control diets, without any matrix values in formulation. The basal diet in this study probably contained sufficient energy for maximal production under the given environmental conditions. Higher energy digestibility brought about by the addition of exogenous enzymes could therefore not stimulate production any further (Farhangi and Carter, 2007; Woest, 2019).

Live weight gain, feed intake, feed conversion ratio and mortality were not affected by either the probiotic or the AGP dietary treatments. These results are in agreement with various other studies where feed additives such as organic acids, probiotics or AGP did not have any effect on weight gain and FCR (Watkins and Kratzer, 1983; Cave, 1984; Izat *et al.*, 1990; Engberg *et al.*, 2000; Panda *et al.*, 2000; Vale *et al.*, 2004; Gunal *et al.*, 2006). However, several other reports showed the beneficial effects of these additives on weight gain and FCR (Henry *et al.*, 1987; Yeo and Kim, 1997). Jayaraman *et al.* (2017) found that both probiotic and AGP were able to increase the body weight and improve the FCR of broilers when compared to the control diet. Probiotics in broiler feed improved growth and reduced mortality (Lutful Kabir, 2009; Park *et al.*, 2018) and improved FCR (Lutful Kabir, 2009; Amerah *et al.*, 2012) when compared to AGP-containing diets. Amerah *et al.* (2012) also found no effect of probiotic on feed intake and body weight of broilers, which is in correspondance with the current study. In the present study, the oberved lack of effects of either probiotic and AGP may be associated with environmental conditions. Healthy chicks that receive all the required nutrients do not respond positively to growth promoters when they are housed under a moderate stocking densities in a clean environment (Anderson *et al.*, 1999; Gunal *et al.*, 2006). No significant difference in mortalities between treatments and overall low mortality numbers may support this theory, together with the fact that all treatments performed above Ross 308 standards.

The body weight of broilers at 35 days-of-age that received the probiotic product was not significantly different from either the Negative Control or the Positive Control. Average cumulative feed conversion ratio from day 0-28 was higher (worse) for the broilers that

received probiotics compared to the two diets that contained AGP. However, birds that received probiotics performed significantly better than both the AGP treatments during the period 28-35 days, resulting in FCR values that did not differ significantly over the overall 35-day period of the trial. In this study, the AGP (virginiamycin) had no effect on the performance traits of male broilers at the end of the 35-day trial period.

5.2 The effect of feed additive on caecal microbe composition and macroscopic gut health

The GIT is host to a complex ecosystem of microbiota and is known to be sensitive to various stressors which can result in a multitude of changes, including alterations to the normal microbiota (Al Fataftah and Abdelqader, 2014). A stable and diverse gut microbiota is as essential for a bird to resist infections as a well-functioning immune system. Whenever a sudden shift in the composition of the microbiome occurs, abnormal and often unfavourable populations of microorganisms tend to dominate the gut (Kogut, 2013). Dysbiosis occurs as a result of inflammation in the intestine together with a disruption in the normal microbiota composition, subsequently leading to reduced digestibility of nutrients and impaired intestinal barrier function (Ducatelle *et al.*, 2018). Undigested food particles; fragile, thin, translucent intestinal walls; watery and foamy intestinal contents; presence of orange mucous; ballooning of the gut and intestinal inflammation, are all signs of dysbiosis in the GIT (Teirlynck *et al.*, 2011). A method for macroscopically scoring gut health to provide an indication of the severity of dysbiosis in the poultry gut has been development by Teirlynck et al., (2011). They found that higher scores were indicative of decreased villi length, thinning of the *tunica muscularis* and an increase in the T-lymphocyte infiltration. In the present study, all treatments including the Negative Control had very low macroscopic gut health scores, indicating that the rearing conditions were good, and birds were generally healthy, depriving the feed additives the opportunity to show any potential beneficial effects.

In this study, there were no significant difference between any of the analysed treatments in the percentage abundance of microbes present in the caeca on a phylum level. Supplementation with a probiotic (NCP) showed numerically the most diverse population of microbes as it had the highest average percentage abundance of other microbes, which could not be identified. There was a trend towards a significant difference in the *Tenericutes* phylum between all treatments. Treatment 3 however, had a significant higher percentage abundance of *Tenericutes*

compared to the Positive Control. According to Knarreborg *et al.* (2008) *Bacillus subtilis* influence the intestinal microbiota, favouring the growth of lactic acid bacteria. *Lactobacillus* and *Bifidobacterium* together with other populations of beneficial bacteria produce increased levels of volatile fatty acids and lactic acid, ultimately decreasing the pH of the GIT leading to the inhibition of undesirable pathogenic bacteria (Park *et al.*, 2018). Similar to a study done by Corrigan *et al.* (2015), the predominant phyla identified in this study were *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Tenericutes*, representing >99% of all sequences. Corrigan *et al.* (2015) found that a mannan-oligosaccharide product had the ability to increase the abundance of *Bacteroidetes* while reducing the abundance of *Firmicutes* when compared to a standard maize-soybean diet without any similar additive. *Bacteroidetes* mainly represents primary fermenters that convert simple sugars derived from breakdown of complex carbohydrates to organic acids, including short chain fatty acids, mainly propionic acid, and hydrogen (Corrigan *et al.*, 2015). The BW results of this study contrasts with results obtained by Singh *et al.* (2013), who found a positive correlation between an increase in the caecal *Firmicute*/*Bacteroidetes* (F/B) ratio and final BW of broilers. Even though the PC had a higher F/B ratio than NCP, there was no significant difference between the final BW of the two treatments.

