The energy sparing effect of guanidinoacetic acid alone or in conjunction with exogenous enzymes in broiler diets

By

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Declaration

I, Julia Zanele Tlou, declare that this dissertation, 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 myself or another individual for a degree at this or any other institution

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ADP	Adenosine diphosphate
AGPs	Antibiotic growth promoters
AME	Apparent metabolisable energy
Arg	Arginine
ATP	Adenosine triphosphate
BW	Body weight
BWG	Body weight gain
CK	Creatine kinase
CP	Crude protein
Cr	Creatine
DM	Dry matter
EFSA	European Food Safety Authority
FCR	Feed conversion ratio
FI	Feed intake
GAA	Guanidinoacetic acid
GAMT	Guanidinoacetate methyltransferase
GIT	Gastro intestinal tract
L–AGAT	L-arginine: glycine amidinotransferase
ME	Metabolisable energy
NSPs	Nonstarch polysaccharides
PCr	Phosphocreatine
SAH	s-adenosyl homocysteine
SAM	s-adenosyl methionine
SAPA	South African Poultry Association
VFA	Volatile fatty acids

Abstract

Feed is the most expensive input in poultry production systems accounting for approximately 70% of the total production costs, with maize and soybean meals contributing the bulk of raw material ingredients used and influence the costs of broiler feed. The aim of this study was to evaluate whether guanidinoacetic acid (GAA) would provide a metabolic compensation to reduced dietary apparent metabolisable energy (AME) by acting as a backup to adenosine triphosphate (ATP) shortage and if such compensation will have a synergistic effect in the presence of nonstarch polysaccharide degrading enzymes (NSPases). Another aim was to evaluate whether NSPases would improve growth of broilers receiving reduced energy in the diet through their effect on feed digestibility. A growth performance trial was conducted using 1920 broiler chickens placed in a 96-pen environmentally controlled broiler facility. Guanidinoacetic acid was included in the feed at 600 g/ton as the commercial product CreAMINO® (Alzchem, Germany) which contains at least 96% GAA, whereas the commercial product Rovabio Advance® (Adisseo, France) was included at a level of 50 g/ton as a source of NSP degrading enzymes. The study consisted of six maize-soybean dietary treatments with 16 replications each. The Positive Control diet was formulated as a standard commercial diet with 2900, 3000 and 3050 kcal/kg (11.70, 12.02 and 11.97 MJ/kg) AME in the starter, grower and finisher phase diets, respectively) without any of the test additives. A Negative Control (NC1) diet contained 65 kcal/kg (or 2%) AME less than the Positive Control. The NC1 diet was then supplemented with either NSP degrading enzymes (Rovabio Advance®) or GAA (CreAMINO®). A second Negative Control (NC2) diet contained 130 kcal/kg (or 4%) AME less than the Positive Control. The last of the treatment diets was similar to the NC2 diet but supplemented with both the test feed additives simultaneously. The test additives were supplemented during the starter, grower and the finisher phases of growth. There was a drop in production performance for the broilers that received NC1 and NC2, evident by significant reductions in body weights and increased feed conversion ratios. Supplementation of NC1 with CreAMINO® significantly improved the body weights of broilers. . No benefit, however, was observed for the NSPase that was included in the diets. It is suggested that CreAMINO® has the potential to contribute at least 65 kcal/g (0.272 MJ/kg) AME in the diet of broilers.

Chapter 1

General introduction

The poultry industry remains the single largest contributor to the agricultural sector in South Africa. Financially, the industry contributed approximately 20.9% of the total agricultural gross production value and 43% animal production value (SAPA, 2018). Globally, poultry feed accounts for the largest share in the overall feed consumption by animals with maize and soybean meal being the main ingredients used (Davids & Meyer, 2017). In South Africa, approximately 39.1% and 60.9% of white and yellow maize respectively, are used as ingredients in the poultry industry (SAPA, 2018). Therefore, any increase in raw material prices places pressure on feed manufacturing companies to increase the nutrient utilisation efficiency of their feed (Botha, 2011).

Energy, expressed as metabolisable energy (ME), accounts for approximately 60-70% of dietary costs in broiler production systems (Kleyn, 2013) and the supply of this energy to muscle tissue plays an important role in determining the performance of broilers (Tossenbeger *et al.,* 2016). The energy concentration of feed ingredients and complete diets is an important consideration, which is one of the main factors affecting broiler production and the economic costs thereof. Diets with higher energy concentrations may allow for more rapid gains or greater quantities of meat to be produced by animals (Abudabos *et al*., 2014).

The need for improved performance has led the poultry industry towards the use of feed additives that improve the digestibility and nutrient availability of feed. Feed additives may be described as chemical and biological supplements, which livestock and poultry producers use to manipulate growth, improve feed efficiency, and reduce mortality through their various mechanisms which include inhibiting bacterial growth and infection (Mann, 1973). Guanidinoacetic acid (GAA), available commercially as the product CreAMINO® (Alzchem, Germany), contains at least 96% GAA (Metwally *et al*., 2015). Guanidinoacetic acid is a natural precursor of creatine, which in its phosphorylated form (phosphocreatine), plays an important role in the high energy metabolism of muscle cells (Heger *et al*., 2014). The creatine/phosphocreatine system functions as a secondary energy reservoir to the ATP/ADP system in order to store and mobilise high energy phosphate groups when required by fast growing tissue, especially muscle tissue (Lemme *et al*., 2007). In general, 66-75% of the daily creatine requirement is synthesised *de novo* in the kidney and liver of vertebrates (Ringel *et al*., 2007) via the methylation of GAA by the enzyme S-adenosylmethionine (Dilger *et al*., 2013). However, this *de novo* synthesis of creatine may be a limiting factor in fast growing or high producing animals fed all-vegetable diets. The main reason is because approximately 1.5-2.0% of this creatine is irreversibly lost and excreted through urine due to its non-enzymatic conversion to creatinine (Michiels *et al*., 2012; Carpena *et al*., 2015; Sharideh *et al*., 2015), thus

creatine stores need to constantly be refilled. Another reason for the insufficient levels of creatine is because, in contrast to animal sources, plant feedstuffs lack either creatine or its immediate precursor, GAA, thus supplementation of these metabolites has proved to be beneficial (Lemme *et al*., 2007; Mousavi *et al*., 2013; Heger *et al*., 2014). The creatine requirement of animals may be age dependent with a higher amount needed by growing animals compared to adults due to the need to supply creatine to growing tissue (Mousavi *et al*., 2013). Furthermore, the regeneration of ATP from the creatine/phosphocreatine system is of utmost importance in maintenance of cardiac muscle energy homeostasis in fast growing animals (Michiels *et al*., 2012; Abudabos *et al*., 2014). Although creatine can be supplemented directly to improve broiler performance and carcass characteristics, it has been shown that GAA inclusion levels of about 0.06-0.12% in the diet is more thermally stable and less expensive compared to creatine itself (Dilger *et al*., 2013; De Groot, 2014; Heger *et al*., 2014). Previous research with GAA as a feed additive in diets of varying energy concentration has consistently improved the feed conversion ratio of broilers, thus indicating better energy utilisation (Tossenberger *et al*., 2016). Carpena *et al*. (2015) showed that supplementation of 0.08% CreAMINO® improved fertility and hatchability in broiler breeders. Yazdi *et al*. (2017) suggested that the increase in the relative weight of breast muscle observed in previous studies in broilers was due to an increase in water uptake and increase in muscle cell volume, influenced by the inclusion of GAA in the diet.

The presence of anti-nutritional factors such as non-starch polysaccharides (NSPs), which form the major carbohydrate structure of plants, represents yet another problem in the broiler industry. These NSPs reduce feed digestibility and subsequent nutrient absorption by increasing the viscosity of digesta and increased feed conversion ratio (FCR) (Nadeem *et al*., 2005; Lee *et al*., 2013; Govil *et al*., 2017). With the presence of NSPs, approximately 400-450 kcal (1.67-1.88 MJ) of energy per kg of feed passes through the digestive system undigested in broilers fed commercial maize-soybean diets (Govil *et al*., 2017). Lee *et al*. (2013) stated that exogenous carbohydrase enzymes (NSPases) counteract NSPs by breaking down the fibre chains in these cell wall structures into smaller fragments and thus lower intestinal viscosity of digesta, improve gut health and improve digestibility of feed and performance of broilers (Nadeem *et al*., 2005; Lee *et al*., 2013). Because many grains used in poultry diets contain a variety of NSPs, the use of products consisting of a mixture of enzymes with varying specificity may be more effective in degradation of NSPs (Lee *et al*., 2013). Simon (1998) stated that in most cases the beneficial effects of exogenous NSP degrading enzymes are greater than expected when used in combination and attributed this to the increased nutrient digestibility or ME content of the diet.

To achieve a profitable balance between feed costs, broiler performance, and quality of product, certain feed additives are available in the market for use in broiler feed rations (Pervez & Sajid, 2011). Nonstarch

polysaccharide degrading enzymes and GAA provide nutritionists with the ability to lower dietary costs by using cheaper raw materials while maintaining growth performance of broilers. To date there is no literature investigating the impact of combining these two feed additives. There is limited research on the energy sparing effect of GAA and needs to be investigated as previous research mostly focused on its ability to spare arginine. It has also been proven that GAA is a suitable substitute for animal based protein sources in broiler diets at standard energy levels, hence the focus of this study was not to evaluate this benefit. The aim of this research project was to test whether GAA and exogenous NSPases can be used to compensate for reduced metabolisable energy when supplemented individually or in combination to broiler diets. To achieve this, the objectives of this trial were to supplement these feed additives at various energy levels and broiler performance was evaluated by comparing body weight gain, voluntary feed intake, feed conversion ratio, and tibia breaking strength after a 35-day rearing period in response to supplementation.

Hypothesis of the study

The first null hypothesis (H_0) of this study was that guanidinoacetic acid does not have the ability to compensate for reduced AME in feed and therefore does not have an energy sparing effect.

The first alternative hypothesis (H_A) was that guanidinoacetic acid can compensate for reduced AME in feed.

The second null hypothesis (H_0) was that exogenous non-starch polysaccharide degrading enzymes do not have energy sparing effects when used in maize-soybean diets.

The second alternative hypothesis (H_A) was that exogenous non-starch polysaccharide degrading enzymes can extract energy from maize-soybean diets and make up for reduced AME.

The third null hypothesis (H_0) was to evaluate whether guanidinoacetic acid in combination with exogenous non-starch polysaccharide degrading enzymes would be able to compensate for reduced metabolisable energy when supplemented together.

The third alternative hypothesis (H_A) was that the combined effect of guanidinoacetic acid and exogenous non-starch polysaccharide degrading enzymes will not compensate for reduced energy in the diet and there will not be an interaction between the two feed additives.

Chapter 2

Literature review

2.1 Introduction

The poultry industry is currently the biggest agricultural sector in South Africa and broiler production has been growing ever since intensive broiler production started in South Africa providing a valuable and affordable source of meat. The most influential factor determining the price of broiler feed is the cost of the raw material ingredients, with maize and soybean meal being the two major components used in the production of broiler feed (Botha, 2011). Feed costs account for approximately 60-75% of the total feed costs in broiler production systems (Proskina & Cerina, 2015). Therefore, nutritionists are tasked to continually produce better and more economical feed (Kabir, 2009). Several additives available in the market could be included in the feed to reduce costs, maintain broiler performance and product quality. The focus of this literature review is to discuss broiler production, feed utilisation and subsequent costs and the importance of feed additives in poultry systems, with emphasis on guanidinoacetic acid and nonstarch polysaccharide degrading enzymes.

