Efficacy of enzymes produced by *Talaromyces versatilis* in releasing energy and amino acids in broiler feeds

By

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Declaration

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

C Woest

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Abstract

Exogenous feed enzymes can limit the negative effect of anti-nutrients such as non-starch polysaccharides present in maize and soybean meal based broiler diets, and thereby increase the digestibility and nutritive value of the feed. A study was conducted to determine efficacy of enzymes produced by *Talaromyces versatilis* in releasing energy and amino acids in broiler feeds, and how that will effect production and slaughter parameters during a 35 day broiler production cycle. The positive control diets were formulated to be lower in energy and amino acid levels than the values used commercially. The negative control 1 diets were further reduced in metabolisable energy, and the negative control 2 diets were reduced in amino acids compared to the positive control diets. The negative control 3 diets were reduced in both metabolisable energy and amino acids compared to the positive control diets. The Rovabio Advance enzyme complex was then added to the positive control, the negative control 1 diets, the negative control 2 diets and the negative control 3 diets. The enzymes supplementation resulted in significant improvements in body weight gain during the final week of the trial when compared to non-supplemented diets. The addition of the enzyme complex to diets with reduced amino acid levels, also resulted in a significant improvement in feed conversion ratio and a tendency to improve body weight gain compared to the positive control, during the final week of the trial. Enzyme addition to the reduced energy and reduced energy and amino acid negative control diets resulted in slight but non-significant improvements in 35 day body weight and body weight gain over the 35 day period. The reduced energy and amino acid negative control diets, also showed slight improvement in feed conversion ratio over the 35 day period, with the addition of the enzyme. No improvements in any of the production parameters were observed, with enzyme addition to the positive control diets. Therefore, it can be concluded from the present study that enzymes produced by *Talaromyces versatilis* can improve production parameters of broilers when added to maize and soybean meal based diets with reduced energy and amino acid levels. Enzyme addition to the positive control diet, significantly improved eviscerated carcass yield compared to the reduced energy and amino acid diets. No other significant improvements were observed in any of the carcass parameters evaluated, due to enzyme addition. Therefore, this study did not deliver significant evidence that enzyme supplementation can improve slaughter parameters of broilers.

List of abbreviations

AA	:	Amino acids
AGP	:	Antibiotic growth promoter
AX	:	Arabinoxylan
BW	:	Body weight
BWG	:	Body weight gain
˚C	:	Degrees Celsius
Ca	:	Calcium
CP	:	Crude protein
Dig.	:	Digestible
DM	:	Dry matter
FCR	:	Feed conversion ratio
FI	:	Feed intake
FTU	:	Phytase unit
g	:	Gram
GLM	:	General linear model
g / t	:	Gram per ton
kg	:	Kilogram
LSM	:	Least square mean
m	:	Meter
m^2	:	Square meter
MJ	:	Mega joules
ME	:	Metabolisable energy
NSP	:	Non-starch polysaccharides
NC	:	Negative control
P	:	Phosphorus
PC	:	Positive control
TRT	:	Treatment
VU	:	Visco unit

Table of contents

List of tables

CHAPTER 1

General Introduction

In the poultry industry, as in many animal production systems, profitability depends mainly on the nutritional value and cost of the feed (Tahir *et al.*, 2008; Bedford and Partridge, 2010). A reduction in feed cost will always be a key objective for both poultry feed producers and integrators (Bao *et al.*, 2013). Feed represents approximately 70% of the total cost of broiler production in South Africa, and maize and soybean meal contribute the bulk of raw materials for broiler feed (Dida, 2016; Davids and Meyer, 2017).

Maize contributes around 65% of apparent metabolisable energy, and soybean meal 80% of crude protein in maize and soybean meal based broiler diets (Cowieson, 2005). Maize and soybean meal poultry feed is generally considered to be of high nutritional value, but 15–25% of the feed consumed will escape digestion (Zanella *et al.*, 1999; Bedford and Partridge, 2010; Cowieson, 2010). When birds are fed a typical maize and soybean meal ration, approximately 400-450 kcal of energy per kg of diet is not available (Cowieson, 2010), partly because the birds lack specific enzymes needed to hydrolyse certain feed components and also because of the presence of anti-nutritional factors interfering with the digestive processes (Tahir *et al.*, 2008; Bedford and Partridge, 2010). When anti-nutritional factors interfere with normal digestion, it may cause decreased production and lower feed efficiency and can also trigger digestive upsets (Bedford and Partridge, 2010). One of the main anti-nutrients that may limit the nutritive value of the feed are the non-starch polysaccharides (NSP) (Tahir *et al.*, 2008), which are complex high molecular weight carbohydrates found in the structure of plant cell walls (Irish and Balnave, 1993). Maize and soybean meal based diets contain varying levels of NSP (Irish and Balnave, 1993). The total NSP content of maize is around 97 g/kg, which includes negligible amounts of soluble NSP, and approximately 8% of insoluble NSP consisting mainly of arabinoxylans (Choct, 2006; Rios *et al.*, 2017). Soybean meal contains approximately 217 g/kg total NSP, of which only 3% is soluble NSP and 16% is insoluble NSP, primarily in the form of galactomannans (Irish and Balnave, 1993; Rios *et al.*, 2017). Non-starch polysaccharides are not digestible by monogastric animals because they lack the digestive capacity of ruminant animals (Meng *et al.*, 2005).

The supplementation of animal feed with exogenous enzymes can increase the efficiency of digestion, and thereby improve the nutritional value of feed ingredients. Since the mid-1980s, feed enzymes have dramatically improved the profitability of commercial poultry production (Bao *et al.*, 2013). Enzymes are now routinely included in broiler chicken diets to improve the digestion of carbohydrate, protein and mineral fractions (Choct, 2006; Francesch and Geraert, 2009). The actual mechanism of action of the enzymes present in commercially available supplemental complexes depend on their efficacy to degrade the different substrates present in feed ingredients (Rios *et al.*, 2017). Enzyme supplementation has been more strongly related to diets containing cereals such as wheat and barley because of the high levels of viscous soluble non-starch polysaccharides they contain (Cowieson, 2005). The inclusion of microbial enzymes in diets containing such grains have been observed to improve the nutritive value of the diets (Choct *et al.*, 2004; Meng *et al.*, 2004; Yang *et al.*, 2008; Romero *et al.*, 2014; Lei *et al.*, 2016; Munyaka *et al.*, 2016; Cozannet *et al.*, 2017; Gonzales-Oritz *et al.*, 2017; Yaghobfar and Kalantar, 2017).

Although both maize and soybean meal are considered highly digestible ingredients (Zanella *et al.*, 1999), their nutritional value can still be improved by a combination of supplemental enzymes (Francesch and Geraert, 2009). Enzymes able to break down the cell wall matrix and the insoluble components may result in easier access of digestive enzymes to the released nutrients, normally encapsulated in cell walls or incorporated into the cell wall (Cowieson, 2005; Choct, 2006). Slominski (2011) stated that the constituent NSP in maize and soybean meal requires a broad range of carbohydrase enzymes, if any beneficial response is to be achieved. Non starch polysaccharide degrading carbohydrase enzymes, particularly xylanases, have long been used in wheat-based diets for poultry to degrade the arabinoxylan chains (Cozannet *et al.*, 2017). The efficacy of xylanase is limited in maize, due to multiple arabinose substitutions present in the xylose backbone (Knudsen, 2014). Enriching a preparation with debranching enzymes, such as arabinofuranosidases represents an efficient way to increase the overall enzyme effect (Cozannet *et al.*, 2017). Arabinofuranosidases can cleave arabinose from the xylose backbone and offer access to xylanase enzymes (De La Mare *et al.*, 2013; Cozannet *et al.*, 2017).

The filamentous fungus *Penicillium funiculosum*, recently renamed *Talaromyces versatilis* (Samson *et al.*, 2011), produces a wide range of cellulotic and hemicellulotic enzymes (Lafond *et al.*, 2014). It can grow efficiently under large-scale industrial conditions to secrete biologically active compounds including several hydrolytic enzymes (Belshaw *et al.*, 2003). This capability is currently utilised to produce a commercialised enzymatic cocktail which is used as feed additive in animal nutrition (De La Mare *et al.*, 2013). The multi carbohydrase enzyme complex is composed of pectinases, cellulases, proteases and arabinofuranosidases (Rios *et al.*, 2017). This multi carbohydrase enzyme complex strain was genetically modified via self-cloning to increase the amount of xylanase and arabinofuranosidase, enhancing its efficacy in breaking down highly substituted arabinoxylans (Lafond *et al.*, 2011; De La Mare *et al.*, 2013). Enzymes that are capable of degrading complex arabinoxylan chains more efficiently, can challenge current feed reformulation to consider all potential benefits and nutrient digestibility (Cozannet *et al.*, 2017).

The objective of the present study was to determine the efficacy of enzymes produced by *Talaromyces versatilis* in releasing energy and amino acids in broiler feeds, and how that would affect broiler production and slaughter parameters during a 35 day broiler production cycle.

The aim of the study is to determine the following hypothesis:

- H_0: Supplementation of broiler diets with enzymes produced by *Talaromyces versatilis* will not improve production and slaughter parameters in broilers during a 35 day production cycle.
- H_A: Supplementation of broiler diets with enzymes produced by *Talaromyces versatilis* will improve production and slaughter parameters in broilers during a 35 day production cycle.

CHAPTER 2

Literature Review

2.1 Introduction

The aim of this section is to review the current literature on the classification of non-starch polysaccharides, with specific reference to the main types present in maize and soybean meal based diets. The main anti-nutritional effects of non-starch polysaccharides will also be discussed. Further to this, the mode of action and types of non-starch polysaccharide degrading enzymes will be discussed. This review will also investigate the inclusion of multi-enzymes in maize soybean meal based diets, and the main factors affecting the efficacy of these exogenous enzymes. The final part of this review will discuss the enzymes produced by the filamentous fungus *Talaromyces versatilis*.

2.2 Classification of non-starch polysaccharides

Non-starch polysaccharides (NSP) can be classified into three main groups, namely cellulose, non-cellulosic polymers and pectic polysaccharides (Bailey, 1973). Arabinoxylans, mixed-linked ß-glucans, mannans, and xyloglucan are part of the non-cellulosic polymers whereas polygalacturonic acids substituted with arabinan, galactan and arabinogalactan are included in the group of pectic polysaccharides (Sinha *et al.*, 2011; Choct, 2015). Fructans, glucomannans and galactomannans belong to the group of NSP that is not as abundant as cellulose, hemicellulose or pectins and serve as the storage polysaccharides within the plants (Sinha *et al.*, 2011).

2.2.1 Cellulose

Cellulose is the most abundant biopolymer in nature, comprising more than 50% of all the vegetative carbon (Choct, 1997; Kumar *et al.*, 2012). It is the most important polysaccharide, which is present in all plant tissue as the basic structural component of plant cell walls (Knudsen, 2014). Cellulose is made up of thousands of ß-(1-4) linked glucopyranosyl units, which generally makes it indigestible for monogastric animals due to the absence of the cellulase enzyme in the digestive tract (Sinha *et al.*, 2011). Separate cellulose chains are held together by

hydrogen bonds and this forms a “ribbonlike” two-fold helix, which helps stabilise the glucan chains (Choct, 1997; Knudsen, 2014).

2.2.2 Non-cellulosic polymers

2.2.2.1 Arabinoxylans

Arabinoxylan (AX) is the main component of hemicellulose in annual plants, accounting for 30–35% of the cell wall material (Bedford and Partridge, 2010). The structure of cereal AX are composed predominantly of two pentoses namely arabinose and xylose, and consists of a linear backbone of (1-4)-ß-D-xylopyranosyl residues, which is mainly substituted with α-L-arabinofuranosyl residues to different degrees at the O-2 and/or O-3 positions (Choct, 1997; De La Mare *et al.*, 2013). This results in four structural elements in the molecular form of AX which are monosubstituted at O-2 or O-3, disubstituted at O-2, 3 or unsubstituted. The relative amount of arabinose and xylose, and the sequence of distribution will differ between cereals, and the ratio of the two pentoses can be used to characterise the structure of AX (Knudsen, 2014).

For most cereals, the primary substitution structure is α-L-arabinose. Arabinoxylan can be divided into water-extractable and water-unextractable fractions. The largest fraction of AX in most cereals is water-unextractable, which results from covalent cross-links and noncovalent interactions with other components (Lei *et al.*, 2016). The water-extractable AX is responsible for viscosity, while the cage effect is caused by the water-unextractable AX (Masey O’Neill *et al.*, 2014). Most of the AX in cereal grains are insoluble in water because they are anchored in the cell walls by alkali-labile esterlike cross links, but those that are not bound to the cell walls can form highly viscous solutions and they can absorb about ten times their weight of water (Choct, 1997).

There are two major types of AX, namely those found in endospermic tissues and those found in non-endospermic tissues. The non-endospermic AX contains some side groups together with the α-L-arabinofuranose side chains. The endospermic xylans present in cereals are extremely branched and double substitution can occur with α-L-arabinofuranose at the positions 2 and 3 (Wilkie, 1979). In the majority of AX, there are several substituent groups attached to xylose,

and they determine the solubility, viscosity and other physiochemical properties (Bedford and Partridge, 2010). The side chains determine the solubility, physical conformation, and reactivity of xylan molecule with other hemicellulosic components, and therefore greatly influence the mode and extent of enzymatic cleavage (Chakdar *et al.*, 2016).

2.2.2.2 Mixed-linked-ß-glucans

Mixed-linked ß-glucans are found in most cereals, and are important constituents of the cell walls accounting for up to 70% of the weight (Choct, 1997; Kumar *et al.*, 2012). Barley, oat and rye grains are major sources, whereas wheat, rice and maize have lower concentrations (Kumar *et al.*, 2012). The structure of these polysaccharides is well established and consists of linear homopolymers of D-glucopyranosyl units that are joined by 2 or 3 consecutive ß-(1-4) linkages separated by a single ß-(1-3) linkage (Knudsen, 2014). Cereal ß-glucans are not digested by the monogastric animal's endogenous enzymes (Kumar *et al.*, 2012), and have a negative effect on bird performance and health (Jacob and Pescatore, 2014).

2.2.2.3 Mannans

Mannans are either glucomannans or galactomannans and are commonly found in a variety of feed ingredients, including soybean meal, which is one of the primary ingredients used in poultry diets (Masey O'Neill *et al.*, 2014; Williams *et al.*, 2014; Rehman *et al.*, 2016).

ß-mannans are a group of related heat-resistant compounds (Odetallah *et al.*, 2005) and can contribute up to 1.3% or 1.6% in the dehulled or nondehulled soybean meal, respectively (Hsiao *et al.*, 2006). β-mannans have been found to be deleterious to animal performance, compromising weight gain and feed conversion (Hsiao *et al.*, 2006). It is considered a nutritional constrained because of its extremely high viscosity in solution (Centeno *et al.*, 2006). ß-mannan is also capable of stimulating the innate immune system and is thus potentially capable of stimulating a non-productive energy draining innate immune response (Hsiao *et al.*, 2006). When ß-mannans are present in feed they can depress growth and feed conversion and increase nitrogen and faecal output, effectively decreasing metabolisable energy (Mussini *et al.*, 2011).

Galactomannans are reserve polysaccharides in the seed endosperm of leguminous plants. They are water-soluble, and provide a water-holding function for the seed by absorbing water. Galactomannans are composed of ß-(1-4)-linked mannan chains with α-(1-6)-linked galactosyl side-groups (Sinha *et al.*, 2011; Kumar *et al.*, 2012; Prajapati *et al.*, 2013). Glucomannans act as storage polysaccharides and are present as a minor component in cereal grains (Sinha *et al.*, 2011; Kumar *et al.*, 2012). Many glucomannans are water-soluble and composed of glucose and mannose monomers as part of a ß-(1-4) linked mannan chain (Kumar *et al.*, 2012; Masey O'Neill *et al.*, 2014).

2.2.2.4 Pectic polysaccharides

Pectic polysaccharides are a heterogenous group of cell wall polysaccharides that are found in the cell walls of the stems and leaves of cereals (Choct, 1997; Knudsen, 2014). Pectic components are highly branched and have heterogeneous monosaccharide compositions consisting mainly of galacturonic acid, galactose, arabinose, xylose, fucose and rhamnose (Al Loman and Ju, 2017). Pectic polysaccharides consist mainly of homogalacturonans, rhamnogalacturonans type I and II, xylogalacturonan and arabinogalactan type I and II (Knudsen, 2014).

Homogalacturonan is the main structure of pectin and is a polymer consisting of α-(1-4)-linked D-galacturonic acids (Al Loman and Ju, 2017). Rhamnogalacturonans type I has a backbone of alternating α-(1-2)-linked L-rhamnose residues and α-(1-4)-linked D-galacturonan residues, with side chains of arabinan, galactans and arabinogalactans. Rhamnogalacturonans type II is a complex polysaccharide that consist of a backbone of α-(1-4)-linked D-galacturonic residues that are substituted with aldehydro- and keto-sugar oligosaccharides. Xylogalacturonan is made up of a homogalacturonan backbone which is substituted by one or more ß-(1-3)-linked D-xylose residues, and is mainly found in reproductive tissue (Knudsen, 2014; Al Loman and Ju, 2017).

Arabinans are polymers that consist of α-(1-5)-linked L-arabinose residues that are substituted with one or more α-arabinofuranosyl residues, while galactans are polymers that consist of (1-4)-ß-D-galactose residues (Choct, 1997; Knudsen, 2014). Arabinogalactan type I and II both

have a linear backbone made up out of ß-(1-4)-linked D-galactosyl residues which may be branched to a high or low degree (Choct, 1997; Knudsen, 2014).