In the present study there was an indication towards a significant difference ($P < 0.10$) in the percentage abundance of *Bacteroidetes* and *Proteobacteria*, having higher percentage abundance, between NCP and PC respectively. In a study done by Sakaridis *et al.* (2018), broilers with the highest count of *Campylobacter* had a lower percentage abundance of *Bacteroidetes* and a higher percentage abundance of *Proteobacteria* when compared to all other birds analysed. This result is similar to the one found in this study, where the PC with AGP, also had a significantly higher percentage abundance of *Campylobacter*, when compared to NCP with the probiotic. According to Miles *et al.* (1984) virginiamycin is known to be active against Gram-positive bacteria of the gut, and not against Gram-negative bacteria with specific reference to *Enterobacteriaceae*. Sub-therapeutic levels of in-feed antibiotics are known to reduce the stability of the gut microflora as well as the *Lactobacillus* population (Chambers and Gong, 2011). Even though there was no significant difference between the probiotic and AGP treatment, the probiotic had numerically higher percentage abundance of *Lactobacillus* when compared to the AGP treatment.

Non-starch polysaccharides present in broiler feed, reduce its digestibility, and bring about several anti-nutritional factors, all influencing growth performance of broilers. In an effort to utilise the full nutritive value of broiler diets, the application of NSP degrading enzymes have become a very popular practice worldwide. The effects of NSPases vary depending on diet type and include reducing the viscosity of digesta in wheat-based diets and limiting the encapsulation effect in maize-soya based diets (Cowieson *et al.*, 2006; Flores *et al.*, 2019). Askelson *et al.* (2018) found that by using a probiotic in combination with exogenous carbohydrase enzymes total Lactic Acid Bacteria increased as compared to the negative control diet, as well as reducing *Clostridium perfringens* to levels similar than when antibiotics were administered. Yacoubi *et al.* (2016) studied the prebiotic effects of arabinoxylans (AX), arabino-xylo-oligosaccharides and xylo-oligosaccharides which are some of the oligosaccharides release by NSPases. Utilisation of these polysaccharides by the gut microbiota may enhance the production of short chain fatty acids (SCFA), particularly butyrate. These effects are reliant on the efficient break-down of the xylan backbone to produce short chain arabinoxylans. Removal of the arabinose by debranching enzymes, Arabinofuranosidases, on the side chains of AX facilitate the enzymatic activity of xylanase. This process allows for improved access of xylanases to the xylose backbone for higher success of hydrolysis. This synergistic effect of carbohydrase complex improves the prebiotic action (Yacoubi *et al.*, 2016). This prebiotic effect of the multi-complex carbohydrase enzyme was not evident in the present study. This may be because the birds were under optimal environmental conditions and showed good health status during the trial, allowing them to perform above that Ross 308 standards.

CHAPTER 6
Conclusion and Recommendations

6.1 Conclusion

The objective of this study was to investigate the administration of probiotic, alone or in combination with a multi complex carbohydrase, as a potential alternative to the use of AGP. For many years sub-therapeutic levels of in feed antibiotics have been widely used in the production of poultry and other livestock. However, in recent years the demand for antibiotic-free livestock production has increased due to consumer and regulatory concerns over the development of antibiotic resistant bacteria.

The addition of supplementation of feed with neither AGP (virginiamycin) nor enzymes produced by *Talaromyces versatilis* resulted in any significant improvements in broiler performance

The addition of a single strain *Bacillus subtilis* as probiotic to broiler feed improved FCR during the last week of the trial. The microbial populations in the GIT of *Bacillus subtilis* fed broilers differed from broilers that received a diet containing AGP. Improved abundance of *Bacteroidetes* may reduce or prevent proliferation of pathogenic bacteria species such as *Campylobacter*. However, the ideal intestinal microbiota for optimal broiler performance is not yet known and further research is needed in this area.

Unfortunately, due to the broilers in the negative control obtaining similar performance results than all other treatments in this study, it is not possible to conclude whether a single strain *Bacillus subtilis* can be used successful, alone or in combination with a multi complex carbohydrase, to replace virginiamycin in the commercial production of broilers.

6.2 Recommendations for further studies

To successfully evaluate the potential of any feed additive to replace AGP, it is important to challenge birds to such an extent that significant differences are created between the negative and positive control.

This was not achieved in the current trial. A possible solution is to create conditions which would impose stress on the birds, such as increasing the stocking density to beyond 22 birds/m^2,

inducing cyclic heat stress or using unbalanced diets with even lower energy and more excessive protein concentrations than what was used in the current trial.

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