2.2 Broiler production

Financially, the poultry industry contributes approximately 20.9% of the total agricultural gross production value (of which 17% is attributed to broiler production) and 43% animal volume production (SAPA, 2018). Globally, poultry feed accounts for the largest share in the overall feed consumption by animals driven by a rapid and constant rise in demand of principal poultry products (DAFF, 2017). According to the Bureau for Food and Agricultural Policy (BFAP, 2019), poultry is the cheapest and most consumed source of animal protein in South Africa, of which about 65% is locally produced. The relatively low price of poultry is a reflection of the high efficiency of production, although rising feed and other input costs force consumer prices to increase (DAFF, 2017).

2.3 Broiler feed utilisation and feed additives

Approximately 76% of birds in the poultry industry in South Africa are broilers and the remaining 24% are used in the table egg industry (SAPA, 2018). Feed is the most expensive input in poultry production systems accounting for approximately 70% of the total production costs. Maize and soybean meal contribute the bulk of raw materials used and influence the costs of broiler feed (Davids & Meyer, 2017). Maize is

produced throughout the world, however, there is stiff competition for it among humans, livestock and the poultry industry because maize is high in energy compared to other cereals (Noboa, 2017). Broiler production accounts for approximately 44% of the feed used in South Africa (BFAP, 2019). According to SAPA (2017), the chicken to maize ratio is an important indicator of profitability in the broiler industry as it measures the efficiency with which poultry utilise feed per kilogram weight gain. The increase in the cost of raw material ingredients over the years has put pressure on feed manufacturers to increase the efficiency of nutrient utilisation of the feed (Botha, 2011). Figure 2.1 shows the changes in which broiler feed price in South Africa over the years. Davids and Meyer (2017) stated that feed conversion ratio (FCR), mortality rates and the production efficiency factor (PEF) are universal measures of technical efficiency in production systems.

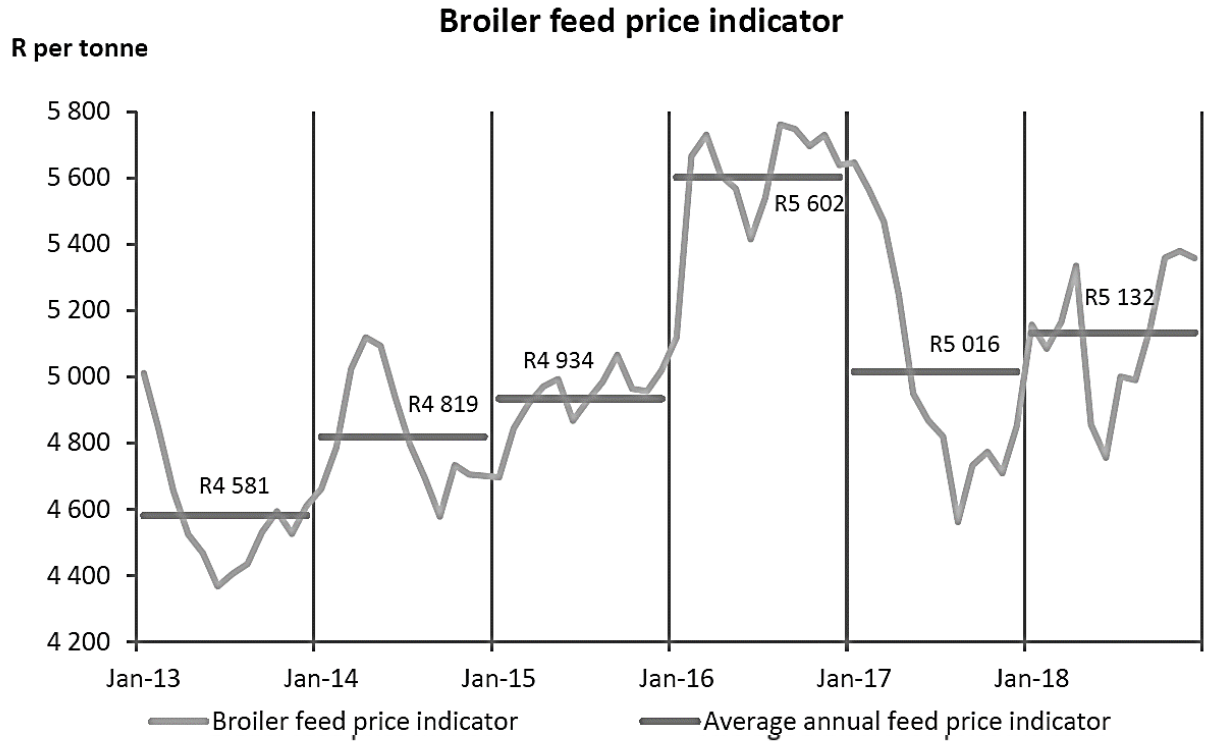

Figure 2.1 Broiler feed price indicator in South Africa from 2013 to 2018 (adapted from SAPA, 2018)

When formulating broiler diets, nutritionists aim to have a favourable balance between feed costs and nutrient requirements in order to optimise the efficiency of nutrient utilisation (Chang'a *et al*., 2019). According to Chang'a *et al.* (2019) broiler performance is affected by, among other factors, the physical form of the diet, nutrient density, and presence of anti-nutritional factors. The authors further stated that compared to pellet feed, mash feed is inferior in quality and utilisation because of its dusty nature, which may reduce feed palatability and intake. Feed raw materials may contain anti-nutritional factors which

interfere with nutrient absorption in broiler diets (Botha, 2011). This leads to some valuable nutrients being wasted because birds are not able to utilise them (Pervez & Sajid, 2011). Certain feed additives such as microbial enzymes that solubilise some cellulose and hemicellulose fractions in feed ingredients, and processing methods such as grinding or pelleting have been found to improve nutrient availability (Pervez & Sajid, 2011; Chang'a *et al*., 2019).

Feed additives may be described as chemical and biological supplements, which livestock and poultry producers use to manipulate growth, improve feed efficiency, and reduce mortality (Mann, 1973). To obtain a profitable balance among the cost of feed, broiler performance, and quality of poultry products, certain additives are available in the market for use in broiler rations (Pervez & Sajid, 2011). Examples of feed additives include antibiotics, essential oils, NSP degrading enzymes, dietary spices, organic acids as well as oligosaccharides and probiotics (Swiatkiewicz *et al*., 2016). Some of these additives are recommended for chemotherapeutic as well as prophylactic use, while others have a reputation for providing a growth promoting effect. However, during the last few decades the extensive use of sub-therapeutic levels of antibiotics in animal feed has been critisised as they pose an increased risk of potential development of resistance in the host against pathogens. This led to a subsequent ban of the use of sub-therapeutic antibiotic levels by the European Union since 2006 (Saeed *et al*., 2017). Amongst other purposes, these sub-therapeutic antibiotics are most commonly used to control disease, increase feed efficiency and increase the rate of gain by animals (Mann, 1973). With its widespread ban, it became important for researchers to find alternatives to antibiotic growth promoters (AGPs) that will boost the health and performance characteristics of livestock (Amad *et al*., 2011). Growth promoters were defined by Alçiçek *et al*. (2003) as those substances that are primarily aimed at improving physical performance such as body weight gain and feed conversion ratios in broilers. As feed costs account for approximately 60-75% of total production costs in poultry systems, Pervez and Sajid (2011) stated that feed additives may provide animal nutritionists with an economical way to reduce these costs while improving feed efficiency and utilisation of nutrients in order to produce high quality protein for human consumption at affordable prices.

2.4 Guanidinoacetic acid

Guanidinoacetic acid (GAA), also known as glucoamine or glucoacetate (Yazdi *et al*., 2017), was first discovered in 1934 as a naturally occurring compound in human beings (Ostojic, 2015). It was used as a therapeutic agent for the treatment of cardiovascular and neuromuscular disorders in humans back in the 1950s (Ostojic, 2015). Figure 2.2 shows the structure of GAA.

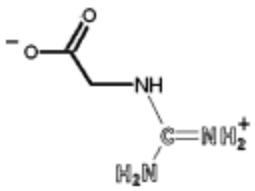

Figure 2.2.The chemical structure of guanidinoacetic acid (adapted from Brosnan *et al.*, 2011)

The only known metabolic function of GAA is its role as the immediate precursor of creatine, which in its phosphorylated form plays a major role in the energy transmission of all living cells as a backup to the adenosine triphosphate (ATP)/adenosine diphosphate (ADP) system. There are two major pathways of ATP synthesis (Camargo, 2015; Noboa, 2017):

- Firstly, through oxidative phosphorylation of nutrients such as carbohydrates, amino acids, and fat in feed;
- Secondly, through substrate-level phosphorylation of intermediates such as creatine via the activity of creatine kinase to produce phosphocreatine. Other examples of substrate level phosphorylation include glycolysis and the Krebs cycle. Substrate level phosphorylation is the most important source of ATP in cells where oxygen is limiting and generally involves the direct transfer of inorganic high energy phosphate groups to ADP to produce ATP.

The creatine/phosphocreatine system thus functions to store and mobilise energy when required at short notice particularly in muscle cells (Michiels *et al*., 2012; Lemme *et al*., 2015; Metwally *et al*., 2015; Yazdi *et al*., 2017). Figure 2.3 shows the metabolic biosynthesis pathway of the creatine/phosphocreatine system.

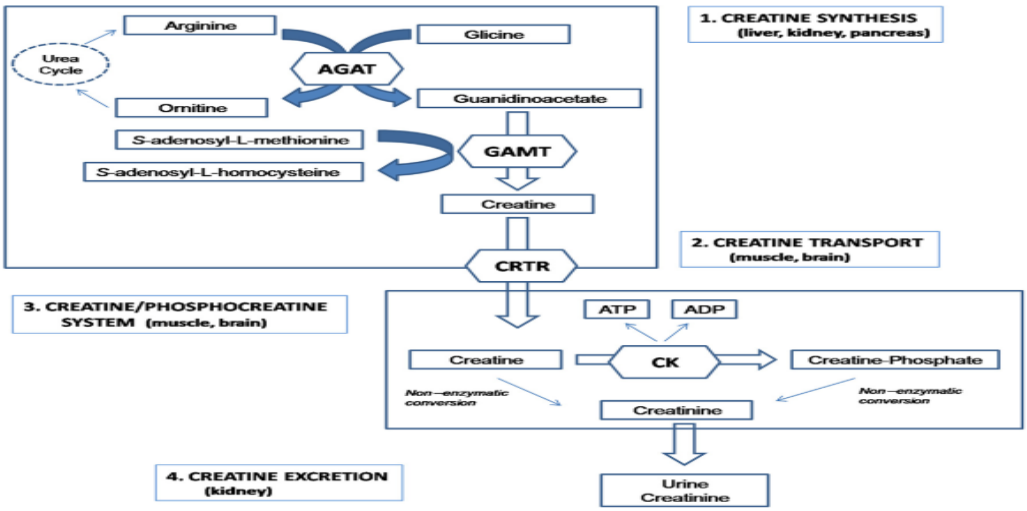

Figure 2.3 Metabolic biosynthesis and function of the creatine/phosphocreatine system (adapted from Fons and Campistol, 2016)

Creatine and its precursor GAA do not occur in plants, therefore, animals reared on vegetable-based diets need to be supplemented with these substrates so as to avoid creatine or arginine deficiency and to promote growth. However, it has been observed that creatine is not an ideal feed additive as it is unstable when stored at high temperatures and low pH conditions. Creatine is also more expensive compared to its derivative GAA (Heger *et al*., 2014). Animal by-products such as fishmeal can also be used to meet the animal's demand for creatine, however these products contain inconsistent and limited amounts of creatine (Carpena *et al*., 2015). Additionally, the dietary contribution of creatine from animal by-products is limited because the inclusion levels in feed are low, there is substantial loss of creatine due to processing and related heat treatment, and like creatine these by-products also tend to be more expensive compared to CreAMINO® (Tossenberger *et al*., 2016).