2.3 Non-starch polysaccharides in maize soybean meal based diets

Even though maize and soybean meal based broiler diets are generally considered to be of high nutritional value (Cowieson, 2010), these raw materials still contain varying levels of non-starch polysaccharides (NSP) that can interfere with digestive processes and lead to decreased production and feed efficiency (Irish and Balnave, 1993; Bedford and Partridge, 2010).

2.3.1 Non-starch polysaccharides in maize

Cereal grains are complex structures, composed of tissues containing cell walls with different properties and composition (Knudsen, 2014). Cereals consist of approximately 10% to 30% NSP, with the majority being arabinoxylans, cellulose and ß-glucans (Choct, 2015). Cereal grains can be classified into two groups, namely viscous and non-viscous cereals (Choct, 2015). Viscous cereals include barley, rye, wheat, while non-viscous cereals include maize, sorghum and rice (Choct, 2015).

Maize and wheat have similar cell wall composition, but the endosperm cell wall that surrounds the cellular endosperm is thinner (Chesson, 2001). The NSP content in maize is on average around 90 g/kg of the dry matter (Knudsen, 2014), which mainly consists of arabinoxylan but also include ß-glucan and cellulose (Chesson, 2001). Even though the NSP content of maize is lower than that of soybean meal, its contribution to the overall NSP level in the feed can be substantial due to the high inclusion rate in maize soybean meal based diets (Cowieson and Adeola, 2005; Meng and Slominski, 2005; Yegani and Korver, 2013). According to Jaworski *et al.* (2015), the NSP composition of the total NSP fraction in maize includes 48.6% arabinoxylans, 21.6% cellulose and 29.8% of other hemicelluloses. Choct (1997) noted that approximately 64% of the NSP in maize kernels is arabinoxylan, with only 2% being water-soluble.

According to Knudsen (1997), the insoluble NSP and cellulose in maize are the most important NSP constituents. The author also stated that the ratio of insoluble NSP to soluble NSP is much higher for maize than wheat, rye, barley or oats. In maize diets, the effect of nutrient encapsulation should be more of an issue than the effect on digesta viscosity (Cowieson and Adeola, 2005; Choct, 2006; Slominski, 2011). The physical barrier created by the cell walls encloses some amounts of starch and protein components, which limits the animal's own digestive enzymes in accessing and fully digesting it. The nutrients then escapes digestion to reach the hindgut and undergo fermentation with a relatively low energy yield (Meng and Slominski, 2005; Kaczmarek *et al.*, 2014).

Maize arabinoxylan also has a much higher degree of substitution compared to wheat (Knudsen, 1997). The insolubility and complexity of maize arabinoxylan structures influence the susceptibility to enzymatic hydrolysis (Malunga and Beta, 2016). Maize arabinoxylan is a highly branched structure heavily decorated with arabinose and several other substituents which can impede xylanases to bind and cleave the β-1,4-linked xylose backbone (Ravn *et al.*, 2018). Addition of supplementary de-branching enzymes may increase the solubilisation capacity of xylanases by removing substituents present on the xylan chain, for example the removal of arabinose by arabinofuranosidases (Ravn *et al.*, 2018).

2.3.2 Non-starch polysaccharides in soybean meal

Seeds and grains of protein crops and feedstuffs have in general a similar structure as cereals. Soybean meal is a by-product from the production of soybean oil, and the concentration of the fibre and protein will consequently be higher than in the grains and seeds (Knudsen, 2014). It contains approximately 35% carbohydrates, of which 14% are soluble sugars and 21% are NSP (Choct, 2015). The carbohydrates in soybean meal consist mainly of pectic polymers.

When evaluating protein ingredients for poultry diets it is essential to not only look at the amino acid composition, but also to take the effects of the NSP content into consideration (Choct, 2015). The plant origin, the variety, the degree of processing as well as the amount of NSP-rich hull that is present in the final product, all influence the NSP content of these ingredients (Kocher *et al.*, 2002). Soybean meal is the by-product after oil extraction of soybeans, and

contains around 48% protein, 35-40% carbohydrates, 7-10% water, 5-6% minerals and less than 1% fat (Choct *et al.*, 2010). Although soybean is the most widely used vegetable protein source for poultry feed, the anti-nutritional activities of these carbohydrates in animal feed are quite often ignored, and it contains nearly as much carbohydrates as protein (Choct *et al.*, 2010).

The nutritive value of soybean meal also depends on the amount of indigestible carbohydrates, in particular, the amount of oligosaccharides and NSP (Kocher *et al.*, 2002). The carbohydrates in soybean meal consist predominantly of NSP and free sugars, such as mono-, di- and oligosaccharides (Choct, 1997). Sucrose is the main soluble sugar in soybean meal, which can contribute as much as 25–35% of the total carbohydrate fraction (Al Loman and Ju, 2017). Oligosaccharides such as stachyose, raffinose and verbascose are found in lower concentrations (Ouhida *et al.*, 2002) and are important to monitor because of their anti-nutritional effects. These galacto-oligosaccharides consist of a terminal sucrose linked galactose residues by α-1,6 linkages (Al Loman and Ju, 2017), and they can be hydrolysed by α-galactosidase to D-galactose and sucrose (LeBlanc *et al.*, 2004).

Soybean consist of between 20-30% NSP, in which approximately 8% are cellulose and the remaining are pectic polysaccharides mainly in the form of rhamnogalacturonans (Choct, 1997). The NSP content of soybean meal consist of 63 g/kg DM soluble NSP and 154 g/kg DM insoluble NSP, with the insoluble component being similar to maize (Knudsen, 1997). According to Al Loman and Ju (2017), an enzyme mixture should at least contain pectinase, xylanase, cellulase and α- galactosidase for effective hydrolysis of all types of carbohydrate in soybean meal. Supplementary accessory enzymes such as α-arabinofuranosidase, endoarabinase, ß-galactosidase and endogalactanase are important for further hydrolysis, as they are involved in degradation of the side chains present in soybean.

2.4 Anti-nutritional effects of non-starch polysaccharides

Non-starch polysaccharides can affect the digestion and absorption of other nutrients either directly or indirectly (Sinha *et al.*, 2011; De Vries *et al.*, 2012). Two models have been suggested for their anti-nutritive role in broiler diets (Căpriță *et al.*, 2010). The first model is by encapsulation in which the NSP coat inhibits the access of digestive enzymes to the starch,

fat and protein with the second model being the fact that the presence of NSP in the intestinal lumen increases the viscosity of the intestinal contents (Căpriță *et al.*, 2010). In addition to direct effects on nutrient digestion and absorption, modifications of the quantity and composition of the intestinal microflora may also be involved in the anti-nutritive effects of NSP (Simon, 1998; Bedford and Apajalahti, 2001). The impact of these anti-nutritive properties on nutrient digestion can be substantial (De Vries *et al.*, 2012).

2.4.1 Encapsulation or cage effect of non-starch polysaccharides

The NSP in cell walls can act as a physical barrier that interferes with digestion of intracellular nutrients, and this effect is also referred to as the cage effect (Khadem *et al.*, 2016). The structural arrangement of NSP in the cell wall can affect digestibility of the NSP-fraction itself as well as that of other nutrients encapsulated in the cell, limiting the accessibility of these nutrients by digestive enzymes (De Vries *et al.*, 2012). Non-starch polysaccharides encapsulates the fat, protein and starch that are in the feed (Pettersson and Åman, 1988; Cowan *et al.*, 1996; Wiseman *et al.*, 2000). This is due to the fact that a diverse amount of enzymes are necessary to break down the intact cell wall to be able to utilise the nutrients (Bedford, 2002).

There is strong evidence that some nutrients in maize are not completely digested in the small intestine and that considerable amounts of starch and protein escape digestion, reach the hindgut, and undergo fermentation with a relatively low energy yield (Carré *et al.*, 1995; Noy and Sklan, 1995). With ground and pelleted feed the gizzard of the bird also fails to develop fully, and as a result many intact particles of feed enter the gut and the contents of some cells may escape digestion (Svihus *et al.*, 1997). Surface activity of NSP can also cause them to bind to the surface of feed particles after ingestion, reducing the accessibility and absorption of nutrients from the diet (Smits and Annison, 1996).

The digesta transit time in poultry is rapid due to a relatively short colon, and consequently the fermentative capacity of this species is limited almost solely to the soluble NSP fraction (De Vries *et al.*, 2012). The digestibility of the NSP fraction from diets containing cereals that are relatively high in soluble NSP, such as barley, wheat and oats are much higher than those that

consist mainly of insoluble NSP such as maize (Jorgensen *et al.*, 1996; Meng and Slominski, 2005).

2.4.2 Effect of non-starch polysaccharides on digesta viscosity

Viscous ileal digesta increases with an increase in the intake of soluble NSP, which decreases the digestion rate and bird performance (Chesson, 2001; Bederska-Łojewska *et al.*, 2017). NSP are solubilised after ingestion, which leads to an increase in the viscosity of the digesta (Classen, 1996). Body weight gain and feed conversion efficiency is negatively correlated with an increase the gut viscosity level (Bedford and Classen, 1993; Bederska-Łojewska *et al.*, 2017).

NSP contribute to the physical properties of the digesta, such as viscosity and hydration properties (Annison and Choct, 1991; Iji, 1999; Choct, 2002). This can affect digesta transit time, bulking properties, microbial activity, gut physiology and function and endogenous losses, again potentially reducing nutrient digestion and absorption (Potkins *et al.*, 1991; Jorgensen *et al.*, 1996; Grala *et al.*, 1998). Diffusion also plays an important role for enzymes, their substrates and the end products to move easily through the intestinal wall. When viscosity increases in the intestines, diffusion occurs at a much slower rate (Annison and Choct, 1991; Bedford, 2002).

The unstirred layer at the mucosal surface of the broiler's digestive tract is also influenced by increased viscosity of the digesta, which will lead to a slower uptake of nutrients (Chesson, 2001). Mucin concentration in the gastro intestinal tract is correlated to dietary NSP intake (Sinha *et al.*, 2011). Young chickens are particularly susceptible to viscous components of the diet, and the only grain that can be used without limitation in the ration is maize (Bederska-Łojewska *et al.*, 2017).

2.4.3 Interaction of non-starch polysaccharides with gut microflora

The gut is the major organ for nutrient digestion, absorption, protection against pathogens, and it is also the largest immunological organ in the body (Choct, 2009). Microflora plays an important role in the health status, nutrition and growth performance of animals by influencing

digestion, intestinal development, absorption of nutrients and the immune system (Yang *et al.*, 2009; Matin *et al.*, 2012). There has to be a balance between possible pathogenic bacteria and the beneficial bacteria, which can be disrupted by factors including age, pH, gastric passage rate, diet, and mucosal secretion as well as disorders that affect the immune system (Matin *et al.*, 2012). This interaction is very complex, and its value is dependent on the circumstances under which the host finds itself as well as the composition and activity of the gut microflora (Yang *et al.*, 2009; Bedford and Cowieson, 2012).

The viscous intestinal environment created by NSP, decreases the rate at which feed moves through the intestines, and impedes rapid digestion of nutrients (Salih *et al.*, 1991; Bederska-Łojewska *et al.*, 2017). Almost 90% of bacteria in the intestinal tract are present in the large intestine (Apajalahti *et al.*, 2007; Parker *et al.*, 2007). Bacteria build-up due to the slower passage rate will lead to the migration of bacteria into the small intestine (Bedford, 2002), and can promote an increase of anaerobic microbes in the upper parts of the gastrointestinal tract (Smits and Annison, 1996; Józefiak *et al.*, 2007). The undigested nutrients leads to the proliferation of pathogenic bacteria (Salih *et al.*, 1991; Bedford and Cowieson, 2012). The bacteria compete with the host, as they are able to utilise the nutrients such as starch and protein (Bedford, 1995).

Undegraded arabinoxylans that reaches the colon stimulate development of residing bacteria such as *Bacteriodes*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, and *Eubacterium* (Riviere *et al.*, 2014). The caeca is also responsible for a high level of fermentation of dietary fibre (Józefiak *et al.*, 2004b). Intestinal bacteria are also able to produce bile acid degrading enzymes which could interfere with lipid digestion in the host (Bedford and Classen, 1993; Bedford, 2002). Since bile acids are also thought to stabilise pancreatic proteases in the intestinal lumen, protein digestion could be compromised. Large amounts of rapidly fermentable substrates can lead to decreased digestion and intestinal disorders (Bedford and Cowieson, 2012).

2.5 Non-starch polysaccharide degrading enzymes

Enzymes became commercially available for use in monogastric animal nutrition at the end of the nineteen eighties, with use continually increasing to the present day (Dos Santos *et al.*,

2017). The most common enzyme in monogastric diets is phytase that is used to increase the hydrolysis phytate to release phosphorus, reducing the need for the addition of expensive inorganic phosphorus sources to the diet. The second most common group is carbohydrase enzymes, initially used in viscous diets with high wheat, barley and rye inclusion and subsequently in maize and sorghum based diets, with the objective of improving nutrient absorption and animal performance (Masey O'Neill *et al.*, 2012).

2.5.1 Mode of action of non-starch polysaccharide degrading enzymes

The main function of exogenous supplementation of carbohydrases is to hydrolyse complex NSP present in plant feedstuffs that monogastric animals are incapable of hydrolysing with their endogenous pool of digestive enzymes (Castillo and Gatlin, 2015). Khadem *et al.* (2016) attributes the effect of NSP hydrolysing enzymes in broiler diets to three mechanisms, firstly through a reduction of digesta viscosity, secondly through disruption of the cell wall structure releasing the encapsulated nutrients also known as the cage effect reduction, and lastly through a prebiotic effect. The three mechanisms will be discussed below.

2.5.1.1 Disruption of cell wall integrity

Complex carbohydrates, that form part of the cell wall, shield substrate from contact with digestive enzymes (Asmare, 2014). NSP degrading enzymes releases nutrients from complex cell wall molecules, thereby improving the access of endogenous enzymes (Govil *et al.*, 2017). The activity of exogenous NSP degrading enzymes creates holes in the cell wall, which allows water hydration and permits pancreatic proteases and amylases to act, enabling better digestion of the starch and protein (Sinha *et al.*, 2011). Meng *et al.* (2005) suggested that the improved energy utilisation in maize soybean meal may be due to both the hydrolysis of the encapsulating cell walls, and the disruption of the cell matrix which results in the release of structural protein.

Carbohydrase supplementation increases digestibility of energy yielding nutrients such as starch and fat, because NSP reduce the capacity for nutrient absorption by reducing enzyme accessibility to substrates (Adeola and Bedford, 2004). In addition, it is possible that carbohydrases act to improve nitrogen and amino acid utilisation as well, by increasing the access to protein for digestive proteases (Tahir *et al.*, 2008).

Carbohydrase enzymes can also increase the availability of minerals in diets, due to the relationship between phytic acid and NSP in cereal grains and legumes. Most of the phosphorus is bound in phytic acid, and this phytic phosphorus and other minerals may be exposed to digestive enzymes when the carbohydrases hydrolysis their substrates (Asmare, 2014). Increased mineral availability may be seen as an indirect response to carbohydrase supplementation (Adeola and Cowieson, 2011).

2.5.1.2 Reduction of digesta viscosity

Non-starch polysaccharide degrading enzymes limit the increase in digesta viscosity through the hydrolysis of plant soluble polysaccharides (Santos *et al.*, 2013; Munyaka *et al.*, 2016; Bederska-Łojewska *et al.*, 2017). The enzymes reduce the thickness of the gut content and increase the nutritive value of the feed, by cleaving the large NSP molecules into smaller polymers, (Annison and Choct, 1991; Choct, 1997).

Studies on monogastric animals have shown that reduced digesta viscosity due to NSP-degrading enzyme supplementation is the main factor responsible for the observed enhanced performance response on feeding plant materials rich in soluble NSPs (Cowieson *et al.*, 2006). In general, maize and soybean meal NSP do not pose a viscosity problem, and the use of a combination of different carbohydrase activities to bring about effective cell wall degradation will be a better suited strategy (Slominski, 2011).

2.5.1.3 Stimulation of bacterial population

Carbohydrase supplementation has also been shown to increase gut health in animals fed high-NSP diets (Castillo and Gatlin, 2015). By reducing digesta viscosity, they encourage slower shedding of microorganisms and a decreased proliferation of harmful bacteria (Vahjen *et al.*, 1998). Exogenous enzymes not only influence the partitioning of nutrients to the host but also, through their action, produce nutrients for specific populations of bacteria, means that they are multifactorial in their effect (Bedford and Cowieson, 2012). Carbohydrases may stimulate and support growth of beneficial bacteria, thereby improving gut and overall health of the animal (Adeola and Cowieson, 2011).

Non-starch polysaccharide degrading enzymes break down plant cell wall carbohydrates and reduce their chain length, producing smaller polymers and oligomers, which can act as substrate for bacterial fermentation. These exogenous enzymes can positively alter volatile fatty acid production and the population profiles of gut-associated microflora (Bedford and Apajalahti, 2001). Carbohydrases improve energy utilisation by shifting absorption of energy-yielding nutrients to the proximal intestine, which decreases host–microbe competition for nutrients and ensures availability of nutrients where absorption efficiency is greatest (Adeola and Cowieson, 2011).

2.5.2 Types of non-starch polysaccharide degrading enzymes

2.5.2.1 Xylanase and debranching enzymes

Xylanases cleave the xylan backbone randomly resulting in non-substituted or branched xylooligosaccharides (Bedford and Partridge, 2010). Xylanases are well-known for their ability to degrade arabinoxylan from wheat (Courtin and Delcour, 2001), but maize arabinoxylan has a higher degree of substitution (Knudsen, 1997). Maize arabinoxylans are insoluble complex structures that are highly branched with several substitutions, and this affects susceptibility to hydrolysis by xylanases (Bunzel, 2009; Knudsen, 2014; Malunga and Beta, 2016). The branched structure prevents xylanases to bind and cleave the β-(1,4)-linked xylose backbone (Ravn *et al.*, 2016). The addition of supplementary de-branching enzymes such as arabinofuranosidases which removes arabinose, may increase the solubilisation capacity of xylanases by removing substituents present on the xylan chain (De La Mare *et al.*, 2013; Cozannet *et al.*, 2017; Ravn *et al.*, 2018).

With regards to feed application, only a partial hydrolysis of xylan is needed for viscosity reduction and thus xylanase addition to feed is already highly effective. However, for complete hydrolysis of the complex structure of xylan, a synergistic action of several hemicellulases is needed (Coughlan *et al.*, 1993). Xylanase may also enhance phytase efficacy by improving access to phytate that would otherwise be encapsulated in cell wall material or by stimulating the ileal brake mechanism and increasing gastric residency of feed (Schramm *et al.*, 2017). An additive effect has been observed when both phytase and carbohydrases were included in broiler diets, compared to including either enzymes independently (Cowieson and Adeola, 2005; Schramm *et al.*, 2017).

2.5.2.2 Cellulase, ß-glucanase and pectinase

Cellulases are glycoside hydrolase enzymes that catalyse the hydrolysis of ß-1,4-glycosidic bonds of cellulose to glucose (Ezeilo *et al.*, 2017). Cellulases can be further subdivided into three main groups namely endo-glucanases, exogluganases and cellobiohydrolases (Igarashi *et al.*, 2008; Ezeilo *et al.*, 2017). Cellulases such as endo-β-1,4 glucanase, are also able to cleave the internal ß-1,4-linkages in β-glucan, and are therefore considered to be a ß-glucanase (Grishutin *et al.*, 2006). Enzymes such as endo-β-1,3-glucanases, endo-β-1,3,1,4-glucanases, and β-glucosidases have the ability to cleave ß-glucosidic bonds in glucans other than cellulose (Grishutin *et al.*, 2006). In a study evaluating the effect of drought affected maize and carbohydrase enzyme mixture consisting of ß-glucanase, cellulase and xylanase inclusion on broiler performance and nutrient digestibility, the results showed no significant variation in broiler body weight or feed conversion ratio (Yoder *et al.*, 2015). The supplementation of an enzyme complex containing phytase, amylase, xylanase, ß-glucanase, pectinase, cellulase and protease, promoted similar performance as the positive control from days 1-21, but only partial improvements during the phase of 22-42 days (Nunes *et al.*, 2015).

Pectinase is oriented to the hydrolysis of 1,4-α-D-galacturonic bonds present in pectic chains (Vieira *et al.*, 2006). When pectinase was included in various enzyme combinations the results have been mixed. It ranged from a possible increase in the AME when pectinase was used in combination with xylanase, glucanase, cellulase, mannanase and galactanase enzymes, to no benefit at all when pectinase was used with glucanase and hemicellulase, to a decrease in AME when the enzyme mixture was used on canola meal as the substrate. The potential benefit of pectinase included in an enzyme combination is dependent on several factors such as the choice and activity of enzymes used, ingredients included in the diets and stage of development of the animal species (Bedford and Partridge, 2010). In a study by Tahir *et al.* (2006) in broilers fed a maize soybean based diet, pectinase alone had no significant effect on any of the parameters measured.

2.5.2.3 ß-Mannanase and α-galactosidase

The mode of action of ß-mannanase in monogastric animals is complex and is linked to the removal of ß-mannans from the animal's diet and it is likely that the beneficial effects of β-

mannanase on energy metabolism may be associated with an increased stimulation of insulin secretion and a blocking of the adverse effect of ß-galactomannan on glucose absorption (Jackson *et al.*, 2004). ß-mannanase targets the galatomannans in the diet, of which soybean meal is the main source (Latham *et al.*, 2016). The mechanism may also be associated with the enzyme's effect on viscosity in the gut, and the release of D-mannose as an energy source (Shastak *et al.*, 2015). ß-mannanase inclusion has been shown to improve body weight and feed conversion ratio in broilers fed a reduced energy diet (Williams *et al.*, 2014; Latham *et al.*, 2016). In a study done with broilers fed diets based on maize and soybean meal, ß-mannanase supplementation however showed no improvement on production performance (Azarfar, 2013).

α-Galactosidase is a glycoside hydrolase enzyme that hydrolyses the bonds of non-reducing α-D-galactose residues in α-D-galactosides from galactoside oligosaccharides, glycoproteins, glycolipids and other galactose-containing molecules (Vieira *et al.*, 2006). A study conducted with broilers on maize and soybean meal diets, α-galactosidase treatments only increased body weight at 21 days, and feed intake at 28 to 37 days of age, respectively. Performance of the birds at 37 days, was however not affected by the enzyme supplementation (Vieira *et al.*, 2006). Zou *et al.* (2013), however, observed α-galactosidase to improve performance of broiler chickens.

2.5.3 Inclusion of multi-enzymes in maize and soybean based broiler diets

Simon *et al.* (1996) stated that only a limited number of glycosidic links need to be broken in order to change the properties of soluble non-starch polysaccharides (NSP). However, to degrade insoluble cell wall materials and to release the entrapped nutrients, a mixture of different enzyme activities are needed. Meng and Slominski (2005) stated that nutrient utilisation of maize and soybean meal diets by broilers could be enhanced when an appropriate combination of multi carbohydrase enzymes are supplemented. There is growing evidence suggesting that the nutritional value of maize and soybean meal based diets can be improved by a combination of supplemental enzymes (Francesch and Geraert, 2009). Slominski (2011) stated that the constituent NSP in maize and soybean meal requires a broad range of carbohydrases, if any response is to be achieved.

In practical diets, which contain a number of plant ingredients and different forms of NSP, further improvements in nutrient utilisation could be achieved using combinations of carbohydrase enzymes, each differing in their substrate preference and mode of action, to target various structures of cell wall polysaccharides (Meng *et al.*, 2005). The beneficial effects of such combinations observed in the broiler industry may have resulted from elimination of the nutrient encapsulating effect of the cell wall polysaccharides and, to some extent, the reduction of intestinal viscosity. The effects of the inclusion of multi-enzymes in maize and soybean meal based broiler diets are discussed below, and summarised in Table 2.1.

In an experiment conducted by Woyengo *et al.* (2010), a multi-carbohydrase enzyme supplement containing cellulose, pectinase, mannanase, galactanase, xylanase, glucanase, amylase and protease improved growth performance and nutrient digestibility and retention. Du Plessis and Jansen van Rensburg (2014) used two enzyme preparations, one being a mixture of amylase, xylanase and protease and the other a ß-mannase product. The addition of the enzyme complexes to the energy restricted diets significantly improved the performance of the broilers, and a positive synergistic effect was evident when combining the two enzyme products. In the study of Cowieson and Bedford (2009), it was found that the supplementation of both ß-glucanase and xylanase to the negative control improved the feed conversion ratio and ileal nutrient digestibility.

The results of an experiment using a multi-enzyme product, allowed for the reduction of the metabolisable energy (ME), crude protein (CP), digestible amino acids (dAA), available phosphorus (P) and calcium (Ca) contents in broiler feed without a negative influence on performance (Francesch and Geraert, 2009). In a study done by De Keyser *et al.* (2016) the effect of NSP enzymes was tested to see if it is possible to save on CP and dAA in broiler feed. Three different multi carbohydrase enzyme products were used, and all of them were able to improve the negative control to the same level as the positive control, in terms of feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR). Klein *et al.* (2015) reported that the individual inclusion of ß-mannanase or a multi-enzyme complex can improve performance in reduced ME diets and when the two are co-administrated it resulted in a more consistent and elevated improvement in growth performance compared to the individual

inclusion in reduced energy diets. Govil *et al.* (2017), showed that the addition of xylanase, ß-mannanase and α-amylase to a low energy diet will improve the overall performance of broilers.

In a study combining carbohydrases and phytase in a diet deficient in ME, CP, Ca and P, the result was a substantial increase in growth performance and utilisation of P, DM, ME and nitrogen (N) in broiler chickens (Lu *et al.*, 2013). Lee *et al.* (2010) evaluated the effect of an enzyme blend which contains carbohydrases and phytase on growth performance and intestinal viscosity in broiler chicks. The average body weights (BW), daily BWG and the FCR of the chicks were improved significantly. BWG (4%) and FCR (7%) were improved significantly in a study combining ß-glucanase and xylanase enzymes (Munyaka *et al.*, 2016). Goli and Shahryar (2015) did a study on the effect of enzyme supplementation on blood biochemical parameters, performance and carcass characteristics in broiler chickens. There were three treatments where the first treatment had no enzymes included, the second had xylanase, ß-glucanase, cellulase and pectinase and the third treatment included xylanase, ß-glucanase and cellulase. The results obtained in the experiments lead to the conclusion that the addition of a multi-enzyme complex can improve broiler performance. Significant improvements in performance were seen in the study by Coppedge *et al.* (2012) where a carbohydrase product containing endo-pentosanase and carbohydrase product containing xylanase, ß-glucanase, α-galactosidase and ß-mannanase, were added to the started and grower periods of their experiment. Increases in processing parameters were also observed, but tended to be less sensitive to the carbohydrase inclusion. In an experiment conducted by Nadeem *et al.* (2005) the results showed that the FI and FCR from days 1-28 and days 1-42 was significantly improved in chicks fed a diet supplemented with a multi-enzyme product. The effects on BWG, dressing percentage and weights of organs, except liver weight, were however found to be non-significant.

When an enzyme complex containing ß-pentosanase, α-amylase, glucanases and galactomannanase was added to a broiler diet it resulted in a slight increase in the dressing percentage and has a positive effect on CP bioavailability and overall broiler performance. The authors also noted that a further increase in the level of enzymes supplied in the diet did not result in further improvements in performance (Abudabos, 2010). In another study, the objective was to determine if the supplementation of ß-mannanase and an enzyme cocktail

containing xylanase, ß-glucanase and α-galactosidase, to a diet also containing phytase would have an effect on the performance of broilers. It was concluded that supplementation of these enzymes improved the performance and processing parameters of male broilers. This data also showed that the intermittent application of enzymes targeting specific substrates determined by dietary ingredient profile could be beneficial (Williams *et al.*, 2014).

Based on the trail by Vieira *et al.* (2015) where an enzyme complex containing exogenous α-amylase and ß-glucanase was supplemented to the diet, it was concluded that it had a partially beneficial impact on the BWG and the FCR of broilers. Yegani and Korver (2013) investigated the effects of three diets containing different enzymes namely, xylanase, α-amylase and protease and lastly xylanase and ß-glucanase. The authors concluded that the enzyme products had no effects on the performance variables, and in some instances had negative impacts. A study performed by Zhu *et al.* (2014) evaluated the effects of xylanase, α-amylase and ß-glucanase supplementation on performance and digestive parameters of broilers fed a maize and soybean based diet from day 1 to day 21 of age. The enzyme supplementation had no effect on the average daily weight gain, FI and FCR. Kaczmarek *et al.* (2014) studied the effects of α-amylase alone or in combination with protease, on nutrient digestion during the first 2 weeks of growth. The results indicated that enzyme supplementation had no effect on either the BWG or the FCR. In an experiment evaluating the effects of a diet containing xylanase, pectinase and α-galactosidase on live performance and carcass yield of broilers, no response was observed to enzyme supplementation (Vieira *et al.*, 2006). Zakaria *et al.* (2010) performed a study to investigate the effect of adding a multi-enzyme feed additive on the performance of broilers, and carcass characteristics. The enzymes used in the study included protease, α-amylase, pectinase, phytase, gluco-amylase and cellulase. It was concluded that the enzyme supplementation elicited few responses in broilers when supplemented at three levels in contrast to a normal maize soybean meal diet.

Table 2.1 Summary of the effect of NSP-degrading enzymes on the performance of broilers fed maize and soybean meal based diets

Enzyme combination	Response	Reference
Cellulose, pectinase, mannanase, galactanase, xylanase, glucanase, amylase, protease	Improved growth performance and digestibility	(Woyengo *et al.*, 2010)
Amylase, xylanase, protease, mannanase	Improved growth performance	(Du Plessis and Jansen van Rensburg, 2014)
Glucanase and xylanase	Improved FCR and digestibility	(Cowieson and Bedford, 2009)
Multi-enzyme combination	Improved growth performance	(Francesch and Geraert, 2009)
Multi-enzyme combination	Improved growth performance	(De Keyser *et al.*, 2016)
Multi-enzyme combination and mannanase	Improved growth performance	(Klein *et al.*, 2015)
Xylanase, mannanase, amylase	Improved growth performance	(Govil *et al.*, 2017)
Multi-enzyme combination	Improved growth performance and digestibility	(Lu *et al.*, 2013)
Multi-enzyme combination	Improved growth performance	(Lee *et al.*, 2010)
Glucanase and xylanase	Improved growth performance	(Munyaka *et al.*, 2016)
Xylanase, glucanase, cellulase, pectinase	Improved growth performance	(Goli and Shahryar, 2015)
Pentosanase, xylanase, glucanase, galactosidase, mannanase	Improved growth and slaughter parameters	(Coppedge *et al.*, 2012)
Multi-enzyme combination	Improved FCR, no effect on BWG or slaughter parameters	(Nadeem *et al.*, 2005)
Pentosanase, amylase, glucanase, galactomannase	Improved growth and slaughter parameters	(Abudabos, 2010)
Mannanase, xylanase, glucanase, galactosidase	Improved growth and slaughter parameters	(Williams *et al.*, 2014)
Amylase, glucanase	Partial growth performance improvement	(Vieira *et al.*, 2015)
Xylanase, amylase, protease, glucanase	No improvement in growth parameters	(Yegani and Korver, 2013)
Xylanase, amylase, glucanase	No improvement in growth parameters	(Zhu *et al.*, 2014)
Amylase, protease	No improvement in growth parameters	(Kaczmarek *et al.*, 2014)
Xylanase, pectinase, galactosidase	No improvement in growth or slaughter parameters	(Vieira *et al.*, 2006)
Protease, amylase, pectinase, gluco-amylase, cellulase	No improvement in growth parameters	(Zakaria *et al.*, 2010)

The goal in using multiple enzymes as diet additives is to target different anti-nutritive compounds in feedstuffs to obtain maximum benefits. The use of multiple carbohydrase enzyme activities may produce greater benefit than each of the enzymes acting individually. However, to maximise the efficacy of enzyme combinations, it is essential to understand how the enzymes work together to hydrolyse their respective substrates (Castillo and Gatlin, 2015). There are conflicting reports in the literature on the ability of single and combinations of enzymes to positively influence growth performance (Coppedge *et al.*, 2012). Despite variances in efficiency, the use of enzyme combinations may provide more potent effects than when used separately (Olukosi *et al.*, 2007).

2.5.4 Factors affecting the efficacy of enzymes

Cowieson *et al.* (2006) stated that one of the primary challenges with respect to enzyme product supplementation is that enzyme addition may not always lead to enhanced growth performance or digestibility of nutrients, and this can be attributed to a wide array of factors. Gracia *et al.* (2003) noted that differences in types and activities, as well as the types of microorganisms being used to produce the enzyme products, can contribute to this variation in results. Other factors might be the level of inclusion (Cowieson and Ravindran, 2008b) and single versus mixture of enzyme activities (Cowieson and Adeola, 2005; Cowieson *et al.*, 2006). Cowieson (2010) reported that the most important factor that influences responses to enzyme products, is the nutritional quality of the diet, with responses expected to be greater in diets that are of lower quality. Ravindran (2013) stated that some of the most important factors contributing to variable bird responses to enzyme supplementation are dietary nutrient density, quality of the dietary ingredients and the age of the birds.

2.5.4.1 Effect of diet nutrient density on efficacy of enzymes

Enzyme effects on performance parameters are not usually observed when standard diets based on balanced and highly digestible nutrients are fed (Moraes *et al.*, 2015). When broilers are fed a theoretically perfect diet it is unlikely that any improvement will be observed by adding an enzyme on top of the diets, as the birds are already demonstrating their full potential which leaves little room for improvement (Sorbara *et al.*, 2009). When improved nutrient utilisation is not accompanied by increased growth performance, it is possible that the control diets were not sufficiently limiting in nutrients to reduce growth (Farhangi and Carter, 2007).

In a study that was conducted to compare the effects of xylanases and ß-glucanase, with α-galactosidase and ß-mannanase at different metabolisable energy concentrations, it was found that the supplementation of these enzymes to the broiler diet could improve the FCR of broilers by improving the energy digestibility and utilization. It was also noted that the addition of xylanases and ß-glucanase to a low energy diet had improved efficiency (Zou *et al.*, 2013). In another study, the combination of xylanase, α-amylase and protease fed at three different levels of metabolisable energy were evaluated. It was concluded that the addition of enzymes allowed for reduced energy levels of broiler diets without having any negative effects on the performance of the broiler chickens (Gitoee *et al.*, 2015). In an experiment where the effects on apparent metabolisable energy was evaluated with enzyme combinations it was found that none of the combinations successfully improved the performance of the standard diet. However, it was found that when enzymes were included in a lower energy diet, the combination of pectinase, protease, and α-amylase significantly improved the ME in comparison to the un-supplemented diet (Kocher *et al.*, 2003).

2.5.4.3 Effect of dietary ingredients on efficacy of enzymes

Bhuiyan *et al.* (2013), conducted an experiment to show the effects of enzyme supplementation to different levels of maize in the diet. The enzymes used in this experiment included xylanase, α-amylase, protease and phytase. The maize were included at three different levels, namely 250 g/kg, 500 g/kg and 750 g/kg. The results indicated that the inclusion of the enzymes to the different levels of maize resulted in a significant increase in FI and BW, but there was no change in the FCR. In the experiment of Meng and Slominski (2005) a multi-carbohydrase cocktail consisting of xylanase, ß-glucanase, pectinase, cellulase, ß-mannanase and galactanase was used in several diets. The four diets used in the study included a semi-purified maize diet, and three diets each containing 30% soybean meal, canola meal or peas in addition to maize. An improvement in BWG and FCR was observed only when the enzymes were included in the maize and soybean meal based diet.