2.4.1 Guanidinoacetic acid metabolism

Guanidinoacetic acid is an intermediary product synthesised from the amino acids, glycine and L-arginine, in a reaction catalyzed by the enzyme L–arginine: glycine amidinotransferase (AGAT), mainly in the kidney and pancreas of all vertebrate animals (EFSA, 2009; Wang *et al*., 2012; Lemme *et al*., 2015). This step of GAA synthesis is the principal regulatory site and rate limiting step in the synthetic pathway of creatine (EFSA, 2009; Noboa, 2017) which in turn regulates the expression of AGAT through a negative feedback mechanism. High levels of creatine down regulate gene expression of AGAT at the transcriptional level (Murakami *et al*., 2014), whereas growth, thyroid and sex hormones have been found to up regulate AGAT and GAMT expression in rats (Brosnan *et al*., 2011; Fons & Campistol, 2016). Dilger *et al*. (2013) supported this notion and stipulated that, with regard to the metabolic regulation of *de novo* GAA synthesis, there seems to be a negative relationship between creatine status and the enzymatic activity of AGAT, which may explain variable responses observed in the Arg-sparing ability of GAA. By this it also lowers the subsequent production of homocysteine (Noboa, 2017). When evaluating the efficiency of dietary GAA utilisation, it is then important to consider its related metabolites, creatine and creatinine (Tossenberger *et al*., 2016), which are affected by metabolic pathways and will be discussed in the following sections.

According to Brosnan *et al*. (2011) there is a high requirement for methylation of GAA to produce creatine as about 63-77% of all labile dietary methyl groups are utilised in the synthesis of creatine in pigs. In humans, about 40% of the dietary methyl groups are used in the production of creatine from GAA and as such, places an appreciable burden on the provision of such methyl groups, either from the dietary substrates (methionine, betaine, or choline) or via *de novo* methylneogenesis, which in turn depends on the amount of B-vitamins available for synthesis (Janicki & Buzala, 2013). Thus, increased methylation demand may also

lead to a deficiency of methionine and possibly choline, folic acid, or vitamin B_{12}. Supplemental creatine has the potential to spare these methyl groups through a negative feedback mechanism on AGAT activity, thus lowering GAA synthesis.

According to Wang *et al*. (2012) the ability of GAA to spare arginine is also beneficial as arginine is able to quench free radicals such as superoxide anions. The excess arginine also serves as a substrate of the nitric oxide synthase family and would thus increase the production of nitric oxide, a free radical which regulates metabolism, contractility and glucose uptake in skeletal muscle. In rats, GAA administration led to a decline in the non-enzymatic antioxidant capacity in the brain. This was assumed to be likely due to oxidation of sulfhydryl groups, thus leading to lower glutathione levels (Michiels *et al*., 2012).

2.4.2 Creatine metabolism

Once synthesised, GAA is transported to the liver where it is methylated by the enzyme s-adenosylmethionine (SAM) at the amidino group in a reaction catalysed by guanidinoacetate methyltransferase (GAMT) to yield creatine (Michiels *et al*., 2012). There is also the subsequent production of approximately 40% s-adenosyl-homocysteine (SAH) as a by-product (Janicki & Buzala, 2013; De Groot, 2014). Figure 2.4 shows the metabolic pathway of creatine synthesis from GAA and its subsequent excretion from the body. S-adenosyl-homocysteine on the other hand, can be reversibly hydrolysed to homocysteine and adenosine, with homocysteine either further catabolised to cysteine or re-methylated to form methionine, or essentially exported to the circulation (Ostojic, 2015). The EFSA (2009) stated that biological methylation and production of SAH are closely linked. Thus, with increased dietary GAA intake the methylation demand increases as well as subsequent production of SAH providing a tool for inducing hyperhomocysteinemia in animals. Tossenberger *et al*. (2016) stated that high plasma homocysteine can also be as a consequence of decreased transfer of methyl group capacity due to either a relative deficiency of methionine, choline, or betaine or a lack of vitamin B_{12} and/or folic acid. Michiels *et al*. (2012) stated in a review that several studies involving labile methyl-group balance and estimates of methylation demand have demonstrated that the methylation of GAA to creatine utilises more SAM than all other methylation reactions combined.

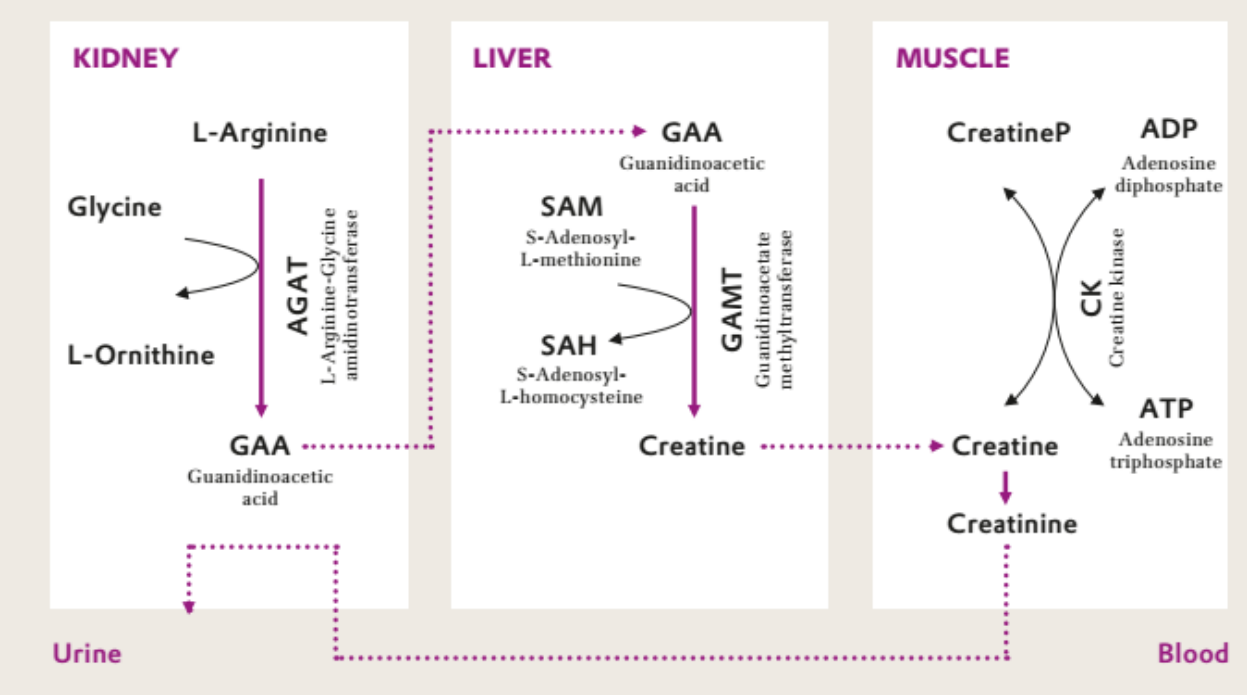

Figure 2.4 The biochemical synthesis of creatine and creatinine (Carpena *et al*., 2015)

Creatine is a nitrogenous organic acid involved in energy metabolism through the creatine /phosphocreatine system in all body cells, but mainly in muscle cells (Yazdi *et al*., 2017). It helps maintain energy balance by accepting high energy phosphate groups from ATP to create phosphocreatine then releases the high energy phosphate groups in the regeneration of ATP when the energy demand is high (De Groot, 2014). Albeit small amounts of GAMT are detectable in the mammalian kidney, creatine is primarily synthesised in the liver, where GAMT has its highest activity. De Groot (2014) stated that in humans for example, GAMT activity is approximately 0.25 µmol creatine/$h^{-1}.g^{-1}$ in the liver whereas in poultry, creatine is synthesised in both the kidney and liver (with GAMT activity being slightly higher in the kidney compared to the liver at 1.2 µmol creatine/$h^{-1}.g^{-1}$ and 1.06 µmol creatine/$h^{-1}.g^{-1}$, respectively). In humans, renal GAA synthesis accounts for approximately 20% of the total GAA production, implying that GAA must be synthesised in other tissues (such as pancreas, liver and muscle) as well, although the renal system clearly plays a major role (Ostojic, 2015).

In general, all vertebrates are able to synthesise approximately 66-75% of their daily creatine requirements *de novo* (Murakami *et al*., 2014). However, it is likely that the daily creatine requirement is proportionally greater in growing animals due to their need to supply creatine for muscle growth in the regeneration of ATP from the creatine /phosphocreatine system. This is in addition to replacing creatine losses from the body in the form of creatinine. Furthermore, there is a need to provide creatine to the growing tissues

(Abudabos *et al*., 2014; Yazdi *et al*., 2017). Therefore, the capacity for *de novo* synthesis may be limiting in high-yielding farm animals, especially in those fed all-vegetable diets (Mousavi *et al*., 2013).

The major portion (>95%) of the creatine pool is located in skeletal muscle tissue and the remainder is inter-alia distributed between the brain, liver, kidneys and testis. However, about 1.5-2% of this creatine/phosphocreatine pool is irreversibly converted to creatinine in the kidney and excreted through urine (Michiels *et al*., 2012; Lemme *et al*., 2015). This non-enzymatic and irreversible conversion of creatine to creatinine is pH and temperature dependent (Fons & Campistol, 2016). Consequently, broilers fed all vegetable based diets may have a creatine deficiency which may depress performance. Therefore, creatine stores need to be replaced. However, creatine itself is not a good feed additive as it is less stable and more expensive than its precursor GAA, thus limiting its use as a direct feed additive.

2.4.3 Functions of the creatine/phosphocreatine system

In growing tissue and cells, creatine helps to maintain energy homeostasis by accepting high energy phosphate groups in a reversible reaction mediated by creatine kinase to produce phosphocreatine, using ATP and ADP as metabolic intermediates (De Groot, 2014). By recycling these high energy phosphate groups to convert ADP back to ATP when the energy demand is high (Metwally *et al*., 2015) this system provides a temporal energy buffer (Wallimann *et al*., 1992). This function was further supported by Carpena *et al*. (2015), whose findings implied that the creatine/phosphocreatine system provides a high energy phosphate buffer in the fusion of multiple cell types including muscle, sperm and/or oocytes. Muscle development was thus improved in the presence of increased phosphocreatine (Carpena *et al*., 2015). According to Yazdi *et al*. (2017) there is a potential increase in water uptake by muscle cells leading to increased muscle cell volume stimulated by the addition of GAA in the diet, which may explain resulting increased breast muscle size. A super-hydrated muscle might stimulate protein anabolism, minimise protein degradation and increase glycogenesis (Michiels *et al*., 2012).