In a study evaluating the effect of drought affected maize and carbohydrase enzyme mixture consisting of ß-glucanase, cellulase and xylanase inclusion on broiler performance and nutrient digestibility, the results showed no significant variation in broiler BW or FCR (Yoder *et al.*, 2015). The response of broiler chickens to two levels of a xylanase and ß-glucanase cocktail,

combined with one of three levels of digestible lysine in the diet were evaluated in an experiment. The enzyme supplementation decreased the FI by 4.67% and improved the FCR by 5.53% between days 1 to 42 without affecting the BWG. A depression in breast weights at day 42 due to 300 g sunflower meal or 8.0 g digestible lysine/kg of diet was compensated for by the enzyme addition. Therefore, there was a significant enzyme x sunflower meal effect (Mushtaq *et al.*, 2008).

Another study done by Cowieson and Ravindran (2008b) tested the sensitivity of broiler in the starter phase to three doses of an enzyme cocktail consisting of xylanase, α-amylase and protease. The results indicated that the supplementation of the control diet with the enzyme cocktail increased the performance in a dose-dependent manner. The dose sensitivity may be related to the concentration of substrates in the diet, ingredient quality and enzyme combinations in the cocktail. Higher doses offered the greatest improvements, but this may not always be the most economically attractive choice.

2.5.4.2 Effect of age of the birds on efficacy of enzymes

According to Figueiredo *et al.* (2012), enzyme supplementation should be beneficial to young and adult chickens. Responses to enzyme supplementation are normally expected to be higher in young broiler chickens, as endogenous enzyme activities in the digestive tract are generally limiting, which could lead to less efficient feed digestion (Olukosi *et al.*, 2007). The digestive enzyme secretion capacity in younger broilers are generally less developed than in adult chickens, and therefore the potential benefits from the addition of feed enzymes has a greater potential to improve digestion (Figueiredo *et al.*, 2012; Ravindran, 2013). The age dependent effect should however be of less significance when the supplemented enzyme activities are not part of the chicken's digestive system, and therefore expected to be complementary to the endogenous digestive enzymes (Aftab, 2012).

The effects of added enzymes may change with bird age (Bedford and Cowieson, 2012) as caecal populations increase in size and variability, and as a consequence fermentation responses to cell wall fragments may be more pronounced in older birds (Wang *et al.*, 2005; Parker *et al.*, 2007). The digestive and microbiota capacity increases with the broiler chicken's age (Bedford,

2000) and it is likely that feed enzymes influence broiler performance through an interaction with microbial populations, which becomes more prolific as the bird ages (Figueiredo *et al.*, 2012). Ravn *et al.* (2018) recently investigated the combined effect of xylanase and arabinofuranosidase debranching enzymes on broiler performance, maize glucurono-arabinoxylan breakdown and caecal microbial fermantation. Enzyme addition resulted in a significant improvement in BW and FCR, which was observed throughout the study, but more pronounced at days 21 and 29. The significantly increased caecal butyrate production most likely contributed to the observed improved gut morphology and broiler performance.

In a study done by Tahir *et al.* (2015) the authors showed that diets containing phytase with either xylanase or a combination of xylanase, protease, and α-amylase showed significant improvement at 35 days, but only a partial improvement at 49 days on the BWG and FCR in broilers. Muller Fernandes *et al.* (2015), found no difference in the effect of different enzymatic supplements at seven days of age, while the addition of enzymatic complexes improved the performance of the broilers at 21 and 35 days when compared to the control, regardless of the enzyme that was used. In an experiment where broilers were fed feeds with reduced mineral and energy levels, the supplementation of two enzyme promoted similar performance as the positive control from days 1-21, but only partial improvements were noted during the phase of 22-42 days. The enzyme combinations did not affect carcass or portion yield (Nunes *et al.*, 2015).

2.6 Enzymes produced by *Talaromyces versatilis*

Benjamin (1955) defined the genus *Talaromyces* as a sexual state of *Penicillium*. The soil deuteromycete *Penicillium funiculosum* was recently renamed as *Talaromyces versatilis*, after phylogenetic information revealed that it is distinct from other *Penicillium* subgenera. Filamentous fungi produce unique sets of enzymes to degrade complex molecules in their surrounding in order to provide them with food sources for growth (De La Mare *et al.*, 2013; Bianco and Perrotta, 2015). These fungi have evolved a complex yet very efficient mechanism for degradation of plant cell walls (Schmoll, 2018).

The filamentous fungus *Talaromyces versatilis* produces a wide range of cellulotic and hemicellulotic enzymes (Lafond *et al.*, 2014) which is utilised industrially to produce a

commercialised multi-enzyme cocktail called “Rovabio Excel” (De La Mare *et al.*, 2013). This product is used as feed additive in animal nutrition for enhancing digestibility of the feed materials that are composed of complex carbohydrates such as cellulose, hemicellulose, arabinoxylan and arabinogalactan, and hence to improve the animal performance and health (Guais *et al.*, 2010).

A recent proteonomic study revealed more than 50 proteins, which included several glycosylhydrolic, hemicellulolytic and proteolytic enzymes (Guais *et al.*, 2008). Several other studies have also been done to study and describe the xylanase (Lafond *et al.*, 2011; Texier *et al.*, 2012; Lafond *et al.*, 2014) and arabinofuranosidase (Guais *et al.*, 2010; De La Mare *et al.*, 2013) enzyme activities of *Talaromyces versatilis*.

The multi-enzyme producing strain was genetically modified via self-cloning to enrich the product by increasing xylanases by 14% and arabinofuranosidases by 65%, with the aim of enhancing its efficacy in breaking down highly substituted arabinoxylans (Guais *et al.*, 2008; Cozannet *et al.*, 2017). The multi-enzyme complex was renamed as “Rovabio Advance” (Adisseo, France), and is composed of xylanases, ß-glucanases, pectinases, cellulases, proteases and arabinofuranosidases (Rios *et al.*, 2017). Although several enzymatic activities are known to be present, the true efficacy of the cocktail is likely depending of the combination of enzymatic activity present in the protein mixture (Guais *et al.*, 2008).

Rios *et al.* (2017) evaluated the effects of the multi-enzyme complex on growth performance, energy and amino acid utilisation in broiler chickens, when added to maize and soybean meal diets. The authors reported that enzyme supplementation led to improvements in feed conversion ratio, digestible energy and digestible amino acids.

2.7 Conclusion

Non-starch polysaccharides (NSP) are one of the main anti-nutrients present in feed ingredients, and can interfere with the digestive processes and decrease the nutritive value of broiler feed. Within NSP, arabinoxylan appears to be the most important factor explaining digestibility impairment. The main anti-nutritional effects of NSP are the increase of digesta viscosity, encapsulation of nutrients and interaction with gut microflora. Even though maize and soybean meal based broiler diets are generally considered to be of high nutritional value, these raw

materials still contain varying levels of NSP that can interfere with digestive processes and lead to decreased production and feed efficiency. The ratio of insoluble to soluble NSP is much higher in maize compared to wheat, and therefore nutrient encapsulation should be more of an issue than the effect on digesta viscosity. Maize arabinoxylan also has a higher degree of substitution compared to wheat. Soybean meal also contains more insoluble than soluble NSP, with the insoluble component being similar to maize. Therefore the constituent NSP in maize and soybean meal diets requires an extensive range of enzymes, if any response is to be achieved. Some of the most important factors contributing to variable responses to enzyme supplementation in broiler diets are dietary nutrient density, quality of the dietary ingredients and age of the birds. The filamentous fungus *Talaromyces versatilis* produces a wide range of cellulotic and hemicellulotic enzymes, which are utilized commercially to produce a commercial multi-enzyme cocktail called “Rovabio Advance” (Adisseo, France). This product is used as a feed additive in animal nutrition for enhancing digestibility of feed materials that are composed of complex carbohydrates, to improve animal performance and health.

CHAPTER 3

Materials and Methods

3.1 Facilities and experimental animals

Experimental procedures were approved by the Animal Ethics Committee of the University of Pretoria (Project number: EC079-15). The trial was conducted at the Wincanton trial facility of Sovereign Foods in Uitenhage, South Africa. The birds were housed in an environmentally controlled house. The house had a solid concrete floor that was evenly covered with pine shavings. The individual pen sizes were 1.04 m x 1.04 m, giving a floor space of 1.08 m^2 per pen.

A total of 2112 male Ross 308 day-old chicks were obtained from Sovereign Foods Hatchery in Uitenhage, South Africa. The house consisted of 96 pens, which were further divided into 12 blocks with a total of 8 pens per block. The birds were randomly divided between the 96 pens, with 22 birds per pen at a stocking density of 20.37 birds/m^2 floor space. Each of the 8 treatments included in the study was repeated once within a block, with a total of 12 replicates per treatment.

3.3 Hygiene and biosecurity

The broiler house was cleaned, washed and disinfected with Vet GL 20 (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. 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.

3.4 General management and vaccinations

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 one bell drinker. The height

of the feeder and drinker were adjusted according to bird growth. The standard heating and lighting programs of Sovereign Foods were followed and can be seen in Table 3.1 and Table 3.2 respectively. The birds had *ad libitum* access to feed and water throughout the trial. To ensure availability of clean water, the bell drinkers were checked and cleaned daily. 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 at day 0, 7, 12 and 17 as indicated below in Table 3.3.

Table 3.1 Temperature profile for trial (degrees Celsius)

Days	Target temperature ˚C
0 - 6	35.0
7 - 13	31.0
14 - 20	27.0
21 - 27	25.0
28 - 35	23.0

Table 3.2 Lighting program for trial (hours)

Days	Time light on	Time 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 route
Day 0	Infectious Bronchitis (IB)	Course spray (Hatchery)
Day 0	New Castle Disease (NCD)	Course spray (Hatchery)
Day 0	Infectious Bursal Disease (IBD) (Gumboro)	Course spray (Hatchery)
Day 7	New Castle Disease (NCD)	Course spray (On farm)
Day 12	Infectious Bursal Disease (IBD) (Gumboro)	Course spray (On farm)
Day 12	New Castle Disease (NCD)	Course spray (On farm)
Day 17	Infectious Bursal Disease (IBD) (Gumboro)	Course spray (On farm)
Day 17	New Castle Disease (NCD)	Course spray (On farm)

3.2 Experimental design and diets

In order to evaluate the efficacy of the multi-enzyme complex produced by *Talaromyces versatalis* (Rovabio Advance, Adisseo, France) in reduced energy and amino acid diets, a study was done using a completely randomized block design. Three feeding phases that were used over a 5 week period. The first phase was a starter diet, which was fed from day 0 to day 14. This was followed by a grower diet, from day 15 to 28. The third and final phase, was fed from day 29 to day 35. The starter diet was fed in the form of crumbs, while the grower and finisher diets were pelleted.

The description of the treatment groups and experimental diets can be seen below in Table 3.4. The positive control diets (PC) were based on a typical South African maize and soybean meal based diet and formulated according to the nutrient specifications of Ross 308. The diets were formulated to be slightly lower in energy and amino acid levels, to ensure that the nutrients were marginally limiting in the feed rations. The negative control 1 diets (NC1) were further reduced in metabolisable energy, and the negative control 2 diets (NC2) were reduced in amino acids compared to the PC diets. The negative control 3 diets (NC3) were reduced in both metabolisable energy and amino acids compared to the PC diets. The Rovabio Advance enzyme complex was then added to the positive control (TRT1), the negative control 1 diets (TRT2), the negative control 2 diets (TRT3) and the negative control 3 diets (TRT4).

Table 3.4 Description of the experimental groups and diets

Experimental groups	Experimental diet description
PC = Positive control	Diet with standard commercial nutrient levels
NC1 = Negative control 1	PC - Metabolisable energy
NC2 = Negative control 2	PC - Digestible amino acids
NC3 = Negative control 3	PC - Metabolisable energy and digestible amino acids
TRT1 = Treatment 1	PC + Rovabio Advance
TRT2 = Treatment 2	NC1 + Rovabio Advance
TRT3 = Treatment 3	NC2 + Rovabio Advance
TRT4 = Treatment 4	NC3 + Rovabio Advance

3.6 Feed formulas

Least cost feed formulation software (Format International, UK) was used to formulate the broiler diets for the starter, grower and finisher phases. The metabolisable energy content, crude protein, and digestible amino acids of the diets were based on a typical South African maize and soybean meal based diet and formulated according to the nutrient specifications of Ross 308. The diets were formulated to be slightly lower in energy and amino acid levels, to ensure that the nutrients were marginally limiting in the feed rations. 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) inclusion level of 100 mg/kg. Dietary treatments 1, 2, 3 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 NSP enzyme (Rovabio Advance, Adisseo, France).

The experimental diets were mixed at Pennville (Pty) Ltd (Pretoria, South Africa). The amounts of all the feed ingredients to be used for the diets were calculated, procured and stored separately. Representative samples of the feed ingredients were collected and analysed prior to feed formulation in order to formulate the diets based on accurate nutrient profiles. The raw material matrixes were updated in the feed formulation software, before final feed formulations were done. Metabolisable energy and digestible amino acids were calculated based on standard procedures (CVB, 2007). The matrix values provided by the enzyme supplier (Adisseo, France), as can be seen in Table 3.5 below, were used to calculate the energy, crude protein and amino acids reductions to be applied to each of the negative control treatment formulations. Tables 3.6

to 3.11 shows the raw material composition and calculated nutrient specifications of all the treatments used during the starter, grower and finisher phases of the trial.

Table 3.5 Recommended enzyme nutritional uplift potential and matrix values (%)

Nutrients	Nutritional Uplift Potential	Matrix values (50 g/ton of feed inclusion)
AME Broiler (MJ/kg)	0.35	7040
Crude protein	0.5	10000
Dig. Lysine	0.02	490
Dig. Methionine	0.01	100
Dig. Cysteine	0.01	200
Dig. Sulphur Amino Acids	0.01	300
Dig. Threonine	0.03	560
Dig. Tryptophan	0.01	140
Dig. Isoleucine	0.02	400
Dig. Arginine	0.02	480
Dig. Valine	0.03	700

In order to minimize variation among dietary treatments, a base mix was calculated for each dietary phase. The part of the formulation that differed for each treatment, was added to the base mix and put through the mixer again to produce each of the 8 different treatments, for the 3 different dietary phases. Samples of each diet were taken from the feed bags as they were filled. These samples were combined and then sub-sampled so that 24 samples of 1 kg each were obtained.

Table 3.6 Raw material composition of the starter diets on an as fed basis (g/kg)

Ingredient	PC	NC1	NC2	NC3	TRT1	TRT2	TRT3	TRT4
Yellow maize	561	578	575	588	561	578	575	588
Soybean oilcake (46.5%)	287	284	276	270	287	284	276	270
Sunflower oilcake	50.0	60.0	50.0	70.0	50.0	60.0	50.0	70.0
Full fat soya	50.0	40.0	50.0	33.0	50.0	40.0	50.0	33.0
Limestone	16.1	16.2	16.2	16.1	16.1	16.2	16.2	16.1
Soya oil	13.0	0.0	10.5	0.0	13.0	0.0	10.5	0.0
Mono dicalcium phosphate	8.40	8.35	8.55	8.50	8.40	8.35	8.55	8.50
Sodium bicarbonate	2.95	3.09	3.02	3.23	2.95	3.09	3.02	3.23
Salt	1.65	1.55	1.61	1.45	1.65	1.55	1.61	1.45
Lysine HCL (78%)	2.70	2.88	2.79	3.09	2.70	2.88	2.79	3.09
Methionine DL (99%)	2.75	2.71	2.69	2.62	2.75	2.71	2.69	2.62
Threonine L (98%)	0.51	0.53	0.37	0.40	0.51	0.53	0.37	0.40
Vitamin & mineral premix	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Choline Chloride (60%)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Clinacox (Coccidiostat)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Zinc bacitracin (AGP)	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67
Phytase enzyme (Axtra Phy 10000)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
NSP enzyme (Rovabio Advance)	0.00	0.00	0.00	0.00	0.05	0.05	0.05	0.05

Vitamin and mineral premix composition of the starter feed in a 1.5 kg unit, with contribution per kg of complete feed: vitamin A: 12 000 IU; vitamin D3: 4 000 IU; vitamin E: 60 IU; vitamin K3: 4 mg; vitamin B1: 4 mg; vitamin B2: 9 mg; vitamin B3: 60 mg; vitamin B5: 15 mg; vitamin B6: 5 mg; vitamin B9: 2 mg; vitamin B12: 0.025 mg; vitamin H: 0.2 mg; antioxidant: 200 mg; Mn: 100 mg; Fe: 70 mg; Zn: 60 mg; Cu: 20 mg; Se: 0.3 mg; I: 1.25 mg. 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.

Table 3.7 Calculated nutrient specifications of the starter diets on an as fed basis (%)

	PC	NC1	NC2	NC3	TRT1	TRT2	TRT3	TRT4
Dry matter	88.73	88.61	88.69	88.63	88.73	88.61	88.69	88.63
AME broiler (MJ/kg)	11.29	10.95	11.30	10.95	11.29	10.95	11.30	10.95
Crude protein	21.75	21.74	21.31	21.31	21.75	21.74	21.31	21.31
Crude fat	4.66	3.25	4.44	3.15	4.66	3.25	4.44	3.15
Crude fat (acid hydrolysis)	5.28	3.86	5.07	3.75	5.28	3.86	5.07	3.75
Fibre	3.64	3.79	3.63	3.91	3.64	3.79	3.63	3.91
Total calcium	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Digestible calcium	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58
Total phosphorous	0.60	0.60	0.59	0.60	0.60	0.60	0.59	0.60
Digestible phosphorous	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Sodium	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
Chloride	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Potassium	1.07	1.07	1.05	1.04	1.07	1.07	1.05	1.04
Lysine	1.34	1.34	1.32	1.32	1.34	1.34	1.32	1.32
Methionine	0.61	0.61	0.60	0.60	0.61	0.61	0.60	0.60
Cysteine	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Sulphur amino acids	0.96	0.96	0.95	0.95	0.96	0.96	0.95	0.95
Threonine	0.81	0.81	0.80	0.79	0.81	0.81	0.80	0.79
Tryptophan	0.25	0.25	0.24	0.24	0.25	0.25	0.24	0.24
Isoleucine	0.92	0.91	0.90	0.89	0.92	0.91	0.90	0.89
Arginine	1.47	1.47	1.44	1.44	1.47	1.47	1.44	1.44
Phenylalanine	1.06	1.06	1.04	1.04	1.06	1.06	1.04	1.04
Leucine	1.80	1.80	1.77	1.76	1.80	1.80	1.77	1.76
Tyrosine	0.77	0.76	0.75	0.74	0.77	0.76	0.75	0.74
Valine	1.02	1.02	1.00	1.00	1.02	1.02	1.00	1.00
Alanine	1.07	1.07	1.05	1.05	1.07	1.07	1.05	1.05
Glutamine	3.87	3.87	3.80	3.80	3.87	3.87	3.80	3.80
Histidine	0.59	0.59	0.58	0.57	0.59	0.59	0.58	0.57
Dig. Lysine	1.16	1.16	1.14	1.14	1.16	1.16	1.14	1.14
Dig. Methionine	0.57	0.57	0.56	0.56	0.57	0.57	0.56	0.56
Dig. Cysteine	0.28	0.28	0.27	0.28	0.28	0.28	0.27	0.28
Dig. sulphur amino acids	0.85	0.85	0.83	0.83	0.85	0.85	0.83	0.83
Dig. Threonine	0.72	0.72	0.69	0.69	0.72	0.72	0.69	0.69
Dig. Tryptophan	0.22	0.21	0.21	0.21	0.22	0.21	0.21	0.21
Dig. Isoleucine	0.80	0.80	0.78	0.78	0.80	0.80	0.78	0.78
Dig. Arginine	1.31	1.31	1.28	1.28	1.31	1.31	1.28	1.28
Dig. Phenylalanine	0.94	0.94	0.92	0.92	0.94	0.94	0.92	0.92
Dig. Histidine	0.51	0.51	0.50	0.50	0.51	0.51	0.50	0.50
Dig. Leucine	1.59	1.59	1.56	1.56	1.59	1.59	1.56	1.56
Dig. Tyrosine	0.67	0.66	0.65	0.64	0.67	0.66	0.65	0.64
Dig. Valine	0.87	0.87	0.85	0.85	0.87	0.87	0.85	0.85
Dig. Alanine	0.89	0.89	0.87	0.88	0.89	0.89	0.87	0.88
Dig. Glutamine	1.93	1.92	1.88	1.86	1.93	1.92	1.88	1.86

Table 3.8 Raw material composition of the grower diets on an as fed basis (g/kg)

Ingredient	PC	NC1	NC2	NC3	TRT1	TRT2	TRT3	TRT4
Yellow maize	611	626	626	639	611	626	626	639
Soybean oilcake (46.5%)	245	238	232	225	245	238	232	225
Sunflower oilcake	50.0	60.0	50.0	70.0	50.0	60.0	50.0	70.0
Full fat soya	50.0	45.0	50.0	35.0	50.0	45.0	50.0	35.0
Limestone	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2
Soya oil	14.0	0.0	11.0	0.0	14.0	0.0	11.0	0.0
Mono dicalcium phosphate	3.50	3.45	3.70	3.60	3.50	3.45	3.70	3.60
Sodium bicarbonate	3.06	3.20	3.16	3.37	3.06	3.20	3.16	3.37
Salt	1.59	1.49	1.53	1.37	1.59	1.49	1.53	1.37
Lysine HCL (78%)	2.72	2.90	2.86	3.16	2.72	2.90	2.86	3.16
Methionine DL (99%)	2.59	2.54	2.54	2.47	2.59	2.54	2.54	2.47
Threonine L (98%)	0.55	0.57	0.44	0.47	0.55	0.57	0.44	0.47
Vitamin & mineral premix	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Choline chloride (60%)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Clinacox (Coccidiostat)	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67
Zinc bacitracin (AGP)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Phytase enzyme (Axtra Phy 10000)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
NSP enzyme (Rovabio Advance)	0.00	0.00	0.00	0.00	0.05	0.05	0.05	0.05

Vitamin and mineral premix composition of the grower feed in a 1.5 kg unit, with contribution per kg of complete feed: vitamin A: 12 000 IU; vitamin D3: 4 000 IU; vitamin E: 60 IU; vitamin K3: 4 mg; vitamin B1: 4 mg; vitamin B2: 9 mg; vitamin B3: 60 mg; vitamin B5: 15 mg; vitamin B6: 5 mg; vitamin B9: 2 mg; vitamin B12: 0.025 mg; vitamin H: 0.2 mg; antioxidant: 200 mg; Mn: 100 mg; Fe: 70 mg; Zn: 60 mg; Cu: 20 mg; Se: 0.3 mg; I: 1.25 mg. 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.

Table 3.9 Calculated nutrient specifications for the grower diets on an as fed basis (%)

	PC	NC1	NC2	NC3	TRT1	TRT2	TRT3	TRT4
Dry matter	88.61	88.48	88.57	88.50	88.61	88.48	88.57	88.50
AME broiler (MJ/kg)	11.65	11.30	11.65	11.30	11.65	11.30	11.65	11.30
Crude protein	20.12	20.12	19.63	19.63	20.12	20.12	19.63	19.63
Crude fat	4.88	3.45	4.61	3.30	4.88	3.45	4.61	3.30
Crude fat (acid hydrolysis)	5.50	4.07	5.24	3.91	5.50	4.07	5.24	3.91
Fibre	3.61	3.78	3.61	3.89	3.61	3.78	3.61	3.89
Total calcium	0.79	0.79	0.79	0.79	0.79	0.79	0.79	0.79
Digestible calcium	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47
Total phosphorous	0.47	0.48	0.47	0.48	0.47	0.48	0.47	0.48
Digestible phosphorous	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36
Sodium	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
Chloride	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Potassium	0.98	0.98	0.95	0.94	0.98	0.98	0.95	0.94
Lysine	1.23	1.23	1.21	1.21	1.23	1.23	1.21	1.21
Methionine	0.58	0.57	0.56	0.56	0.58	0.57	0.56	0.56
Cysteine	0.33	0.33	0.32	0.33	0.33	0.33	0.32	0.33
Sulphur amino acids	0.91	0.91	0.89	0.89	0.91	0.91	0.89	0.89
Threonine	0.75	0.75	0.73	0.73	0.75	0.75	0.73	0.73
Tryptophan	0.23	0.22	0.22	0.22	0.23	0.22	0.22	0.22
Isoleucine	0.84	0.84	0.82	0.81	0.84	0.84	0.82	0.81
Arginine	1.34	1.34	1.30	1.31	1.34	1.34	1.30	1.31
Phenylalanine	0.98	0.98	0.95	0.95	0.98	0.98	0.95	0.95
Leucine	1.69	1.69	1.66	1.65	1.69	1.69	1.66	1.65
Tyrosine	0.71	0.70	0.69	0.68	0.71	0.70	0.69	0.68
Valine	0.95	0.94	0.92	0.92	0.95	0.94	0.92	0.92
Alanine	1.00	1.01	0.99	0.99	1.00	1.01	0.99	0.99
Glutamine	3.58	3.58	3.49	3.50	3.58	3.58	3.49	3.50
Histidine	0.59	0.59	0.58	0.57	0.59	0.59	0.58	0.57
Dig. Lysine	1.06	1.06	1.04	1.04	1.06	1.06	1.04	1.04
Dig. Methionine	0.53	0.53	0.52	0.52	0.53	0.53	0.52	0.52
Dig. Cysteine	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26
Dig. sulphur amino acids	0.79	0.80	0.78	0.78	0.79	0.80	0.78	0.78
Dig. threonine	0.67	0.67	0.64	0.64	0.67	0.67	0.64	0.64
Dig. tryptophan	0.20	0.19	0.19	0.19	0.20	0.19	0.19	0.19
Dig. isoleucine	0.73	0.73	0.71	0.70	0.73	0.73	0.71	0.70
Dig. Arginine	1.19	1.19	1.16	1.16	1.19	1.19	1.16	1.16
Dig. phenylalanine	0.87	0.86	0.84	0.84	0.87	0.86	0.84	0.84
Dig. histidine	0.47	0.47	0.46	0.46	0.47	0.47	0.46	0.46
Dig. Leucine	1.49	1.49	1.46	1.46	1.49	1.49	1.46	1.46
Dig. Tyrosine	0.61	0.61	0.59	0.59	0.61	0.61	0.59	0.59
Dig. Valine	0.80	0.80	0.78	0.78	0.80	0.80	0.78	0.78
Dig. alanine	0.84	0.84	0.82	0.83	0.84	0.84	0.82	0.83
Dig. glutamine	1.75	1.73	1.69	1.67	1.75	1.73	1.69	1.67

Table 3.10 Raw material composition of finisher diets on an as fed basis (g/kg)

Ingredient	PC	NC1	NC2	NC3	TRT1	TRT2	TRT3	TRT4
Yellow maize	622	639	636	650	622	639	636	650
Soybean oilcake (46.5%)	206	202	194	188	206	202	194	188
Sunflower oilcake	50.0	60.0	50.0	70.0	50.0	60.0	50.0	70.0
Full fat soya	80.0	70.5	80.0	63.5	80.0	70.5	80.0	63.5
Limestone	12.7	12.7	12.8	12.7	12.7	12.7	12.8	12.7
Soya oil	13.5	0.0	10.5	0.0	13.5	0.0	10.5	0.0
Mono dicalcium phosphate	2.55	2.50	2.70	2.65	2.55	2.50	2.70	2.65
Sodium bicarbonate	3.10	3.24	3.18	3.39	3.10	3.24	3.18	3.39
Salt	1.57	1.47	1.52	1.36	1.57	1.47	1.52	1.36
Lysine HCL (78%)	2.75	2.94	2.85	3.16	2.75	2.94	2.85	3.16
Methionine DL (99%)	2.45	2.41	2.40	2.33	2.45	2.41	2.40	2.33
Threonine L (98%)	0.55	0.57	0.42	0.44	0.55	0.57	0.42	0.44
Vitamin & mineral premix	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride (60%)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Clinacox (Coccidiostat)	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67
Zinc bacitracin (AGP)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Phytase enzyme (Axtra Phy 10000)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
NSP enzyme (Rovabio Advance)	0.00	0.00	0.00	0.00	0.05	0.05	0.05	0.05

Vitamin and mineral premix composition of the finisher feed in a 1.5 kg unit, with contribution per kg of complete feed: vitamin A: 10 000 IU; vitamin D3: 3 000 IU; vitamin E: 60 IU; vitamin K3: 3 mg; vitamin B1: 2 mg; vitamin B2: 7.5 mg; vitamin B3: 50 mg; vitamin B5: 13 mg; vitamin B6: 5 mg; vitamin B9: 1.5 mg; vitamin B12: 0.025 mg; vitamin H: 0.12 mg; antioxidant: 200 mg; Mn: 100 mg; Fe: 40 mg; Zn: 50 mg; Cu: 15 mg; Se: 0.3 mg; I: 1.25 mg. 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.

Table 3.11 Calculated nutrient specifications for the finisher diets on an as fed basis (%)

	PC	NC1	NC2	NC3	TRT1	TRT2	TRT3	TRT4
Dry matter	88.60	88.47	88.56	88.50	88.60	88.47	88.56	88.50
AME broiler (MJ/kg)	11.85	11.50	11.85	11.50	11.85	11.50	11.85	11.50
Crude protein	19.42	19.42	18.99	18.97	19.42	19.42	18.99	18.97
Crude fat	5.36	3.91	5.10	3.81	5.36	3.91	5.10	3.81
Crude fat (acid hydrolysis)	6.03	4.57	5.76	4.46	6.03	4.57	5.76	4.46
Fibre	3.65	3.81	3.65	3.93	3.65	3.81	3.65	3.93
Total calcium	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Digestible calcium	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
Total phosphorous	0.45	0.45	0.44	0.45	0.45	0.45	0.44	0.45
Digestible phosphorous	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
Sodium	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
Chloride	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Potassium	0.94	0.94	0.92	0.91	0.94	0.94	0.92	0.91
Lysine	1.19	1.19	1.16	1.16	1.19	1.19	1.16	1.16
Methionine	0.55	0.55	0.54	0.54	0.55	0.55	0.54	0.54
Cysteine	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32
Sulphur amino acids	0.87	0.87	0.86	0.86	0.87	0.87	0.86	0.86
Threonine	0.72	0.72	0.70	0.70	0.72	0.72	0.70	0.70
Tryptophan	0.22	0.22	0.21	0.21	0.22	0.22	0.21	0.21
Isoleucine	0.81	0.80	0.79	0.78	0.81	0.80	0.79	0.78
Arginine	1.29	1.29	1.26	1.25	1.29	1.29	1.26	1.25
Phenylalanine	0.94	0.94	0.92	0.92	0.94	0.94	0.92	0.92
Leucine	1.64	1.64	1.61	1.60	1.64	1.64	1.61	1.60
Tyrosine	0.68	0.67	0.66	0.65	0.68	0.67	0.66	0.65
Valine	0.91	0.91	0.89	0.89	0.91	0.91	0.89	0.89
Alanine	0.98	0.98	0.96	0.96	0.98	0.98	0.96	0.96
Glutamine	3.46	3.46	3.38	3.38	3.46	3.46	3.38	3.38
Histidine	0.59	0.59	0.58	0.57	0.59	0.59	0.58	0.57
Dig. Lysine	1.02	1.02	1.00	1.00	1.02	1.02	1.00	1.00
Dig. methionine	0.51	0.51	0.50	0.50	0.51	0.51	0.50	0.50
Dig. cysteine	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Dig. sulphur amino acids	0.76	0.77	0.75	0.75	0.76	0.77	0.75	0.75
Dig. threonine	0.64	0.64	0.62	0.61	0.64	0.64	0.62	0.61
Dig. tryptophan	0.19	0.18	0.18	0.18	0.19	0.18	0.18	0.18
Dig. isoleucine	0.70	0.69	0.68	0.67	0.70	0.69	0.68	0.67
Dig. arginine	1.15	1.14	1.12	1.12	1.15	1.14	1.12	1.12
Dig. phenylalanine	0.83	0.83	0.81	0.81	0.83	0.83	0.81	0.81
Dig. histidine	0.45	0.45	0.44	0.44	0.45	0.45	0.44	0.44
Dig. leucine	1.44	1.45	1.42	1.41	1.44	1.45	1.42	1.41
Dig. tyrosine	0.59	0.58	0.57	0.56	0.59	0.58	0.57	0.56
Dig. Valine	0.77	0.77	0.75	0.75	0.77	0.77	0.75	0.75
Dig. alanine	0.81	0.82	0.80	0.80	0.81	0.82	0.80	0.80
Dig. glutamine	1.67	1.65	1.62	1.59	1.67	1.65	1.62	1.59

3.7 Analysis of feed samples

Representative samples of the 24 different feeds (8 experimental diets for each of the three phases) were collected after the production of the feed and during feeding, before the birds had access to the feed. One representative sample of each of the 24 different feeds, was analysed for their nutritional content and determine the accuracy of the formulated diets. The analysis was done at the Chem Nutri Analytical laboratory (Johannesburg, South Africa). Samples were ground and analysed for dry matter, ash, crude protein, ether extract, crude fibre, calcium, phosphorus, sodium, chloride and potassium.

Dry matter and ash content of the feed samples were determined following the AOAC's official method of analysis (AOAC, 2000, Official method of analysis 942.05). Moisture was determined according to the AOAC's official method of analysis (AOAC, 2000, Official method of analysis 943.01). Crude fibre was analysed following the AOAC's official method of analysis (AOAC, 2000, Official method of analysis 962.09). The crude fat content was determined according to the AOAC's official method of analysis (AOAC, 2000, Official method of analysis 920.39). Crude protein was analysed following the AOAC's official method of analysis (AOAC, 2000, Official method of analysis 988.05). The phosphorus and chloride content in the feed were determined using the AOAC's official method of analysis for phosphorus (AOAC, 2000, Official method of analysis 965.17) and chloride (AOAC, 2000, Official method of analysis 969.10). The calcium, sodium and potassium content in the feed were determined using the AOAC's official method of analysis (AOAC, 2000, Official method of analysis 935.13).

Representative samples of the 24 different feeds (8 treatments and 3 phases) were also send to the Carat Laboratory (Adisseo, France) for analysis of gross energy and total amino acids. Gross energy was determined using an isoperibol oxygen bomb calorimeter. Total amino acids were determined according to the European Union's official method of analysis (European Union, 2009, Z100), using a liquid chromatography amino acid analyzer. Representative samples of the feed were analysed for phytase and xylanase activity at the Carat Laboratory (Adisseo, France), using a spectrometer and viscometer respectively. A phytase unit (FTU) unit is defined as the amount of enzyme that liberates one micromole inorganic phosphate per minute from sodium phytate at pH 5.5 and 37^{0}C. For xylanase one viscosimetry unit (VU) is the amount of

enzyme which hydrolyzes the substrate (wheat arabinoxylan), reducing the viscosity of the solution to give a change in relative fluidity of one unit per minute at 30^{0}C and pH 5.5.

3.8 Production parameters

3.8.1 Body weight and body weight gain

The body weights of all the chickens were determined at day old, day 7, 14, 21, 28 and 35, on a per pen basis. Average body weight (g / bird), weekly body weight gain (g / bird / day) and body weight gain (g / bird) for the overall trial period was calculated for each pen.