The second main function of the creatine/ phosphocreatine system is that it serves as a "spatial energy buffer" or rather an "energy transport system" by serving as a transport mechanism for the high phosphate groups from mitochondrial sites of production to the cytoplasmic sites of high ATP consumption via the subcellular compartmentalised creatine kinase (CK) isoenzymes (Wallimann *et al*., 1992; Metwally *et al*., 2015). Creatine and phosphocreatine are smaller than ADP and ATP and can therefore more easily diffuse through cellular membranes and accumulate to higher concentrations in cells without affecting regulatory feedback mechanisms (Longo *et al*., 2011). Creatine kinase is comprised of four isoenzymes detected in the cytosol of muscle and brain, as well as in the mitochondria of muscle and all other tissues. Thus, it is

upon these compartments of CK that the phosphocreatine "energy transport/shuttle" theory is based (De Groot, 2014).

Wallimann *et al*. (1992) stated that the third function of the creatine/phosphocreatine system is to prevent an increase in the level of intracellular or free ADP which would hinder ATP-dependent processes, thus avoiding activation of cellular ATPases as well as a net loss of adenine nucleotides (Janicki & Buzala, 2013). This function is achieved by the net release of inorganic phosphate (Pi) from the creatine/phosphocreatine system to regenerate ATP, thus establishing a system capable of constantly regenerating ATP from ADP in tissues with high energy requirements, such as the muscle (Longo *et al*., 2011). For example, during the first phases of muscle exercise, Pi increases proportionately with the amount of phosphocreatine hydrolysed, while the levels of ATP and ADP remain stable (Wallimann & Hemmer, 1994). Therefore, without a functioning phosphocreatine shuttling system to maintain energy levels, muscles would not be able to contract properly and there would be little to no tissue growth in poultry (De Groot, 2014).

The fourth main function of the creatine/phosphocreatine system as stated by Wallimann *et al*. (1992) is that it provides a mechanism for proton buffering. Since the CK reaction towards regeneration of ATP does not only use ADP but also protons (H+), both products of ATP hydrolysis, a close coupling of CK with ATP enzymes prevents local or global acidification of muscle cells and it also increases the concentration of ADP during exercise (Janicki & Buzala, 2013). In other words, the creatine/phosphocreatine system prevents a rapid fall in ATP levels and thus, a buildup of ADP levels during cellular work, while avoiding intracellular acidification due to ATP hydrolysis (Wallimann & Hemmer, 1994). This was in line with the findings by Michiels *et al*. (2012) who observed the highest phosphocreatine: ATP ratio with an increase in supplemental GAA. The authors concluded that the buffering capacity of the phosphocreatine system for ATP hydrolysis was increased and agreed with the notion that creatine loaded muscles have the capacity for improved growth and work, beneficial for both skeletal muscle growth and contraction in muscle such as the heart.

2.4.4 Other functions of guanidinoacetic acid

Guanidioacetic acid is beneficial in broiler diets due to its ability to spare arginine (Arg), which is considered to be the fifth limiting amino acid in typical maize-soybean diets for broilers (Dilger *et al*., 2013; Abudabos *et al*., 2014). Dilger *et al*. (2013) found that the addition of GAA improved the growth performance of birds fed Arg-deficient diets, thus theoretically allowing the Arg that would have been used to synthesise GAA to be available for other functions in the body such as protein accretion and nitric oxide synthesis. Also, in commercial protein-reduced maize-soybean diets, Arg becomes the third limiting amino

acid and a dietary source of GAA (CreAMINO®) has been shown to increase muscle protein with a subsequent decrease in muscle fat, thus resulting in increased muscle mass (De Groot, 2014). In context of the metabolic regulation of the *de novo* GAA synthesis, the existence of a negative feedback mechanism of creatine towards L-AGAT enzyme-activity, may help explain the contradictory responses observed with regard to the Arg sparing ability of GAA (Dilger *et al*., 2013).

In their study with broiler breeders, Carpena *et al*. (2015) found that including increasing levels of GAA in the diet of broiler breeders improved the fertility and hatchability of breeders and feed conversion efficiency of their progenies up to 0.08%. The authors stated that this improvement in fertility with increased supplementation was associated with creatine use by spermatozoa, which improved flagella motility and thus improving access to the ovum, an important factor in oocyte fertilisation and the improved feed efficiency of the unsupplemented progeny due to the increased creatine content in eggs of the meat-type breeders that received GAA. Sharideh *et al*. (2015) proposed that as a precursor of creatine, it is probable that supplementary GAA increased the uterine phosphocreatine content, which in turn contributed to ATP availability to mitochondria for sperm metabolism requirements.

At a molecular level, GAA seems to play a role in the up-regulation of growth promoting genes, namely myogenin and insulin growth-like factor 1 (IGF-1), which in turn stimulates skeletal muscle development. Insulin growth-like factor-1 stimulates protein anabolism and muscular development, whereas myogenin is one of the vital genes concerned with muscle fibre formation (Metwally *et al*., 2015). In the same study by Metwally *et al.* (2015), it was stated that GAA down-regulates expression of myostatin, a potent muscle growth inhibitor expressed both in embryonic and adult pectoral muscle. Therefore, GAA may reduce the metabolic load caused by protein degradation in broilers fed Arg deficient diets.

Guanidinoacetic acid may reduce non-enzymatic antioxidant capacity of cells (Emami *et al*., 2017) possibly through the oxidation of sulfhydryl groups, thus leading to lower levels of glutathione (Michiels *et al*., 2012).

2.5 Carbohydrates in broiler feed

Carbohydrates constitute about 60-90% of plant dry matter and comprise a diverse group of molecules with a range of chemical, physiological, metabolic, and energetic properties (Kleyn 2013; Ochoa *et al*., 2014). Carbohydrates can be classified into sugars, compounds strictly with less than 10 building blocks, and non-sugars, also known as structural carbohydrates (McDonald *et al*., 2010; Kleyn 2013). Figure 2.5 shows a detailed classification of carbohydrates. Oligosaccharides can be defined as all sugars, except monosaccharides, which are made up of only one building block (Kleyn, 2013). Polysaccharides on the other hand can be further divided into starch (highly digestible and made of α-glycosidic bonds) and

nonstarch polysaccharides (the most abundant polysaccharides of plant cell walls). These nonstarch polysaccharides include hemicellulose, cellulose, lignin, and pectins (Ochoa *et al*., 2014).

Carbohydrates represent a major part of animal diets, making up to 70% of the diets, as a high-yielding source of energy (Kleyn, 2013; Ochoa *et al*., 2014). In poultry feed, starch contributes approximately 60% towards the apparent metabolisable energy (AME) in complete diets (Cowieson, 2005). Regardless of its dietary source, the starch molecule is composed primarily of glucose polymers, amylose (mostly linear in structure) and amylopectin (bush-like structure consisting of α-1, 6 glucose branches) (McDonald *et al*., 2010). The proportions of amylose to amylopectin *per se*, depend on the raw material source and starch digestibility itself, and are directly correlated to the amount of amylopectin present due to its side chains, making it less vulnerable for attack by endogenous amylase enzymes (Stefanello *et al*., 2015). Therefore, the more amylopectin present the lower the digestibility of starch. Figure 2.6 shows the different structures of amylose compared to amylopectin.

Cowieson (2005) described starch digestion as a simple process achieved by endogenous secretion of α-amylase and maltase activity. Approximately 90-95% of starch is digested in the small intestine (Williams *et al*., 1997). However, poultry do not secrete the endogenous enzymes required to break down the β-glycosidic linkages of non-starch polysaccharides (NSPs) which form the fibre background of plant cell walls (Kleyn, 2013). Compared to wheat, on a dry matter basis maize contains a higher amount of starch (690-730 g/kg versus 651 g/kg in wheat) and contains lower levels of NSPs, 68-97 g/kg vs. 119 g/kg, respectively (Kleyn, 2013; Stefanello *et al*., 2015).

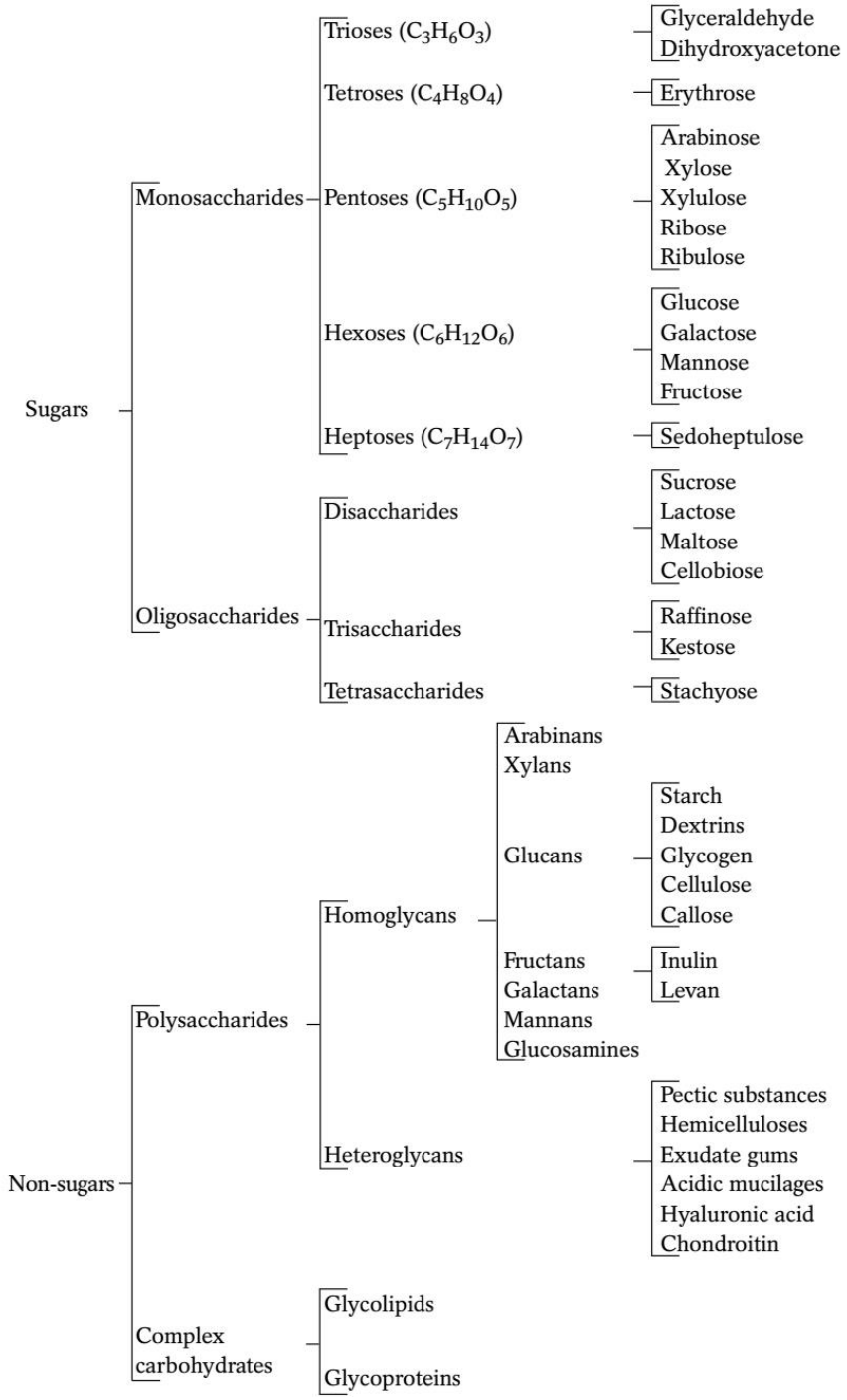

Figure 2.5 Classification of carbohydrates into different groups (McDonald *et al.*, 2010)