3.8.2 Feed intake

The amount of feed consumed by the chickens was determined on day 7, 14, 21, 28 and 35 on a per pen basis. The average feed intake for all birds was calculated on a weekly and cumulative basis. With every change to the next phase, the left over feed from the previous phase was weighed back and discarded.

3.8.3 Mortality corrected feed conversion ratio

The body weight and the amount of feed consumed on day 7, 14, 21, 28 and 35, were used to calculate the feed conversion ratio (unit of feed consumed per unit of live mass gained) per treatment group. The feed conversion ratios were corrected for mortalities. The weight of the dead birds in every experimental group for every week was calculated and added to the live weight on day 7, 14, 21, 28 and 35. The total feed consumed was divided by the total body weight gained on the days mentioned.

3.9 Carcass parameters

The average weight of the broilers were calculated per pen at 35 days of age. A total of 2 chickens were selected per pen, with weights within a 100 grams range of the average of the pen. A total of 24 birds were therefore selected per treatment group.

Carcass weight was measured after manual evisceration by the removal of the head, feet and viscera. Whole carcass weight was expressed as a percentage of live body weight to calculate dressing percentage. Thereafter the carcass was manually dissected, and the weights of the breasts, drumsticks and wings were measured and also expressed as a percentage of live body weight. The processing of the carcasses was done immediately after slaughter, to limit potential changes in moisture content.

3.10 Statistical analysis

Data were analysed statistically as a randomized block design with the GLM model (SAS, 2018) for the average effects over time. Repeated Measures Analysis of Variance with the GLM model was used for repeated week or period measures. Means and standard errors were calculated and significance of difference ($P < 0.05$) was determined by Fischer's test (Samuels, 1989).

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 (growth and carcass parameters)

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

Standard chi-square tests were used for the mortality data, and the data were analysed with the frequency model of SAS (2018). The level of statistical significance was $P < 0.05$.

Chapter 4

Results

4.1 Feed analysis

The results of all feed analysis are summarized in Table 4.1 (broiler starter feed), Table 4.2 (broiler grower feed) and Table 4.3 (broiler finisher feed).

Table 4.1 Chemical analysis of the broiler starter feeds on a dry matter basis (%)

Nutrients	PC	NC1	NC2	NC3	TRT1	TRT2	TRT3	TRT4
Dry matter	91.0	90.2	89.9	89.8	90.6	89.8	90.3	89.8
Gross energy (MJ/kg)	17.31	16.73	17.16	16.75	17.13	16.87	17.12	16.73
Crude protein	22.3	21.4	19.9	20.1	21.4	21.6	20.9	20.9
Crude fat	4.90	4.60	6.60	4.50	6.50	4.70	5.60	4.40
Ash	5.21	5.57	4.85	4.81	5.25	4.78	5.63	5.47
Total calcium	0.99	1.05	0.79	0.88	0.96	0.89	0.98	0.98
Total phosphorus	0.60	0.63	0.57	0.57	0.60	0.60	0.61	0.62
Sodium	0.16	0.20	0.15	0.15	0.16	0.16	0.16	0.17
Chloride	0.19	0.22	0.19	0.19	0.20	0.19	0.21	0.21
Potassium	1.00	1.04	0.90	0.92	0.96	0.97	0.97	0.94
Lysine	1.27	1.24	1.26	1.26	1.29	1.31	1.30	1.35
Methionine	0.56	0.54	0.68	0.65	0.65	0.67	0.68	0.65
Cysteine	0.33	0.31	0.30	0.31	0.31	0.32	0.31	0.34
Threonine	0.85	0.83	0.79	0.79	0.83	0.85	0.83	0.84
Isoleucine	0.94	0.91	0.85	0.85	0.89	0.90	0.89	0.93
Arginine	1.45	1.41	1.34	1.33	1.39	1.41	1.42	1.42
Phenylalanine	1.04	1.01	0.92	0.94	0.98	1.00	0.98	0.98
Leucine	1.84	1.80	1.65	1.64	1.70	1.74	1.71	1.75
Tyrosine	0.74	0.71	0.66	0.66	0.69	0.70	0.69	0.68
Valine	1.06	1.02	0.98	0.97	1.02	1.03	1.02	1.03
Alanine	1.06	1.03	0.97	0.97	0.99	1.01	1.01	1.04
Glutamine	4.10	3.98	3.68	3.69	3.83	3.90	3.85	3.85
Histidine	0.54	0.53	0.49	0.49	0.51	0.52	0.52	0.54
Glycine	0.88	0.85	0.82	0.83	0.86	0.88	0.87	0.89
Serine	1.06	1.03	0.96	0.95	1.00	1.02	1.01	1.03
Xylanase VU/kg[1]	0	0	0	0	3504	4047	3907	4180
Phytase FTU/kg[2]	1045	1060	1548	1102	1142	1567	1664	1360

PC = Positive control, TRT1 = PC + Rovabio Advance 50 g/ t of feed, NC1 = Negative control 1 (PC - ME), TRT2 = NC1 + Rovabio Advance 50 g/ t of feed, NC2 = Negative control 2 (PC - Amino acids), TRT3 = NC2 + Rovabio Advance 50 g/ t of feed, NC3 = Negative control 3 (Positive control - ME and amino acids), TRT4 = NC3 + Rovabio Advance 50 g/ t of feed

[1] VU/kg = Visco units of xylanase per kg of feed, [2] FTU/kg = Phytase units per kg of feed

Table 4.2 Chemical analysis of the broiler grower feeds on a dry matter basis (%)

Nutrients	PC	NC1	NC2	NC3	Trt1	Trt2	Trt3	Trt4
Dry matter	89.2	88.7	89.7	89.1	89.5	89.1	89.4	89.3
Gross energy (MJ/kg)	17.24	17.01	17.18	17.22	17.05	17.03	17.11	16.91
Crude protein	20.5	20.1	19.8	20.0	20.6	20.4	19.5	19.4
Crude fat	6.00	4.30	6.40	5.50	6.60	6.20	6.90	5.60
Ash	5.17	4.65	4.85	4.88	4.61	4.61	4.80	4.78
Total calcium	0.87	0.84	0.86	0.80	0.87	0.77	0.85	0.87
Total phosphorus	0.52	0.46	0.50	0.45	0.47	0.43	0.47	0.49
Sodium	0.19	0.17	0.18	0.17	0.17	0.16	0.18	0.18
Chloride	0.21	0.22	0.20	0.20	0.20	0.20	0.20	0.21
Potassium	1.01	0.95	0.91	0.87	0.96	0.90	0.93	0.92
Lysine	1.26	1.24	1.22	1.23	1.29	1.27	1.20	1.21
Methionine	0.61	0.57	0.58	0.57	0.63	0.60	0.57	0.57
Cysteine	0.34	0.32	0.33	0.33	0.34	0.34	0.32	0.31
Threonine	0.83	0.82	0.78	0.79	0.81	0.82	0.77	0.76
Isoleucine	0.90	0.88	0.87	0.86	0.91	0.90	0.85	0.87
Arginine	1.33	1.31	1.31	1.31	1.34	1.38	1.29	1.31
Phenylalanine	0.95	0.95	0.92	0.94	0.96	0.96	0.93	0.93
Leucine	1.75	1.74	1.73	1.70	1.73	1.76	1.73	1.72
Tyrosine	0.68	0.68	0.68	0.67	0.70	0.70	0.66	0.65
Valine	1.00	0.99	0.97	0.96	0.99	1.00	0.96	0.97
Alanine	1.03	1.02	1.01	1.00	1.01	1.03	0.99	1.00
Glutamine	3.72	3.69	3.65	3.62	3.71	3.73	3.55	3.64
Histidine	0.53	0.51	0.51	0.51	0.53	0.54	0.50	0.50
Glycine	0.84	0.83	0.81	0.82	0.83	0.85	0.80	0.82
Serine	0.99	0.98	0.98	0.95	0.99	0.99	0.95	0.95
Xylanase VU/kg[1]	0	0	0	0	3210	2979	3098	3335
Phytase FTU/kg[2]	1341	1393	1243	1429	1367	1486	1463	1197

PC = Positive control, TRT1 = PC + Rovabio Advance 50 g/ t of feed, NC1 = Negative control 1 (PC - ME), TRT2 = NC1 + Rovabio Advance 50 g/ t of feed, NC2 = Negative control 2 (PC - Amino acids), TRT3 = NC2 + Rovabio Advance 50 g/ t of feed, NC3 = Negative control 3 (Positive control - ME and amino acids), TRT4 = NC3 + Rovabio Advance 50 g/ t of feed

[1] VU/kg = Visco units of xylanase per kg of feed, [2] FTU/kg = Phytase units per kg of feed

Table 4.3 Chemical analysis of the finisher feeds on a dry matter basis (%)

Nutrients	PC	NC1	NC2	NC3	Trt1	Trt2	Trt3	Trt4
Dry matter	89.2	88.7	89.7	89.1	89.5	89.1	89.4	89.3
Gross energy (MJ/kg)	17.14	17.01	17.25	17.05	17.47	17.15	17.39	17.08
Crude protein	18.8	19.1	18.5	18.4	18.9	18.8	18.3	18.6
Crude fat	6.00	4.30	6.40	5.50	6.60	6.20	6.90	5.60
Ash	4.76	4.44	4.66	4.18	4.18	4.64	4.20	4.74
Total calcium	0.82	0.69	0.73	0.69	0.71	0.70	0.76	0.71
Total phosphorus	0.51	0.45	0.46	0.46	0.43	0.45	0.47	0.44
Sodium	0.18	0.16	0.17	0.16	0.16	0.17	0.17	0.17
Chloride	0.21	0.21	0.21	0.20	0.20	0.28	0.28	0.27
Potassium	0.86	0.80	0.82	0.81	0.80	0.82	0.82	0.79
Lysine	1.12	1.17	1.17	1.12	1.15	1.17	1.15	1.15
Methionine	0.53	0.59	0.55	0.57	0.57	0.55	0.56	0.51
Cysteine	0.30	0.31	0.30	0.30	0.30	0.31	0.30	0.31
Threonine	0.75	0.76	0.74	0.74	0.77	0.78	0.73	0.75
Isoleucine	0.83	0.82	0.83	0.81	0.83	0.86	0.83	0.82
Arginine	1.21	1.24	1.24	1.18	1.23	1.27	1.22	1.24
Phenylalanine	0.89	0.88	0.89	0.85	0.89	0.92	0.89	0.87
Leucine	1.69	1.63	1.67	1.65	1.67	1.71	1.66	1.65
Tyrosine	0.65	0.64	0.66	0.63	0.66	0.68	0.65	0.64
Valine	0.92	0.92	0.94	0.92	0.94	0.96	0.93	0.93
Alanine	0.97	0.96	0.98	0.96	0.97	0.99	0.97	0.97
Glutamine	3.45	3.42	3.47	3.42	3.47	3.55	3.44	3.48
Histidine	0.49	0.48	0.49	0.47	0.50	0.49	0.48	0.48
Glycine	0.78	0.78	0.79	0.78	0.79	0.81	0.78	0.79
Serine	0.92	0.91	0.92	0.90	0.93	0.95	0.91	0.91
Xylanase VU/kg[1]	0	0	0	0	3122	3275	3084	2773
Phytase FTU/kg[2]	1316	1266	1228	1335	1318	1188	1326	1149

PC = Positive control, TRT1 = PC + Rovabio Advance 50 g/ t of feed, NC1 = Negative control 1 (PC - ME), TRT2 = NC1 + Rovabio Advance 50 g/ t of feed, NC2 = Negative control 2 (PC - Amino acids), TRT3 = NC2 + Rovabio Advance 50 g/ t of feed, NC3 = Negative control 3 (Positive control - ME and amino acids), TRT4 = NC3 + Rovabio Advance 50 g/ t of feed

[1] VU/kg = Visco units of xylanase per kg of feed, [2] FTU/kg = Phytase units per kg of feed

4.2 Production parameters

4.2.1 Body weight

The weekly broiler body weights, are shown below in Table 4.4. Chickens were weighed at the start of the trial (day 0), and thereafter weighing occurred on a weekly basis (days 7, 14, 21 and 28) with the last weighing done at the end of the trial (day 35). Chick weight did not differ significantly ($P > 0.05$) between the treatments at the start of the trial (day 0) and at the end of the first week (day 7). The body weight of the broilers were above the Ross 308 breed standards (Aviagen, 2014) for all the treatments throughout the trial.

The body weight of the broilers in TRT1, was significantly higher ($P < 0.05$) than the reduced energy NC1 and TRT2 at day 14, but did not differ significantly ($P > 0.05$) from the PC. At days 21 and 28 the body weight of the broilers in the PC was significantly higher ($P < 0.05$) than the NC1 and TRT2, with the body weight of the broilers in TRT1 being significantly higher ($P < 0.05$) than the NC1 at day 28. The body weight of the broilers in NC1 was significantly lower ($P < 0.05$) than the PC at day 35. At 35 days, the body weight of the broilers in TRT 2 was not significantly different ($P > 0.05$) from the PC, TRT1 or NC1.

The body weight of the broilers in NC2 was significantly lower ($P < 0.05$) than the PC on day 28, with TRT3 being significantly lower ($P < 0.05$) than the PC on days 21 and 28. NC2 and TRT3 broiler body weight were however not significantly different from the PC at day 35 ($P > 0.05$). The body weight of the broilers in the PC and TRT1, were significantly higher ($P < 0.05$) than the body weight for NC3 and TRT4 at days 14 and 28. At day 21, the body weight of the broilers in NC3 and TRT4 were significantly lower ($P < 0.05$) than the PC, with the body weight of the broilers in NC3 also being significantly lower ($P < 0.05$) than TRT1. At day 35, only the body weight of the broilers in NC3 was significantly lower ($P < 0.05$) than the PC diet, with the body weight of the broilers in TRT4 being similar to both the PC and TRT1.

Table 4.4 The average weekly body weight of the broilers (g / bird) for the different treatments from day 0 to 35

Treatments	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
PC	45.8	207.7	552.5^{ab}	1165.8^{a}	1942.2^{a}	2626.4^{a}
TRT1	45.7	209.5	556.8^{a}	1152.6^{ab}	1906.8^{ac}	2600.8^{ab}
NC1	45.8	208.3	539.7^{b}	1135.5^{b}	1862.0^{b}	2555.5^{b}
TRT2	45.8	207.9	541.8^{b}	1130.2^{b}	1885.5^{cb}	2572.9^{ab}

Treatments	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
PC	45.8	207.7	552.5	1165.8^{a}	1942.2^{a}	2626.4
TRT1	45.7	209.5	556.8	1152.6^{ab}	1906.8^{ab}	2600.8
NC2	45.8	212.0	552.3	1167.8^{a}	1885.3^{b}	2574.9
TRT3	45.6	209.0	543.9	1137.6^{b}	1880.8^{b}	2616.3

Treatments	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
PC	45.8	207.7	552.5^{a}	1165.8^{a}	1942.2^{a}	2626.4^{a}
TRT1	45.7	209.5	556.8^{a}	1152.6^{ac}	1906.8^{a}	2600.8^{ab}
NC3	45.8	206.5	539.3^{b}	1124.5^{b}	1852.9^{b}	2544.0^{b}
TRT4	45.8	209.3	539.2^{b}	1131.4^{bc}	1850.7^{b}	2567.3^{ab}
SEM	0.057	2.432	4.690	9.688	13.26	21.40

Enzyme inclusion	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
0	45.8	208.6	546.0	1148.4	1885.6	2575.2
1	45.7	208.9	545.4	1138.0	1881.0	2589.3
SEM	0.028	1.216	2.345	4.844	6.632	10.70

^{abc} Column means with common superscript did not differ significantly for the least square means (P > 0.05)

PC = Positive control

TRT1 = PC + Rovabio Advance 50 g/ t of feed

NC1 = Negative control (Positive control - ME)

TRT2 = NC1 + Rovabio Advance 50 g/ t of feed

NC2 = Negative control (Positive control - Amino acids)

TRT3 = NC2 + Rovabio Advance 50 g/ t of feed

NC3 = Negative control (Positive control - ME and amino acids)

TRT4 = NC3 + Rovabio Advance 50 g/ t of feed

4.2.2 Body weight gain

The weekly and overall body weight gain of the broilers, are shown below in Table 4.5. No significant differences ($P > 0.05$) were observed from day 0 to 7, between any of the treatments.

The body weight gain of the broilers in NC1 was significantly lower ($P < 0.05$) than the PC for week 2 (days 8-14), week 4 (days 22 to 28) as well as the overall period (days 0-35). The body weight gain of the broilers in the PC was also significantly higher ($P < 0.05$) than TRT2 during week 3 (days 15 to 21), but not for the overall period (days 0-35). The body weight gain of the broilers in TRT1 was significantly higher ($P < 0.05$) than NC1 and TRT2 during week 2 (days 8 to 14).

The body weight gain of the broilers in TRT1 was significantly higher ($P < 0.05$) than TRT3 for week 2 (days 8 to 14), while the body weight gain of the broilers in the PC was significantly higher ($P < 0.05$) than the NC2 treatment during week 4 (days 22 to 28). There were no significant differences ($P > 0.05$) between the body weight gain of the broilers in the PC, TRT1, NC2 and TRT3 for the overall period (days 0 to 35). The body weight gain of TRT3 tended ($P < 0.1$) to be higher than the PC, during the final week of the trial.

The body weight gain of the broilers in the PC and TRT1, were significantly higher ($P < 0.05$) than both the NC3 and TRT4 during week 2 (days 8 to 14), while only the body weight gain of the broilers in the PC was significantly higher ($P < 0.05$) than the NC3 and TRT4 during week 3 (days 15 to 21) and week 4 (days 22 to 28). The body weight gain of the broilers in the PC was also significantly higher ($P < 0.05$) than the NC3 treatment for the overall period (days 0 to 35), but did not differ significantly from TRT4.

Enzyme addition resulted in a significant improvement ($P < 0.05$) in body weight gain of the broilers during the final week of the trial period in comparison to the non-supplemented diets.

Table 4.5 The average weekly body weight gain of the broilers (g / bird / week) for the different treatments from day 0 to 35 and total body weight gain

Treatments	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35	Day 0-35
PC	161.9	342.3^{ac}	613.3^{a}	762.5^{a}	668.1	2579.2^{a}
TRT1	163.3	347.3^{a}	593.4^{ab}	744.9^{ab}	686.6	2553.9^{ab}
NC1	162.5	331.4^{b}	595.8^{ab}	712.7^{b}	640.3	2507.8^{b}
TRT2	162.1	333.8^{cb}	588.5^{b}	732.7^{ab}	657.2	2525.5^{ab}

Treatments	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35	Day 0-35
PC	161.9	342.3^{ab}	613.3	762.5^{a}	668.1	2579.2
TRT1	163.3	347.3^{a}	593.4	744.9^{ab}	686.6	2553.9
NC2	166.0	338.6^{ab}	613.3	712.9^{b}	674.0	2527.8
TRT3	162.7	334.9^{b}	593.7	729.6^{ab}	720.4	2569.0

Treatments	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35	Day 0-35
PC	161.9	342.3^{a}	613.3^{a}	762.5^{a}	668.1	2579.2^{a}
TRT1	163.3	347.3^{a}	593.4^{ab}	744.9^{ab}	686.6	2553.9^{ab}
NC3	160.4	331.0^{b}	583.2^{b}	719.0^{b}	668.1	2496.3^{b}
TRT4	163.2	329.0^{b}	592.2^{b}	714.9^{b}	701.7	2520.4^{ab}
SEM	2.437	3.149	7.114	13.05	20.47	21.24

Enzyme inclusion	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35	Day 0-35
0	162.7	335.9	601.4	726.8	662.6^{b}	2527.8
1	162.8	336.3	592.0	730.5	691.5^{a}	2542.2
SEM	1.218	1.575	3.557	6.524	10.23	10.62

^{abc} Column means with common superscript did not differ significantly for the least square means (P > 0.05)

PC = Positive control

TRT1 = PC + Rovabio Advance 50 g/ t of feed

NC1 = Negative control (Positive control - ME)

TRT2 = NC1 + Rovabio Advance 50 g/ t of feed

NC2 = Negative control (Positive control - Amino acids)

TRT3 = NC2 + Rovabio Advance 50 g/ t of feed

NC3 = Negative control (Positive control - ME and amino acids)

TRT4 = NC3 + Rovabio Advance 50 g/ t of feed

4.2.3 Weekly feed intake

The weekly feed intake of the broilers in the different treatments, are shown below in Table 4.6. The weekly feed intake of the broilers was not significantly different ($P > 0.05$) between the PC and the TRT1, NC1, TRT2, NC3 and TRT4 treatments for any of the weeks during the trial period. The feed intake of the broilers in the PC was however significantly higher ($P < 0.05$) than the NC2 and TRT3 treatments during the final week of the trial.

4.2.4 Cumulative feed intake

The cumulative feed intake of the broilers in the different treatments, are shown below in Table 4.7. The cumulative feed intake of the broilers was not significantly different ($P > 0.05$) between the PC and the TRT1, NC1, TRT2, TRT3, NC3 and TRT4 treatments for any of the periods during the trial period. The cumulative feed intake of the broilers in the PC was however significantly higher ($P < 0.05$) than the NC2 treatment for the cumulative period of day 0 to 35.

Table 4.6 The average weekly feed intake of the broilers (g / bird / week) for the different treatments from day 0 to 35

Treatments	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35
PC	172.2	436.2	811.8	838.8	1529.1
TRT1	172.0	434.9	805.7	827.7	1481.4
NC1	170.7	434.7	803.0	830.1	1489.4
TRT2	170.4	437.3	803.9	840.8	1512.2

Treatments	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35
PC	172.2	436.2	811.8	838.8	1529.1[a]
TRT1	172.0	434.9	805.7	827.7	1481.4[ab]
NC2	172.0	435.9	812.4	820.5	1453.3[b]
TRT3	173.3	436.5	803.8	829.2	1472.7[b]

Treatments	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35
PC	172.2	436.2	811.8	838.8	1529.1
TRT1	172.0	434.9	805.7	827.7	1481.4
NC3	172.1	438.6	805.3	839.8	1501.6
TRT4	174.6	442.1	809.3	833.1	1488.2
SEM	2.001	3.637	6.481	6.794	17.36

Enzyme inclusion	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35
0	171.8	436.4	808.1	832.3	1493.4
1	172.6	437.7	805.7	832.7	1488.6
SEM	1.000	1.818	3.240	3.397	8.682

[ab] Column means with common superscript did not differ significantly for the least square means (P > 0.05)

PC = Positive contro

TRT1 = PC + Rovabio Advance 50 g/ t of feed

NC1 = Negative control (Positive control - ME)

TRT2 = NC1 + Rovabio Advance 50 g/ t of feed

NC2 = Negative control (Positive control - Amino acids)

TRT3 = NC2 + Rovabio Advance 50 g/ t of feed

NC3 = Negative control (Positive control - ME and amino acids)

TRT4 = NC3 + Rovabio Advance 50 g/ t of feed

Table 4.7 The average cumulative feed intake of the broilers (g / bird) for the different treatments from day 0 to 35

Treatments	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-35
PC	172.2	610.5	1422.3	2278.0	3826.3
TRT1	172.0	606.8	1415.1	2254.0	3744.0
NC1	170.7	605.4	1408.4	2255.9	3808.6
TRT2	170.4	607.7	1411.6	2280.3	3828.9

Treatments	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-35
PC	172.2	610.5	1422.3	2278.0	3826.3[a]
TRT1	172.0	606.8	1415.1	2254.0	3744.0[ab]
NC2	172.0	609.3	1424.1	2250.2	3722.5[b]
TRT3	173.3	609.8	1413.6	2260.0	3750.8[ab]

Treatments	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-35
PC	172.2	610.5	1422.3	2278.0	3826.3
TRT1	172.0	606.8	1415.1	2254.0	3744.0
NC3	172.1	612.1	1419.8	2271.3	3800.5
TRT4	174.6	617.4	1426.6	2265.2	3771.1
SEM	2.001	4.857	10.24	17.31	32.73

Enzyme inclusion	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-35
0	171.8	609.3	1418.6	2263.8	3789.5
1	172.6	610.4	1416.7	2264.9	3773.7
SEM	1.000	2.429	5.118	8.656	16.37

[ab] Column means with common superscript did not differ significantly for the least square means (P > 0.05)

PC = Positive control

TRT1 = PC + Rovabio Advance 50 g/ t of feed

NC1 = Negative control (Positive control - ME)

TRT2 = NC1 + Rovabio Advance 50 g/ t of feed

NC2 = Negative control (Positive control - Amino acids)

TRT3 = NC2 + Rovabio Advance 50 g/ t of feed

NC3 = Negative control (Positive control - ME and amino acids)

TRT4 = NC3 + Rovabio Advance 50 g/ t of feed

4.2.5 Mortality

The chi-square frequency analysis of the total mortalities of the broilers for the different treatments, are shown below in Table 4.8.The mortality of the broilers were not significantly affected by treatment or addition of the enzyme during the trial.

Table 4.8 Chi-Square frequency analysis of total mortalities of the broilers for the different treatments from day 0 to 35

Treatments	Enzyme inclusion	Frequency	Percentage
PC	0	8	3.0
TRT1	1	7	2.7
NC1	0	10	3.8
TRT2	1	9	3.4
NC2	0	7	2.7
TRT3	1	9	3.4
NC3	0	10	3.8
TRT4	1	6	2.3

PC = Positive control

TRT1 = PC + Rovabio Advance 50 g/ t of feed

NC1 = Negative control (Positive control - ME)

TRT2 = NC1 + Rovabio Advance 50 g/ t of feed

NC2 = Negative control (Positive control - Amino acids)

TRT3 = NC2 + Rovabio Advance 50 g/ t of feed

NC3 = Negative control (Positive control - ME and amino acids)

TRT4 = NC3 + Rovabio Advance 50 g/ t of feed

4.2.6 Weekly feed conversion ratio

The weekly feed conversion ratio (FCR) of the broilers in the different treatments, are shown below in Table 4.9. There were no significant differences ($P > 0.05$) for FCR of the broilers between any of the treatments during the first week of the trial.

The FCR of the broilers in TRT1 did not differ significantly ($P > 0.05$) from the broilers of the PC, for any of the weeks during the trial period. The broilers in NC 1, performed significantly worse ($P < 0.05$) than the broilers of the PC during weeks 2 and 4, and the broilers of TRT1 in week 2. The broilers in TRT2 had a significantly worse ($P < 0.05$) FCR than the broilers of the PC during weeks 2 and 3, and the broilers of TRT1 in week 2.

The broilers in the NC2 treatment, performed significantly worse ($P < 0.05$) in terms of FCR than the broilers of the PC in week 4. The broilers of TRT3 performed significantly worse ($P < 0.05$) in week 2, compared to the broilers of the PC and TRT1. The broilers of TRT3 however performed significantly better ($P < 0.05$) in the last 7 days of the trial compared to the PC treatment.

The broilers in NC3 and TRT4 performed significantly worse ($P < 0.05$) than the broilers of the PC in weeks 2, 3 and 4 and significantly worse ($P < 0.05$) than the broilers of TRT1 in weeks 2 and 4.

Table 4.9 The average weekly feed conversion ratio of the broilers (g feed intake / g body weight gain) for the different treatments from day 0 to 35

Treatments	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35
PC	1.07	1.27[b]	1.32[b]	1.08[b]	2.25
TRT1	1.04	1.25[b]	1.35[ab]	1.10[ab]	2.16
NC1	1.05	1.31[a]	1.35[ab]	1.13[a]	2.17
TRT2	1.05	1.31[a]	1.37[a]	1.13[ab]	2.21

Treatments	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35
PC	1.07	1.27[b]	1.32	1.08[b]	2.25[a]
TRT1	1.04	1.25[b]	1.35	1.10[b]	2.16[ab]
NC2	1.04	1.28[ab]	1.32	1.15[a]	2.13[ab]
TRT3	1.06	1.30[a]	1.36	1.12[ab]	2.03[b]

Treatments	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35
PC	1.07	1.27[a]	1.32[a]	1.08[a]	2.25
TRT1	1.04	1.25[a]	1.35[ab]	1.10[a]	2.16
NC3	1.07	1.32[b]	1.38[b]	1.16[b]	2.20
TRT4	1.06	1.34[b]	1.37[b]	1.16[b]	2.11
SEM	0.012	0.010	0.015	0.015	0.060

Enzyme inclusion	Day 0-7	Day 8-14	Day 15-21	Day 22-28	Day 29-35
0	1.06	1.29	1.34	1.13	2.19
1	1.05	1.30	1.36	1.13	2.13
SEM	0.006	0.005	0.008	0.007	0.030

[ab] Column means with common superscript did not differ significantly for the least square means (P > 0.05)

PC = Positive control

TRT1 = PC + Rovabio Advance 50 g/ t of feed

NC1 = Negative control (Positive control - ME)

TRT2 = NC1 + Rovabio Advance 50 g/ t of feed

NC2 = Negative control (Positive control - Amino acids)

TRT3 = NC2 + Rovabio Advance 50 g/ t of feed

NC3 = Negative control (Positive control - ME and amino acids)

TRT4 = NC3 + Rovabio Advance 50 g/ t of feed

4.2.7 Cumulative feed conversion ratio

The cumulative feed conversion ratio (CFCR) of the broilers for the different treatments, are shown below in Table 4.10. The broilers performed better than Ross 308 breed standards (Aviagen, 2014) in terms of CFCR for all the treatments throughout the trial. The PC fed broilers performed significantly better than the broilers of NC1 in terms of CFCR over 14, 21 and 28 days, but not for the overall period of day 0 to 35. The broilers of the PC also performed significantly better than broilers of TRT2, over 14, 21 and 28 days but not for the overall period of 35 days. The CFCR of the broilers in TRT1 was significantly better than the broilers in NC1 over 14 and 28 days, and CFCR of the broilers in TRT2 over 14, 21 and 28 days, but not for the overall period of 35 days.

The CFCR of the broilers in NC2, did not differ significantly from the CFCR of the broilers in the PC or TRT1 for any of the cumulative periods. The CFCR of the broilers in TRT3 was significantly worse than the broilers in the PC over 14, 21 and 28 days, but not the overall period. The CFCR of the broilers in TRT3 was also worse than the broilers in TRT1 over 14 days, and broilers in NC2 over 21 days, but not the overall period.

The CFCR of the broilers in NC3, was significantly worse than the broilers of the PC over 14, 21, 28 and 35 days. The CFCR of the broilers in NC3 treatment was also significantly worse than the broilers of TRT1 for all the cumulative periods. The CFCR of the broilers in TRT4 was significantly worse than the broilers in the PC and TRT1 over 14, 21 and 28 days, but not for the overall cumulative period of 35 days.

Table 4.10 The average cumulative feed conversion ratio of the broilers (g feed intake / g body weight gain) for the different treatments from day 0 to 35

Treatments	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-35
PC	1.07	1.20^{b}	1.27^{c}	1.19^{b}	1.47
TRT1	1.04	1.18^{b}	1.27^{bc}	1.20^{b}	1.46
NC1	1.05	1.23^{a}	1.29^{ab}	1.23^{a}	1.48
TRT2	1.05	1.23^{a}	1.30^{a}	1.23^{a}	1.49

Treatments	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-35
PC	1.07	1.20^{b}	1.27^{b}	1.19^{b}	1.47
TRT1	1.04	1.18^{b}	1.27^{ab}	1.20^{ab}	1.46
NC2	1.04	1.20^{ab}	1.26^{b}	1.22^{ab}	1.46
TRT3	1.06	1.22^{a}	1.29^{a}	1.22^{a}	1.45

Treatments	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-35
PC	1.07	1.20^{b}	1.27^{b}	1.19^{b}	1.47^{b}
TRT1	1.04	1.18^{b}	1.27^{b}	1.20^{b}	1.46^{b}
NC3	1.07	1.23^{a}	1.31^{a}	1.25^{a}	1.51^{a}
TRT4	1.06	1.25^{a}	1.31^{a}	1.25^{a}	1.49^{ab}
SEM	0.012	0.007	0.008	0.007	0.014

Enzyme inclusion	Day 0-7	Day 0-14	Day 0-21	Day 0-28	Day 0-35
0	1.06	1.22	1.28	1.22	1.48
1	1.05	1.22	1.30	1.23	1.47
SEM	0.006	0.003	0.004	0.004	0.007

^{abc} Column means with common superscript did not differ significantly for the least square means (P > 0.05)

PC = Positive control

TRT1 = PC + Rovabio Advance 50 g/ t of feed

NC1 = Negative control (Positive control - ME)

TRT2 = NC1 + Rovabio Advance 50 g/ t of feed

NC2 = Negative control (Positive control - Amino acids)

TRT3 = NC2 + Rovabio Advance 50 g/ t of feed

NC3 = Negative control (Positive control - ME and amino acids)

TRT4 = NC3 + Rovabio Advance 50 g/ t of feed

4.3 Carcass Parameters

No significant differences ($P > 0.05$) were observed for any of the carcass parameters when comparing the broilers of the PC to the broilers of TRT1. The eviscerated carcass yield of the broilers in TRT1 was significantly better ($P < 0.05$) than the broilers of NC3, and the broilers of TRT4. The broilers in the PC and TRT1 showed significantly better ($P < 0.05$) drumstick yield, than broilers of NC3 and TRT4. The broilers of NC1, and TRT2, had significantly better ($P < 0.05$) breast yield than all the other treatments. There were no significant differences ($P > 0.05$) observed in the yield of the wings and thighs among the broilers in any of the treatment groups.

Table 4.11 Carcass parameters as a percentage of live weight of the broilers for the different treatments

Treatments	Eviserated	Wings	Thighs	Drumsticks	Breasts
PC	74.0	7.4	21.6	9.5	18.4[b]
TRT1	74.5	7.7	21.7	9.5	18.4[b]
NC1	74.2	7.4	21.2	9.3	19.5[a]
TRT2	74.4	7.6	21.4	9.3	19.5[a]

Treatments	Eviserated	Wings	Thighs	Drumstick	Breast
PC	74.0	7.4	21.6	9.5	18.4
TRT1	74.5	7.7	21.7	9.5	18.4
NC2	74.3	7.7	21.6	9.4	19.0
TRT3	74.2	7.5	22.0	9.3	18.8

Treatments	Eviserated	Wings	Thighs	Drumstick	Breast
PC	74.0[ab]	7.4	21.6	9.5[a]	18.4
TRT1	74.5[a]	7.7	21.7	9.5[a]	18.4
NC3	73.7[b]	7.7	21.5	9.1[b]	18.5
TRT4	73.5[b]	7.7	21.3	9.1[b]	18.3
SEM	0.002	0.001	0.002	0.001	0.002

Enzyme inclusion	Eviserated	Wings	Thighs	Drumstick	Breast
0	74.1	7.6	21.5	9.3	18.8
1	74.2	7.6	21.6	9.3	18.7
Standard error of means	0.001	0.001	0.001	0.001	0.001

[ab] Column means with common superscript did not differ significantly for the least square means (P > 0.05)

PC = Positive control

TRT1 = PC + Rovabio Advance 50 g/ t of feed

NC1 = Negative control (Positive control - ME)

TRT2 = NC1 + Rovabio Advance 50 g/ t of feed

NC2 = Negative control (Positive control - amino acids)

TRT3 = NC2 + Rovabio Advance 50 g/ t of feed

NC3 = Negative control (Positive control - ME and amino acids)

TRT4 = NC3 + Rovabio Advance 50 g/ t of feed

Chapter 5

Discussion

In the poultry industry profitability is mainly dependent on the cost and nutritive value of the feed (Tahir *et al.*, 2008; Bedford and Partridge, 2010). One of the main anti-nutrients that may limit the nutritive value of the feed are the non-starch polysaccharides (NSP) (Tahir *et al.*, 2008). Monogastric animals lack the digestive capacity to digest NSP (Meng *et al.*, 2005). Slominski (2011) stated that the constituent NSP in maize and soybean meal requires a broad range of carbohydrases, if any beneficial response is to be achieved.