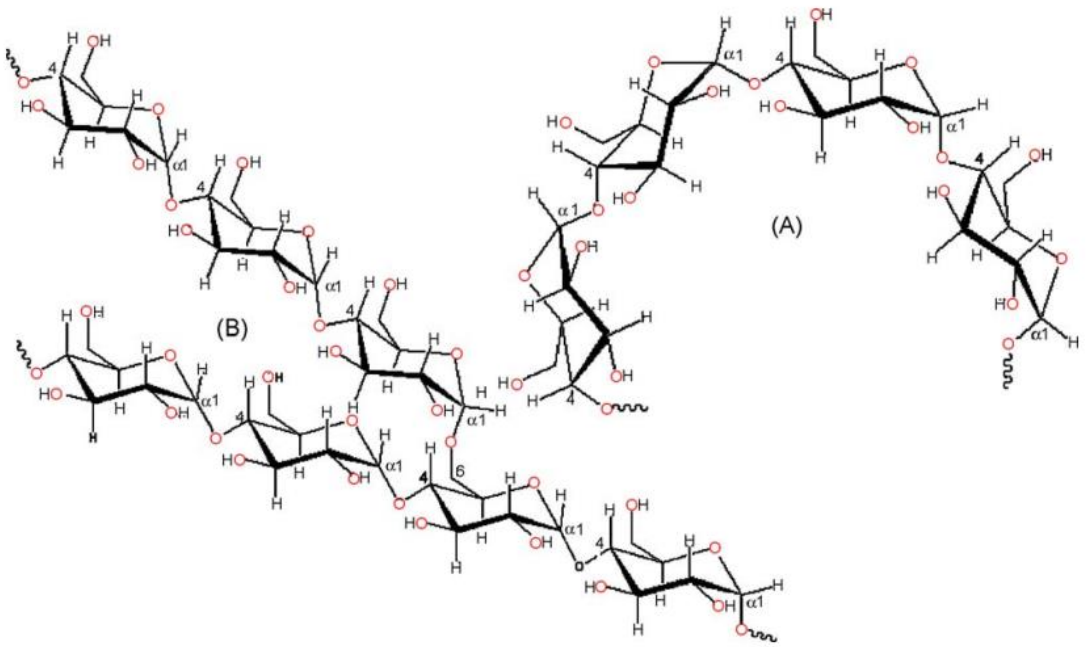

Figure 2.6 Basic representations of (A) amylose and (B) amylopectin structures (Cowieson, 2005)

2.5.1 Classification of non-starch polysaccharides

Nonstarch polysaccharides (NSPs) comprise the major components of dietary fibre, principally found in plant cell walls. They are macromolecules of a large number of glucose monomers linked together by glycosidic bonds (Ochoa *et al*., 2014). Nonstarch polysaccharides can essentially be divided into two main classes:

- The first group includes the water-soluble fraction (also known as non-cellulosic polysaccharides), which has a tendency to form a gel-like viscous consistency in the digestive tract (Kleyn, 2013). This fraction includes β-glucans and arabinoxylans. Water soluble NSPs affect the interaction between digestive enzymes, nutrients and other substrates. They hinder digesta movement and consequently, the transportation of hydrolysis products to the intestinal mucosa (Slominski, 2011; Lee *et al*., 2013). In pigs, the increased viscosity in the gastrointestinal tract (GIT) caused by β-glucans and pentosans may affect the natural sieving of particles by causing physiological and morphological changes in the intestine (Annison & Choct, 1991). Thus, large particles are consequently suspended in the viscous digesta and pass through the duodenum instead of falling to the base of the stomach, resulting in less efficient digestion. The increase in viscosity disturbs peristaltic movement of the GIT, and also affects the pancreatic secretion of enzymes, thus impeding digesta movement and transport of hydrolysis products to the intestinal mucosa (Slominski, 2011). In poultry, however, the viscous nature of the digesta caused by the chemical

cross-linking between the water soluble NSPs subsequently results in increased water intake and undesirable microbial fermentation in the ileum ensues. Increasing moisture content in the excreta results in sticky droppings and potential diarrhoea, this ultimately causes problems with litter waste management (Nadeem *et al*., 2005; Lee *et al*., 2013). However, the most adverse effect of water-soluble NSPs is the reduced access of endogenous enzymes to feed particles and nutrients trapped in the carbohydrate-water matrix, hence reducing feed digestibility and animal performance (Nadeem *et al*., 2005; Slominski, 2011). The increase in wet and sticky droppings may potentially cause leg problems due to its effect on litter waste (Lee *et al*., 2013). Wet litter can lead to ulcerative conditions of the skin on feet; this condition is normally called footpad dermatitis or hock-burn (Botha, 2011).

- The second group consists of insoluble NSPs. This group of NSPs is biologically inert and are also known as cellulosic NSPs. Examples include pectins such as arabinoxylans (bound to cellulose in the plant cell wall), 1,4-β-arabinogalactans and ramnogalacturonans, the so-called "soya pectin" (Vahjen *et al*., 2005; O'Neill *et al*., 2014). Meng and Slominski (2005) stated that poultry can potentially digest water-soluble NSPs but lack the enzymes to digest insoluble NSPs so it is most likely that they pass through the birds unchanged, thus most of the anti-nutritive effects are associated with the soluble fraction (Williams *et al*., 1997; Botha, 2011).

A more detailed classification of NSPs is displayed in Figure 2.7.

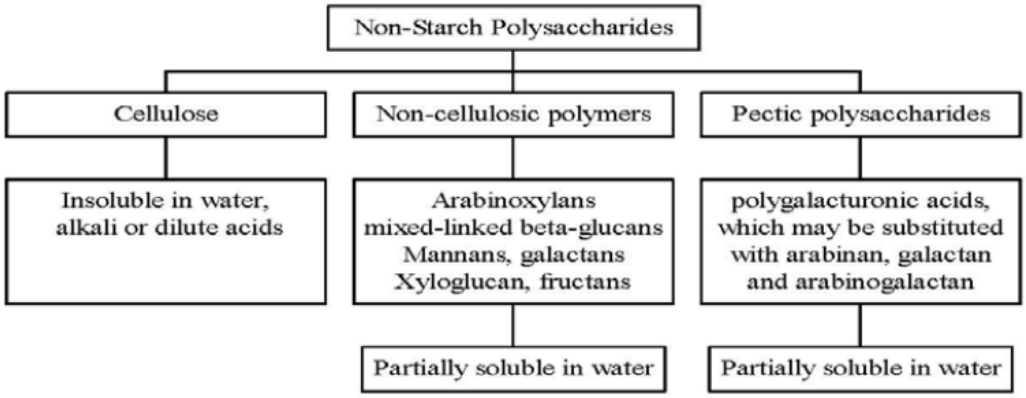

Figure 2.7 Classification of non-starch polysaccharides (adapted from Choct *et al*., 2010)

2.5.2 The effect of non-starch polysaccharides on digestion

Cereal grains are carbohydrate concentrates of which the main component, starch, provides the bulk of energy in monogastric diets and is located intracellularly in the endosperm (McDonald *et al*., 2010; O'Neill *et al*., 2014). The term dietary fibre was initially coined by Hipsley in 1953 as a definition of non-digestible components that make up the cell wall of plant material, however this term was later refined to describe a

complex mixture of carbohydrate polymers associated with a number of other nonstarch or rather non-carbohydrate components such as NSPs, lignin, proteins, fatty acids and waxes (Lee *et al*., 2010). The fibre component of cereal grains primarily consists of insoluble NSPs, forming part of the cell wall structure, thus encapsulating starch, proteins, fat and other nutrients (Choct, 2006; Aok, 2012). This is referred to as the cage effect (Rios *et al*., 2017) and is the primary mechanism by which NSPs exert their effect on nutrient digestion (Simon, 1998). This cage effect limits the animal's own digestive enzymes from accessing and fully digesting the intracellular starch and protein (Meng & Slominski, 2005). Ochoa *et al*. (2014) estimated that cereal grains contain approximately 10-30% NSPs. Poultry are able to synthesise a number of enzymes, including amylases, proteases and lipases to digest starch, proteins, as well as fats, respectively. They do not however, have the necessary enzymes to digest dietary fibre (Nadeem *et al*., 2005; Kleyn, 2013) or rather they do not produce sufficient enzymes to breakdown non-starch polysaccharides present in cell walls of cereal grains (Khattak *et al*., 2006).

In grain legumes used as protein concentrates, such as soybean meal or canola meal, pectic polysaccharides including arabinogalactans, rhamnogalacturonans (type one and two), as well as xylogalactoronans form the majority of NSPs in these diets (Vahjen *et al*., 2005; Choct 2006; Aok, 2012). These, including the raffinose family of oligosaccharides also found in soybean meal, are insoluble and indigestible to monogastric animals (Ochoa *et al*., 2014). Pèron *et al*. (2011) stated that high levels of insoluble NSPs result in increased water-holding capacity, reduced access of digestive enzymes to nutrients (nutrient packaging), and increased endogenous loss of nutrients. Diets containing high concentrations of NSPs may lead to an enlargement of the bird's pancreas, thus reflecting increased secretion of endogenous enzymes by the pancreas. Ultimately, high levels of dietary NSPs will result in reduced animal performance and nutrient utilisation, and will also stimulate undesirable microbial growth in the GIT (Botha, 2011; Péron *et al*., 2011). According to Lee *et al*. (2013), a decrease in passage rate of digesta such as caused by high NSP levels, reduces the oxygen tension in the small intestine, thus producing a favourable environment for anaerobic fermentative microorganisms. An indirect effect of NSPs on the efficient use of nutrients in broilers is related to the activity of gut microbiota as measured by the concentration of volatile fatty acids (VFAs) in the intestinal tract, the result being bacterial colonisation of the epithelium of the small intestine and caeca (Segobola, 2016). It was initially thought that elevated VFAs production, as a consequence of microbial fermentation, would translate to increased dietary energy content. However, due to the drastic change in the intestinal ecosystem, the net effect was reduced nutrient digestion accompanied by poor bird performance (Choct *et al*., 2010). Colonisation of *Salmonella faecium* species, intestinal microbes capable of degrading bile salts, increased when a wheat-based diet was fed to broilers, which resulted in reduced fat digestion due to insufficient bile salts (Ward, 1996). Choct *et al*. (2010) stated that slow moving digesta

with low oxygen tension in the small intestine provides a relatively stable environment whereby fermentative microbiota can establish in the gut. High bacterial populations irritate and cause thickening of the mucosal lining in the gut, damage microvilli and reduce nutrient absorption**.** Insufficient bile salts also have a negative effect on protein digestion because bile acids stabilise pancreatic proteases in the intestinal lumen. Therefore, protein digestion will be compromised due to a lack of bile acids (Ward, 1996). The subsequent release of low molecular oligosaccharides and monomers hastens microbial fermentation and increases intestinal osmotic pressure, which are both known to impede nutrient absorption (Vahjen *et al*., 2005). This was in agreement with Lee *et al*. (2010) who further stated that higher counts of anaerobic bacteria have been found in birds fed high levels of NSPs, which was presumed to be as a consequence of the increased intestinal viscosity. This therefore implies that as the level of water soluble NSPs increases in the diet, the relative digestibility of the feed decreases. This theory was supported by Williams *et al*. (1997) who investigated the effect of wheat arabinoxylans on endogenous secretions and protein digestibility and found that at lower concentrations (15 g/kg) arabinoxylans caused an increase in endogenous amino acid losses. The authors also observed in this study that at higher concentrations of 35 g/kg, a direct inhibition of protein degradation and consequently, amino acid absorption, occurred. Segobola (2016) also stated that the concentration of water-soluble NSPs is inversely correlated to the MEn content in broiler diets.

Another way in which NSPs affect nutrient digestion is thought to be the manner in which they exert their antinutritional effect by means of forming a gel-like consistency in the gastro-intestinal tract. This leads to an increase in gut viscosity and a reduction in rate of passage of digesta, which in turn, results in changes in gut morphology and physiology (Annison & Choct, 1991; Choct *et al*., 2010). Because poultry have a fast rate of passage of food, it is also likely that the insoluble NSPs pass through the GIT of the bird undigested and biologically inactive (Annison & Choct, 1991). Cereal grains like maize do not present viscosity values as high as barley or wheat due to the negligible quantities of β-glucans and the low content of soluble pentosans in the grain (Gracia *et al*., 2003).

2.5.3 Non-starch polysaccharides in maize-soybean diets

Maize is one of the most important cereal grains used in animal feed, serves as a highly digestible source of energy (Govil *et al*., 2017) and presents fewer problems when included in the diet. Maize is the main source of dietary energy in poultry diets and contains approximately 690-730 g starch/kg dry matter (DM) (McDonald *et al*., 2010; Stefanello *et al*., 2017). Soybean meal, a by-product of the oil extraction industry with approximately 15-18% polysaccharides (Waldroup *et al*., 2006), is the most widely used vegetable protein source in monogastric diets because of its high protein content of approximately 500 g/kg and

favourable amino acid composition (Vahjen *et al*., 2005). It also serves as the standard by which other vegetable proteins are evaluated (Choct *et al*., 2010).

Albeit maize-soybean meal diets are highly digestible, the possibility of improving energy and protein digestibilities exist because these raw materials do contain some anti-nutritional factors that inhibit full digestion of nutrients (Tahir *et al*., 2006). Soybean meal contains substantial amounts of inhibitory, goitrogenic, allergenic and anti-coagulant factors, however, all of these factors are inactivated during heat processing (Botha, 2011). The most important anti-nutritional factors that both these feed ingredients contain are low molecular weight NSPs forming part of the cell wall structure, which can impede normal digestive and absorptive processes of carbohydrates, proteins and other intracellular nutrients such as minerals (Govil *et al*., 2017). In maize, the quantity of these NSPs ranges from 68-97 g/kg DM, whereas soybean meal contains about 160-300 g/kg DM NSPs (Vahjen *et al*., 2005; Stefanello *et al*., 2017). Table 2.1 shows the quantities of NSPs in different raw materials (Kleyn, 2013). In their experiment, Meng and Slominski (2005) measured the total as well as water soluble NSPs in the feed ingredients and diets used in their experiments. Maize contained the lowest NSPs amongst all the feed ingredients (Table 2.2). According to Tahir *et al*. (2006) approximately 10% of protein in soybean meal is located within the cell wall matrix and can only be made available for broiler chickens by degradation of NSPs.

Table 2.1 The level of non-starch polysaccharides in feed ingredients in g/kg DM (Kleyn, 2013)

Ingredient	**CF**	**NDF**	**NSP**
Wheat	23	110	110
Barley	52	150	147
Oats	91	248	253
Wheat bran	85	390	360
Maize gluten	74	360	368
Soya hulls	354	599	868
Soybean meal	85	189	190
Maize	26	100	70

CF= crude fibre; NDF=neutral detergent fibre; NSP= non-starch polysaccharides

Table 2.2 Total and water soluble non-starch polysaccharide (NSP) contents of feed ingredients and experimental diets (Meng and Slominski, 2005)

	Total NSP (mg/g)	Water-Soluble NSP (mg/g)	Water-Soluble NSP (% of the total NSP)
Ingredient			
Maize	76.3	6.4	8.4
Soybean Meal	136.7	13.4	9.8
Canola Meal	174.5	14.3	8.2
Peas	124.7	5.9	4.7
Diet			
Maize	51	4.3	8.5
Maize-Soybean	90.1	8.4	9.3
Maize-Canola	95.1	8.4	8.8
Maize-Peas	79.1	5.3	6.7

Within the same plant species there is great variation in the NSP content affected by plant genotype and the environment in which they were cultivated (Rios *et al*., 2017).

2.5.4 Exogenous enzymes in broiler diets

Some valuable nutrients in feed are wasted because of the broiler's inability to digest them fully. Reasons for poor digestion may be a lack of digestive enzymes, rapid feed passage rate through the GIT reducing time for digestive activity, subclinical infections and/or inadequate processing of feed ingredients (Pervez & Sajid, 2011). Therefore, feeding exogenous dietary enzymes to poultry has been one of the major nutritional advances over the last 50 years. According to Khattak *et al*. (2006) the theory of feeding enzymes to poultry is a concept based on the fact that plants contain certain compounds that are either indigestible for the animal or hinder the animal's digestive system. Often this would be because the animal cannot produce the necessary enzymes to degrade the compounds hindering digestion. Nutritionists are now able to assist the animals by identifying indigestible compounds and feeding suitable enzymes produced by microorganisms, carefully selected for the task and cultured under controlled conditions. Govil *et al*. (2017) stated that approximately 400-450 kcal (1.67-1.88 MJ) of energy per kg feed intake is not digested when birds are fed typical maize-soybean meal diets without exogenous enzyme supplementation. Therefore, exogenous NSP degrading enzymes may prove to be beneficial in monogastric diets. Two types of NSP-degrading enzyme preparations have been made commercially available; firstly, there are mono/single-enzyme preparations which comprise only a single enzyme and, secondly, multi-enzyme complexes (or cocktails), which are processed fermentation surfactants of selected fungal strains (Stefanello *et al*., 2015). Previous research has demonstrated positive responses to both single- (Gracia *et al*., 2003; Cho & Kim, 2013) and multi-enzyme cocktail (Cowieson & Adeola 2005; Cowieson 2005; Lee *et al*., 2010; Narasimha

et al., 2013; Stefanello *et al*., 2015) in poultry diets. Despite of all the evidence of the beneficial effects of exogenous enzymes on broiler performance, there were, however, also studies conducted where no significant improvement in performance characteristics were found despite significant improvements in nutrient digestibility (Meng and Slominski 2005; Tahir *et al*., 2006; Vieira *et al*., 2006; Waldroup *et al*., 2006; Duorado *et al*., 2009; Amerah 2015). This implies that albeit feed digestibility was improved it was not to the point where performance parameters were affected, or that sufficient nutrients were already available to the broilers to sustain potential performance (West *et al*., 2007). Thus, more research needs to be conducted to investigate the point at which increased nutrient digestion translates to improved growth response. Table 2.3 displays a summary of the beneficial effects of exogenous NSP enzymes used individually or in combination in maize-soybean diets observed in previous studies with broilers.

An increase in the economic value of commercial diets that are supplemented with enzymes can be achieved by:

- The net release of available phosphorus from phytate hydrolysis (Zeng *et al*., 2015).
- The removal of the nutrient encapsulating effect of the cell walls (i.e. the carbohydrate-protein bonds) and therefore, enhanced energy as well as amino acid availability (Slominski, 2011).
- The solubilisation of cell wall NSPs for more efficient hindgut fermentation, and consequently, improved overall dietary energy utilisation. The latter being achieved by production of volatile fatty acids through fermentation, which are absorbed and in turn stimulate hormonal feedback mechanisms that delay gastric emptying and can also be used as energy (Stefanello *et al*., 2017).
- The elimination of anti-nutritive properties of certain dietary NSPs through hydrolysis to prebiotic type xylo-oligomers, which in turn indirectly benefit digestion by facilitating intestinal development and health in young birds (Narasimha *et al*., 2013).

Table 2.3 Effect of supplementing exogenous non-starch polysaccharide degrading enzymes on broilers receiving commercial diets

Enzyme Used	Enzyme Effect	Reference
α - Amylase	↑NSP digestibility; improved FCR	(Gracia *et al*.,2003)
α - Amylase, β - xylanase, phytase and protease	↑BWG; improved FCR	(Coweison & Adeola, 2005)
β-Glucanase, , β - xylanase, cellulase, pectinase, and protease	Improved FCR overall	(Nadeem *et al*., 2005)
β-Glucanase, , β - xylanase, cellulase, pectinase, mannase and galactanase	↑CP digestibility; ↑NSP digestibility; no significant results with respect to growth	(Xeng & Slominski, 2005)
α - Galactosidase	No significant results	(Waldroup *et al*., 2006)
α - Amylase, β - xylanase, phytase and protease	↑BWG; ↑organic matter digestibility	(Coweison *et al*., 2006)
Pectinase, cellulose and hemicellulase	↑CP digestibility; ↑NSP digestibility; ↑ carcass parameters produced	(Tahir *et al*., 2006)
Xylanase, pectinase and α - galactosidase	No significant results	(Viera *et al*., 2006)
Xylanase and β-glucanase	No significant results	(West *et al*., 2007)
α - Amylase, β - xylanase, phytase and protease	↑NSP digestibility; improved growth performance	(Olukosi *et al*., 2007)
β-Glucanase, , β - xylanase, cellulase, pectinase, mannase and galactanase	↑ Tibia breaking strength; ↑ash content of bones; ↓intestinal viscosity by at least 12.4%	(Lee *et al*., 2010)
β-Glucanase, , β - xylanase, cellulase, β - mannase and α - galactanase	Improved FCR; ↓excrement output	(Zou *et al*.,2013)
β - Mannase	↑BWG, ↑nutrient digestibility; ↑breast meat weight; improved FCR	(Cho & Kim, 2013)
α - Amylase, β - xylanase and phytase	↑starch digestibility; ↑BWG; improved FCR; improved AMEn	(Stefanello *et al*., 2015)
α - Amylase, β - xylanase and protease	↑nutrient digestibility; ↑BWG; improved FCR; improved AMEn	(Amera *et al*., 2017)
Xylanase and β-glucanase	↑Ileal digestibility; improved FCR	(Rios et al., 2017)
Mannanase, α – amylase and β - xylanase	↑BWG; improved FCR	(Govil *et al*., 2017)

AA = amino acids, NSP = nonstarch polysaccharides, CP = crude protein, BW = body weight, AME_n = nitrogen corrected apparent metabolisable energy, FCR = feed conversion ratio, and VFA = volatile fatty acids

Meng and Slominski (2005) investigated the effect of cell wall degrading enzyme supplementation on different broiler diets. The authors observed an increase in NSP digestibility due to enzyme supplementation in the maize-based diet used in the study and suggested that the production of free sugars, oligosaccharides,

or low-molecular weight polysaccharides might have resulted from the additional enzyme activity, and that these constituents could have been available for microbial fermentation in the caeca, which essentially resulted in synthesis of volatile fatty acids (VFAs). The authors further stated that these VFAs would then have been absorbed and used by the bird, contributing to a small increase in AMEn content of the maize diet. Based on this evidence, the authors concluded that NSP-degrading enzymes have the ability to improve the nutritive value of maize. It may be difficult, however, to say whether oligosaccharides should be regarded as "nutrients" or "anti-nutrients" depending on what researchers seek to achieve. Choct *et al*. (2010) stated that researchers who look for the prebiotic properties of oligosaccharides often report changes in the quantity and profile of the intestinal microflora, indicative of a beneficial effect, whereas those who investigate the performance related parameters argue that an elevated level of oligosaccharides in poultry diets elevates fluid retention, hydrogen production and diarrhea thus leading to impaired nutrient utilisation.