Enzyme complexes that include debranching enzymes, such as arabinofuranosidases, can increase the overall enzyme effect (Cozannet *et al.*, 2017). Arabinofuranosidases can cleave arabinose from the xylose backbone and offer access to endo-xylanase activity (De La Mare *et al.*, 2013; Cozannet *et al.*, 2017). The filamentous fungus *Penicillium funiculosum,* recently renamed *Talaromyces versatilis* (Samson *et al.*, 2011), produces a wide range of cellulotic and hemicellulotic enzymes, including pectinases, cellulases, proteases and arabinofuranosidases (Lafond *et al.*, 2014; Rios *et al.*, 2017). Enzymes that are capable of degrading complex arabinoxylan chains more efficiently, can challenge current feed formulation to consider all potential benefits and digestibility of nutrients (Cozannet *et al.*, 2017).

The objective of the present study was to determine the efficacy of enzymes produced by *Talaromyces versatilis* in releasing energy and amino acids in broiler feeds, and how that would affect broiler production and slaughter parameters during a 35 day broiler production cycle.

5.1 Ration evaluation

The analysed nutrient levels were close to the calculated values (see Tables 4.1, 4.2 and 4.3). There were limitations with regards to the feed analysis as dietary energy was analysed by means of measuring gross energy, while metabolisable energy was used in the formulations. The analysed gross energy of all the reduced energy negative control diets (NC1, NC3) and experimental diets (TRT2 and TRT4), analysed lower than the positive control diets, as well as the treatments where only amino acids levels were reduced (NC2 and TRT3).

Even though all the diets were formulated based on digestible amino acids, the analysed total amino acids of all the final feeds, were similar to the calculated total amino acid levels of all the different formulated feeds. Variation in results of the total amino could be ascribed to practical constraints during the process of mixing smaller amounts of trial feed whilst adding small amounts of synthetic amino acids. The analysed results could have possibly been improved by increasing the number of samples analysed per feed, and thereby increasing the repeatability of the analysis. The recovery of enzyme activity for both enzymes were above the guaranteed minimums, as specified by the respective suppliers.

5.2 Production parameters

In the present study, enzyme addition to the positive control diets did not improve any of the production parameters (see Tables 4.4 to 4.10). These findings are in agreement with the results of similar studies (Kocher *et al.*, 2002; Vieira *et al.*, 2006; Kaczmarek *et al.*, 2009; Kaczmarek *et al.*, 2014), where no improvements in production parameters were observed when enzyme combinations were added on top of the positive control treatments. In an experiment by Kocher *et al.* (2003) where the effects of different enzyme combinations on apparent metabolisable were evaluated, it was found that none of the combinations successfully improved the performance of the standard diet. When the enzymes were included in a lower energy diet in the same study, the authors observed that the combination of pectinase, protease, and amylase significantly improved the apparent metabolisable energy in comparison to the un-supplemented diet.

When improved nutrient utilisation due to enzyme addition is not accompanied by increased growth performance, it is possible that the control diets were not sufficiently limiting in nutrients to reduce growth (Farhangi and Carter, 2007). Enzyme effects on performance parameters are not usually observed when standard diets based on balanced and high digestible nutrients are fed (Moraes *et al.*, 2015). When feeding a theoretically perfect diet to broilers, it is unlikely that any improvement will be observed by adding an enzyme on top of the diets (Sorbara *et al.*, 2009), as the diet already allows the bird to perform close to its genetic potential.

In the present study, the body weight and body weight gain of the reduced energy negative control treatment was significantly lower than the positive control ($P < 0.05$), but did not differ significantly ($P > 0.05$) in terms of feed intake or feed conversion ratio (see Tables 4.4, 4.5, 4.9 and 4.10). Therefore it is possible that the reduction in metabolisable energy was not sufficiently limiting to reduce growth performance sufficiently. According to Leeson *et al.* (1996), broilers fed marginal nutrient reduced diets tend to increase their feed intake as dietary energy is reduced, which was not observed in the present study (see Tables 4.6 and 4.7). In a recent study by Plumstead *et al.* (2007), the authors similarly reported that the feed intake of broilers was not affected by the dietary metabolisable energy density. When the Rovabio Advance enzyme was added to the reduced energy negative control diets, the feed intake, body weights at 35 days as well as the body weight gain and feed conversion ratio over 35 days were not significantly different ($P > 0.05$) from the positive control (see Tables 4.4 to 4.7, 4.9 and 4.10). There were however small, but non-significant improvements in 35 day body weight and body weight gain over 35 days, when the enzyme were added to the reduced energy negative control treatment (see Tables 4.4 and 4.5). These observations are similar to the results of a study by Vieira *et al.* (2015) , where the addition of an enzyme complex to reduced energy diets led to partial improvements in body weight gain and feed conversion ratio, compared to the positive control diets. Such improvements might still be of economic importance in a commercial broiler operation, even though these effects are small and difficult to detect in a small-scale experiment (Meng *et al.*, 2005).

Results of similar studies concerning multiple enzyme combinations to reduced energy maize and soybean meal based diets are conflicting, with some studies observing improvements to similar levels as the positive control (Du Plessis and Jansen van Rensburg, 2014; Klein *et al.*, 2015; Govil *et al.*, 2017), some showing partial improvements compared to the negative control (Zakaria *et al.*, 2010; Vieira *et al.*, 2015), and others finding no significant differences compared to the negative control (Yu *et al.*, 2007; Cowieson and Ravindran, 2008a; Cowieson *et al.*, 2010). In a study evaluating the effects of three different enzyme combinations, Yegani and Korver (2013) found no improvement and in some instances negative effects were observed on performance variables due to enzyme supplementation.

An important factor to consider in the present study is that the reduction of energy in the reduced energy treatments, were obtained by removing the soybean oil and lowering the full fat soybean meal levels in the formulations. This also led to a lower level of crude fat in the final diets. According to Bao *et al.* (2013), reduced dietary fat concentration, can depress the performance of broilers and Cowieson *et al.* (2010) suggested that minimum fat concentrations could maximise bio efficacy of NSP enzymes in maize and soybean meal based diets.

Enzyme addition to the reduced amino acid negative control, showed a significant improvement in feed conversion ratio ($P < 0.05$) and a tendency to improve body weight gain ($P < 0.1$) compared to the positive control, during the final week of the present trial (see Tables 4.5 and 4.9). Rios *et al.* (2017) observed similar improvements in feed conversion ratio when broilers were fed maize and soybean meal diets supplemented with the same enzyme complex from *Talaromyces versatilis* as was used in the present study. This also coincides with a study by Tahir *et al.* (2008), where a combination of pectinase, cellulase and hemicellulase improved body weight gain in crude protein reduced diets from day 15 to 27.

Enhanced amino acid utilisation with enzyme supplementation is likely due to an improvement in the digestibility (Zanella *et al.*, 1999; Rutherfurd *et al.*, 2007; Cowieson and Ravindran, 2008a), as well as a reduction in endogenous losses (Cowieson and Ravindran, 2008b). Alterations in the secretions of endogenous enzymes and the microbial populations in the intestinal environment of the broiler chicken, can also contribute to the observed improvement in amino acid digestibility (Choct, 1997; Cowieson and Ravindran, 2008a; Cowieson, 2010). The effects of added enzymes may change with broiler age (Bedford and Cowieson, 2012) as caecal populations increase in size and variability, and as a consequence fermentation responses to cell wall fragments may be more pronounced in older birds (Wang *et al.*, 2005; Parker *et al.*, 2007).

In this study significantly lower feed intake ($P < 0.05$) was observed for broilers that received the reduced amino acids for the cumulative period of 35 days (see Tables 4.6 and 4.7) and the reduced amino acid plus enzyme treatment during the final week of the trial (see Tables 4.6), compared to the positive control. This observation is contradictory to the results of the studies

of De Keyser *et al.* (2016) and Tahir *et al.* (2008), where a dietary reduction in crude protein and amino acids did not affect feed intake. The body weight at day 35 and body weight gain from day 0 to 35 of the broilers fed the amino acid reduced treatments in the present study, did not differ significantly from the positive control treatment ($P > 0.05$), which confirms that the growth was not impacted by the reduction in amino acids or feed intake (see Tables 4.4 and 4.5).

Decreasing the crude protein to metabolisable energy ratio in diets that contain adequate amounts of crude protein and amino acids, have been shown to improve feed conversion ratio and growth rate in broilers (Hidalgo *et al.*, 2004; Saleh *et al.*, 2004; Dozier *et al.*, 2006; Dozier *et al.*, 2007). When compared to body weight gain, protein gain decreases with increase in body weight, and therefore amino acid requirements decrease with age and body weight (Baker, 2009). Genetic differences also needs to be considered as the Ross 308 strain has been shown to react differently to Cobb 500 when dietary protein was reduced, by lowering feed intake after 21 days of age (Kemp *et al.*, 2005; Berhe and Gous, 2008).

In the present study, the body weight, body weight gain and feed conversion ratio over the 35-day period of broilers that received the reduced energy and amino acids negative control diets were significantly worse ($P < 0.05$) compared to the positive control (see Tables 4.4, 4.5 and 4.10). However, feed intake did not differ significantly ($P > 0.05$) between these groups of birds (see Tables 4.6 and 4.7). According to Leeson and Summers (1997), broiler chickens can compensate for lower nutrient concentrations in the diet by increasing their feed intake. The results in the present study regarding feed intake is contradictory to the findings of Leeson *et al.* (1996), where broilers fed diets with marginally reduced nutrient densities, tended to increase their feed intake. It is possible that the diets in the current study were not sufficiently limiting in nutrients to stimulate an increase in feed intake. The average feed intakes of the broilers in the present study were also above the breed standards for the Ross 308 strain (Aviagen, 2014). The theoretical maximum of feed intake is determined by the capacity of the digestive system (Tallentire *et al.*, 2018), and the broiler chickens might experience a physical limitation when attempting to consume more of a low density diet (Kamran *et al.*, 2008).

When the Rovabio Advance enzyme was added to the reduced energy and amino acid diet in the present study, the body weight at 35 days and body weight gain and feed conversion over the 35-day period, were not significantly different ($P > 0.05$) from the positive control (see Tables 4.4, 4.5 and 4.10). When maize and soybean meal based diets containing reduced levels of energy and amino acids were supplemented with an enzyme complex from *Talaromyces versatilis*, Rios *et al.* (2017) noted that the feed conversion ratio of the broilers improved to similar levels than the positive control. Contradictory to the results in the present study, Meng and Slominski (2005) observed no significant differences in production parameters when a multicarbohydrase cocktail consisting of xylanase, glucanase, pectinase, cellulase, mannanase and galactanase, was added to an energy and amino acid reduced maize soybean meal diet. In a study by Cowieson and Ravindran (2008b), using an enzyme cocktail consisting of xylanase, α-amylase and protease in a dose dependent manner, significant differences were observed at double the recommended dose, but not at the single dose.

Enzyme addition resulted in a significant improvement ($P < 0.05$) in body weight gain during the final week of the trial period in comparison to non-supplemented diets (see Table 4.5). However, no significant differences for body weight gain was observed during any of the other periods, indicating a possible age effect ($P > 0.05$). Similar results have been observed in other studies (Alam *et al.*, 2003; Gracia *et al.*, 2003; Józefiak *et al.*, 2004a; Figueiredo *et al.*, 2012; Yegani and Korver, 2013). It is likely that feed enzymes influence broiler performance through an interaction with microbial populations, which becomes more prolific as the bird ages (Figueiredo *et al.*, 2012). Ravn *et al.* (2018) also recently showed that the supplementation of a maize and soybean meal broiler diets with a combination of xylanase and arabinofuranosidase enzymes resulted in significant improvements in growth performance and caecal butyrate production, with the effects being more pronounced at days 21 and 29.

5.3 Carcass parameters

In the present study, no significant improvements ($P > 0.05$) in any of the carcass parameters were observed with enzyme addition to the positive control diet (see Table 4.11). These results are in agreement with the results from similar studies (Zakaria *et al.*, 2010; Azarfar, 2013; Muller Fernandes *et al.*, 2015). Vieira *et al.* (2006) reported similar results for the yields of the commercial cuts, but observed contradictory results for carcass yield with a decrease when enzymes were added to the positive control.

Broilers from the reduced energy negative control group, and the reduced energy plus Rovabio Advance treatment, had significantly better breast yield than those from any of the other treatments ($P < 0.05$). Klein *et al.* (2015) and Coppedge *et al.* (2012) observed no significant difference for breast yield when comparing energy reduced negative control diets, with or without enzyme supplementation, to the positive control diets. The results from the present study is also contradictory to the results from Williams *et al.* (2014), where the reduction in energy decreased all processing parameters evaluated. Govil *et al.* (2017) observed no significant differences in carcass yield when comparing an energy reduced negative control to the positive control, but yields were significantly improved by the addition of a multi-enzyme product. The increased breast yield observed in the present study by reducing the energy levels, can be explained by an increased crude protein and amino acids to energy ratio, which has also been observed in other studies (Corzo *et al.*, 2005; Kidd *et al.*, 2005; Dozier *et al.*, 2006; Dozier *et al.*, 2007; Widyaratne and Drew, 2011).

No significant differences were observed for any of the carcass parameters when comparing the reduced amino acid negative control and Rovabio Advance reduced amino acid treatment, to the positive control ($P > 0.05$). These observations are contradictory to the results from a study by Tahir *et al.* (2008), where a reduction in crude protein levels decreased carcass and breast yield, and enzyme addition restored the yield to similar levels than the positive control.

Enzyme addition to the positive control diets resulted in significantly improved eviscerated carcass yield compared to the reduced energy and amino acid negative control, and the reduced energy and amino acid plus Rovabio Advance treatment ($P < 0.05$). The positive control with and without enzyme, also showed significantly better drumstick yield, than the reduced energy and amino acid negative control, and the reduced energy and amino acid plus Rovabio Advance treatment ($P < 0.05$). Coppedge *et al.* (2012) observed no significant difference for carcass yield when comparing energy and amino acids reduced negative control diets, with or without enzyme supplementation, to the positive control diets. There were no significant differences ($P > 0.05$) in the yield of the wings and thighs among the treatment groups, observed in the present study.

Chapter 6

Conclusion

Exogenous feed enzymes are important tools in monogastric animal nutrition as they can limit the effect of anti-nutrients present in the raw materials, and thereby increase the digestibility and nutritive value of the feed. One of the main anti-nutrients in maize and soybean meal based broiler diets are the non-starch polysaccharides, which requires a broad range of carbohydrase enzymes if any beneficial response is to be achieved.

The addition of enzymes produced by *Talaromyces versatilis* to broiler feed, resulted in significant improvements in body weight gain during the final week of a broiler growth performance trial. The addition of the enzyme complex to diets with reduced amino acid levels, also resulted in a significant improvement in feed conversion ratio and a tendency to improve body weight gain compared to the positive control, during the final week of the trial. Enzyme addition to the reduced energy and reduced energy and amino acid negative control diets resulted in slight but non-significant improvements in final body weight and body weight gain. Supplementation of the enzymes also slightly improved broiler feed conversion ratio over the 35-day period for birds that received the reduced energy and amino acid diets. No improvements in any of the production parameters were observed, with enzyme addition to the positive control diets.

Enzyme addition to the positive control diet, significantly improved eviscerated carcass yield compared to the reduced energy and amino acid diets. No other significant improvements were observed in any of the carcass parameters evaluated, due to enzyme addition. Therefore this study did not deliver significant evidence that enzyme supplementation can improve carcass parameters of broilers. It can, however, be concluded from the present study that enzymes produced by *Talaromyces versatilis* may improve production parameters of broilers when added to maize and soybean meal based diets with reduced energy and amino acid levels.

Chapter 7

Critical review and recommendations

Further studies on the efficacy of enzymes produced by *Talaromyces versatilis* in broilers fed maize and soybean meal diets are recommended, taking into account the following factors:

1. Commercial broiler diets are normally formulated to contain metabolisable energy, crude protein and amino acid levels that are above the requirements of the broiler chickens to ensure optimal performance. Careful consideration should be taken to ensure that the diet specification selected for trial purposes, are limiting enough to allow significantly lower performance of negative control diets which will facilitate the accurate evaluation of the efficacy of supplemented enzymes. The above is especially relevant when using highly digestible raw materials such as maize and soybean meal. Broilers that are fed marginal nutrient reduced diets tend to increase their feed intake, and this effect was not observed in the current study.

2. Further to that, it is recommended that the full matrix reduction in metabolisable energy and crude protein and amino acids is applied, as specified by the supplier. Separate reductions in only metabolisable energy or crude protein and amino acids did not lead to the expected reduction in all production parameters. Reductions in either metabolisable energy or crude protein and amino acids, can lead to a sum-optimal ratio in these nutrients, as the enzyme complex is expected to affect the digestibility of all nutrients to some extent.

3. Raw material inclusion levels should also be taken into consideration during formulation, and it is recommended that between treatment differences in oil and crude fat levels should be limited as much as possible to prevent extra-caloric effects. The use of inert diluents should also be considered to decrease the between treatment variation in raw materials and nutrients.

4. The analysed results of the feed samples could be improved by increasing the number of samples analysed per feed, and thereby increasing the repeatability of the analysis.

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