It should be noted that due to enzyme characteristics such as substrate affinity, range of pH activity, or susceptibility to cereal endogenous inhibitors, the bio-efficacy of carbohydrate degrading enzymes can vary widely (Péron *et al*., 2011). Other factors that may cause variation in response to supplementation with enzymes include, feed processing, individual enzyme molecule properties, breed and age of birds (Amerah, 2015). Rios *et al*. (2017) stated that enzymes have an active site specification, therefore, in order to observe the benefit of supplementation the enzymes need to be able to reach the specific site of attachment.

An enzyme cocktail containing several enzyme activities is most preferred in broiler diets because traditional broiler diets rarely contain a single raw material ingredient but are made up of different cereals with different kinds of NSPs at variable levels (Nadeem *et al*., 2005). Therefore, enzymes with many different activities are capable of complementing each other by targeting different feed components (Olukosi *et al*., 2007). This may lead to either additive or synergistic interactions between the enzymes and the diet. There are several possibilities in which multiple enzyme cocktails may be additive ranging from working in synergism (one + one = three) to being antagonistic (one + one = 0.75) or anything in between (Kleyn, 2013). Kutlu *et al*. (2019) tested whether a multi-enzyme (Rovabio Advance®) produced by a single fungi would maintain the performance of broilers receiving maize-soya based diets with almost 3% reduced nutrient density. The results of their study showed that a 3.5% improvement in broiler performance of birds supplemented with Rovabio Advance® compared to the negative control group.

Although multiple enzyme complexes are preferred, understanding the actions of these enzymes is being complicated by a number of confounding factors, as summarised below:

- Even if an enzyme cocktail is included, if the nutrient levels in the diet are sufficient or too high above to meet the requirements of the animal, there will be no animal response to addition of the enzyme cocktail (West *et al*., 2007; Kleyn, 2013).
- *In vitro* measurements of enzyme efficacy are hardly precise as they only offer a measure of digestibility and do not take into account what else happens in the animal's body (Kleyn, 2013). For instance, enzymes play an important role in reducing endogenous protein and energy loss, gut health and environmental pollution which are difficult to measure (Choct, 2006; Aok, 2012).
- Enzymes act differently across different ages of animals and tend to be more effective in younger animals due to their poorly developed digestive system. According to Leslie *et al*. (2007), the immature gut lacks the competency to fully digest feedstuffs and subsequently absorb digesta particles because of a lack of brush border enzymes, inadequate maintenance of absorptive mechanisms, and low surface area caused by immature villus length. The pancreatic enzymes required to initiate digestion in the intestinal lumen are also limited in both volume and activity.
- Many of the 'real' effects of enzymes are not measured or reported upon, for example, improvements in gut health, as measured by intestinal lumen thickness or crypt development, does not only benefit the animal from a nutritional point of view but also lead to an improvement in the animal's immunity (Slominski, 2011; Zou *et al*., 2013).
- Measuring small statistical effects (1-2%) is difficult and requires large numbers of replicates of the data (Kleyn, 2013).

2.6 Conclusion

Guanidinoacetic acid (GAA) is the only immediate metabolic precursor for creatine synthesis which in its phosphorylated form provides a source of backup or supplementary energy to fast growing tissues by donating high energy phosphate groups. Previous research has proven the efficacy of GAA to be a suitable substitute for creatine supplementation as it is much cheaper and more stable than creatine itself and able to spare arginine which is the fifth limiting amino acid in maize-soybean meal diets, thus making it available for other functions in the body such as protein accretion. Other studies have shown that GAA is also a suitable feed additive that can be used in all vegetable diets to broilers to replace fish meal and poultry-by products while maintaining growth performance in standard maize-soybean meal diets. Supplementation of broiler diets with GAA is very necessary as about 1.8-2% of the creatine synthesised endogenously is irreversibly lost through urine by conversion once it is converted to creatinine. Although maize-soybean meal diets are considered to be highly digestible, approximately 1.67-1.88 MJ/kg energy lost through faeces due to undigested nutrients encapsulated by nonstarch polysaccharides (NSPs) in typical maize-soybean

meal diets without enzyme supplementation. Nonstarch polysaccharide, defined as low molecular weight compounds, exert their effect by increasing the viscosity of digesta, encapsulation of cell wall nutrients such as proteins, energy and minerals, and also by interacting with gut microflora. This can be can be rectified by supplementation of broiler diets with exogenous NSP degrading enzymes produced by fungi such as *Talaromyces versalitis* which have been proven to improve feed digestion and essentially better nutrient utilisation. The benefits of GAA and NSPase supplementation of broiler diets that contain marginal nutrient s have been well established in terms of improving growth by sparing arginine or supplying an energy source in all vegetable diets and improving nutrient digestion, respectively.. There is limiting data however, on the interaction of GAA and/or NSPases with metabolisable energy concentrations and no previous study has been done investigating the effects of combining the two feed additives. Therefore the purpose of this study was to investigate whether these feed additives would provide such effects when supplemented in reduced energy diets when used individually or in combination in maize-soybean meal diets.

Chapter 3

Materials and Methods

The trial was conducted at the Hillcrest Experimental Farm (University of Pretoria) in an environmentally controlled broiler house, divided into 96 identical concrete floored pens. All animal procedures were reviewed and approved by the Animal Ethics of the University of Pretoria (approval number EC065-17).

3.1 Animals and housing

One thousand nine hundred and twenty (1920) healthy Ross 308 male were selected immediately post hatch at Eagles Pride hatchery, using feather sexing, while discarding any bird showing any visible sign of deformity or weakness. The selected birds were delivered at the Experimental Farm the following morning after being vaccinated for Newcastle Disease and Gumboro at the hatchery. Upon arrival, groups of 20 birds were randomly selected, weighed, allocated to a pen and received numbered neck-tags corresponding to their respective pens. The trial house contained 96 pens in total and 20 birds were placed per pen (1.5 m × 1.5 m), which was in line with the recommendation of 15 birds per square meter stocking density by the South African Poultry Association (SAPA, 2012) code of practice for broiler production. New and clean pine shavings were used to provide floor bedding material.

Each pen had two fountain drinkers during the brooding phase to supplement the nipple drinkers and ensure adequate water intake by the chicks. The fountain drinkers were removed once the birds were more acquainted with the nipple drinkers as their primary water source. The pens were fitted with five nipple drinkers connected to a municipal water line, which was flushed before the birds arrived to ensure fresh water supply. During the first week the height of the water line was adjusted to be in line with the bird's eye, where after the water line was regularly adjusted according to the broiler height so that the birds would slightly tilt their heads while standing to drink water. While providing the birds with easy access to water throughout rearing, it also assisted to minimise water spillage and leaks. The starter feed was weighed a day prior to placement of the birds and allowed to warm up to room temperature. Each pen was supplied with two tube feeders and two extra pan feeders during the brooding phase to allow easy access to feed and sufficient feed intake during this phase. The water and feed in fountain drinkers and pan feeders were replaced twice daily during the brooding phase. The pan feeders were removed along with the fountain drinkers at day seven. The height of the tube feeders was adjusted according to the average height of the birds. Feed was provided *ad libitum* throughout the trial.

The temperature, lighting program and ventilation were set according to the Ross 308 broiler management manual (Aviagen, 2017), all controlled by a Skov system fitted in the house. A brooding temperature of 32°C was initially set then gradually decreased and recorded daily (while monitoring bird behaviour) to reach about 25°C, with electric heaters acting as the main source of heat throughout the entire house. House environment was controlled by a combination of electric heaters, automated electric exhaust and stirring fans and mist sprayers. Minimum ventilation was always maintained to ensure clean air inside the house and to also prevent accumulation of toxic gases such as ammonia. Apart from automatic recordings, temperature readings were manually taken at least four times a day during brooding and twice daily thereafter. The lighting program was set to initially allow for 20 hours of light during the brooding phase and gradually decreased to 16 hours when the birds reached 7 days of age.

A standard vaccination program was followed in which the birds received live vaccines immediately post hatch against Newcastle Disease and Gumboro (Infectious Bronchitis) at the hatchery, by means of spray vaccines. On days 14 and 18 the birds received booster vaccines against Newcastle and Gumboro diseases. The water lines were lifted to deprive the birds of water for approximately two and a half hours (bird behaviour was monitored for level of thirst). The vaccines were mixed into the water according to manufacturer's recommendations and then supplied to the birds in fountain drinkers for approximately an hour to ensure adequate intake of the vaccine by all birds in each pen. Thereafter the drinker lines were lowered again to the appropriate height to supply *ad libitum* water.

3.2 Experimental design and treatments

A randomized complete block design with six dietary treatments was followed. Each treatment was replicated 16 times where one pen, containing 20 birds, was considered an experimental unit (pen replicate).

Trial feed was formulated based on Ross 308 broiler nutrient specifications recommended by Aviagen (2017) to meet or exceed daily nutrient requirements of the chicks. All feed was mixed at the Wisium Feedmill (Johannesburg, South Africa) using a three-ton scale mixer. A three-phase feeding program was followed, whereby the starter feed was fed from day one to day 14 in crumbled form; the grower feed was fed from day 14 to day 26 in pelleted form; and the finisher, also in pelleted form, was fed from day 26 to day 34.

The trial constituted six dietary treatments. Treatment 1 was a standard commercial energy diet and thus formulated to have the highest energy content in the study (2900, 3000 and 3050 kcal/kg or 11.70, 11.97, and 12.02 MJ/kg AME in the starter, grower and finisher phase diets, respectively) and contained none of the test feed additives. The energy level of the other treatments was reduced adjusting the relative quantities

of maize, wheat bran, sunflower oilcake and soya oil in the diets. The energy concentration in Treatments 2 (Negative Control, NC1), 3 (NC1+NSPase) and 4 (NC1+CreAMINO®) was reduced by 65 kcal (0.272 MJ) per kilogram of feed. Finally, the energy values of Treatments 5 (Negative Control, NC2) and 6 (NC2+NSPase+CreAMINO®) were reduced by 130 kcal (0.544 MJ) per kilogram of feed. A summary of the dietary treatments used is displayed in Table 3.1.

A commercial NSP degrading enzyme complex, Rovabio Advance® (Adisseo, France), containing endo-1,4-beta-xylanase (EC 3.2.1.8) and endo-1,3(4)-beta-glucanase (EC 3.2.1.6) as main ingredients, was added to Treatments 3 and 6 at the manufacturer's recommendation of 50 mg/kg to evaluate its ability to compensate for reduced energy through the activity of the enzymes on feed digestibility. For the context of this study, Rovabio Advance® is also referred to as NSPases when describing the experimental diets where the feed additive was used.

CreAMINO® (Alzchem, Germany) which contains about 96% guanidinoacetic acid (Heger *et al*., 2014), was added to diets 4 and 6 to evaluate whether it can compensate for reduced dietary energy. Treatment 6 included a combination of both feed additives to determine if there would be any synergistic interaction between CreAMINO® and Rovabio Advance®. The CreAMINO® was supplied by Alzchem (Germany) added at 0.6 g/kg (or 0. 06% in the diet), in accordance with the manufacturer's guideline. The percentage of GAA in the diet was subsequently calculated by multiplying the analysed value of CreAMINO® in the diet by 0.96. Both additives were supplied in powder form. Upon manufacturing, samples of each of the dietary treatments weighing approximately 500 g were collected and sent to Evonik Animal & Health in Germany to determine the level of energy, crude protein, amino acids, as well as the minerals such as calcium, phosphorus, and sodium in the diets. Only the Positive Control (Treatment 1), Treatment 4 (NC1 + CreAMINO®) and Treatment 6 (NC2 + NSPase + CreAMINO®) were analysed. The raw material compositions, nutrient formulations and analyses of the treatment diets used in the trial are listed in Tables 3.2-3.10. Analyses of feed samples are described in Section 3.3.

Table 3.1 Description of the treatment groups and experimental diets

Treatment[1]	**Rovabio Advance®**	**CreAMINO®**
1. Positive Control	–	–
2. Negative Control (-65 kcal/kg, NC1)	–	–
3. NC1 + NSPase	0.05 g/kg	–
4. NC1) + CreAMINO®	–	0.6 g/kg
5. Negative Control (-130 kcal/kg, NC2)	–	–
6. NC2 + NSPase + CreAMINO®	0.05 g/kg	0.6 g/kg

[1]NC1= Negative Control 1 with 65 kcal/kg (0.272MJ/kg) AME per kg feed less than the Positive Control; NC2 = Negative Control 2 with 130 kcal/kg (0.544MJ/kg) AME per kg feed less than the Positive Control; NSPase = nonstarch polysaccharide degrading enzymes

The diets were fortified with Phyzyme (Danisco Animal Nutrition, Denmark), a commercial phytase enzyme, with an enzyme activity of approximately 10 000 phytase units. One phytase unit is defined as the amount of enzyme required to liberate 1 µmol of inorganic phosphate from phytate, under normal conditions. The diets also contained a coccidiostat (Salinomycin) and Zinc Bacitracin to minimise development of disease and improve growth, as well as vitamin and mineral premixes produced by DSM (Johannesburg, South Africa) to meet the micro nutrient requirements of the broilers.

Table 3.2 Raw material composition (%) of the starter diets (on an as fed basis)

	Treatments					
	1 ***Positive Control***	**2** ***Negative Control (-65 kcal/kg, NC1)***	**3** ***NC1 + NSPase***	**4** ***NC1) + CreAMINO®***	**5** ***Negative Control (-130 kcal/kg, NC2)***	**6** ***NC2 + NSPase + CreAMINO®***
Maize, 7.7% CP	61.48	56.77	56.78	56.83	53.22	53.25
Soya oilcake, 50% CP	30.44	32.00	32.00	32.03	29.91	30.07
Sunflower oilcake (36 % CP)	4.00	4.00	4.00	4.00	4.00	4.00
Wheat bran	-	3.52	3.51	3.36	9.10	8.86
Limestone ($CaCO_3$)	1.11	1.13	1.13	1.12	1.17	1.17
Monocalciumphosphate	1.06	0.97	0.97	0.97	0.88	0.88
Sodium bicarbonate	0.49	0.37	0.37	0.37	0.34	0.34
Biolys® (Lysine)	0.34	0.25	0.25	0.25	0.32	0.32
MetAMINO® (Methionine)	0.27	0.25	0.25	0.25	0.27	0.27
Premix Poultry	0.30	0.30	0.30	0.30	0.30	0.30
Salt (NaCl)	0.19	0.19	0.19	0.19	0.18	0.19
Choline Cloride 60%	0.11	0.10	0.10	0.10	0.11	0.11
ThreAMINO® (Threonine)	0.07	0.04	0.04	0.04	0.06	0.06
Salinomycin 60ppm	0.05	0.05	0.05	0.05	0.05	0.05
Zinc Bacitracin 75ppm	0.05	0.05	0.05	0.05	0.05	0.05
ValAMINO (L-Valine)	0.04	-	-	-	0.03	0.02
Phyzyme XP 10000 TPT 100g/t	0.01	0.01	0.01	0.01	0.01	0.01
Rovabio Advance® 50g	-	-	0.005	-	-	0.005
CreAMINO®	-	-	-	0.06	-	0.06

CP= crude protein, NSPase= nonstarch polysaccharide degrading enzyme, 65 kcal /kg = 0.272MJ/ kg feed, 130 kcal /kg = 0.544MJ/kg feed

Table 3.3 Calculated nutrient concentrations in the starter diets (on an as is basis)

		Treatments					
		1	**2**	**3**	**4**	**5**	**6**
		Positive Control	***Negative Control (-65 kcal/kg, NC1)***	***NC1 + NSPase***	***NC1) + CreAMINO®***	***Negative Control (-130 kcal/kg, NC2)***	***NC2 + NSPase + CreAMINO®***
Dry matter	%	89.20	89.20	89.20	89.21	89.15	89.16
Moisture	%	10.80	10.80	10.80	10.79	10.85	10.84
Crude protein	%	22.57	23.41	23.41	23.53	23.06	23.23
Crude fibre	%	2.73	3.01	3.01	3.00	3.41	3.39
Ether extract	%	3.04	2.99	2.99	2.99	3.00	3.00
Ash	%	6.13	6.16	6.16	6.16	6.17	6.17
NDF	%	11.22	12.21	12.2	12.16	13.84	13.76
ADF	%	3.86	4.25	4.25	4.23	4.73	4.71
ADL	%	0.81	0.91	0.91	0.91	1.07	1.07
Starch	%	40.45	38.08	38.08	38.09	36.86	36.83
Sugars	%	4.94	5.26	5.26	5.26	5.33	5.34
NFE	%	54.74	53.63	53.64	53.53	53.50	53.37
C18:2 in total FA	%	53.49	53.65	53.65	53.61	53.69	53.65
C18:2 %	%	1.42	1.39	1.39	1.39	1.40	1.39
Choline	mg/kg	1 700	1 700	1 700	1 700	1 700	1 700
AMEn Poultry	MJ/kg	12.13	11.86	11.86	11.86	11.59	11.59
AMEn Poultry	kcal/kg	2 900	2 835	2 835	2 835	2 770	2 770
Ca	%	0.95	0.95	0.95	0.95	0.95	0.95
P (Total)	%	0.82	0.83	0.83	0.83	0.84	0.84
av P Poult Coeff	%	28.0	27.0	27.0	27.0	27.0	27.0
av P Poultry	%	0.53	0.52	0.52	0.52	0.50	0.50
Sodium (Na)	%	0.23	0.19	0.19	0.19	0.18	0.18
Chlorine (Cl)	%	0.18	0.18	0.18	0.18	0.18	0.18
Sulphur (S)	%	0.21	0.21	0.21	0.21	0.21	0.21
Magnesium (Mg)	%	0.16	0.18	0.18	0.18	0.19	0.19
Potassium (K)	%	0.83	0.89	0.89	0.89	0.91	0.91
Electrolyte Balance	mEq/kg	260	260	260	260	260	260
av P Poultry Coeff	%	64.0	64.0	64.0	64.0	64.0	64.0
av P Poultry Phytase	%	0.50	0.50	0.50	0.50	0.50	0.50
Lysine	%	1.34	1.35	1.35	1.35	1.35	1.35
Methionine	%	0.62	0.61	0.61	0.61	0.61	0.61
Cysteine	%	0.36	0.37	0.37	0.37	0.37	0.37
Methionine+Cysteine	%	0.98	0.98	0.98	0.98	0.99	0.99
Threonine	%	0.9	0.91	0.91	0.91	0.91	0.91
Tryptophan	%	0.27	0.29	0.29	0.29	0.29	0.29
Arginine	%	1.49	1.57	1.57	1.61	1.54	1.58
Isoleucine	%	0.94	0.98	0.98	0.98	0.95	0.95
Leucine	%	1.85	1.90	1.90	1.90	1.84	1.84
Valine	%	1.08	1.09	1.09	1.09	1.10	1.10
Histidine	%	0.57	0.60	0.60	0.60	0.58	0.59
Phenylalanine	%	1.07	1.12	1.12	1.12	1.08	1.09
Tyrosine	%	0.71	0.74	0.74	0.74	0.72	0.72
Glycine	%	0.89	0.93	0.93	0.93	0.92	0.92
Serine	%	1.02	1.07	1.07	1.07	1.04	1.04
Proline	%	1.22	1.26	1.26	1.26	1.24	1.24
Alanine	%	1.05	1.08	1.08	1.08	1.06	1.06
Asparagine	%	2.12	2.22	2.22	2.22	2.15	2.16
Glutamine	%	3.80	3.97	3.97	3.97	3.90	3.91
EAA	%	11.21	11.52	11.52	11.56	11.34	11.4
NEAA	%	10.11	10.54	10.54	10.54	10.32	10.34
EAA ratio total AA	%	48.00	48.00	48.00	48.00	48.00	48.00
NEAA ratio total AA	%	49.00	49.00	49.00	49.00	49.00	49.00

ADF= acid detergent fibre; ADL= acid detergent lignin; av P= available phosphorus; AMEn= apparent metabolisable energy corrected for nitrogen; EAA= essential amino acids; FA= fatty acids; NDF= neutral detergent fibre; NEAA= non-essential amino acids; NFE= nitrogen free extract; NSPase= nonstarch polysaccharide degrading enzyme. 65 kcal /kg = 0.272MJ/ kg feed, 130 kcal /kg = 0.544MJ/kg feed; All amino acids are formulated on total values.

Table 3.4 Analysed nutrient concentrations in the starter diets (on an as is basis)

		Treatments		
		1	**4**	**6**
		Positive Control	***NC1 + CreAMINO®***	***NC2 + NSPase+ CreAMINO®***
Dry matter	%	88.74	88.92	89.00
Crude protein	%	23.85	23.88	23.16
Crude fibre	%	2.70	3.91	3.61
Ether extract	%	3.20	2.71	2.78
Ash	%	6.70	5.41	5.48
NDF	%	9.50	11.93	12.75
ADF	%	3.50	4.71	4.39
Starch	%	38.50	37.50	39.20
Sugars	%	3.90	4.93	4.67
AME Poultry	MJ/kg	11.70	11.50	11.67
AME Poultry	kcal/kg	2795.63	2747.12	2786.28
Calcium (Ca)	%	-	0.75	0.73
Phosphorus (P)	%	0.65	0.63	0.63
Sodium (Na)	%	-	0.15	0.15
Magnesium (Mg)	%	-	0.20	0.22
Potassium (K)	%	-	0.99	0.96
Lysine	%	1.41	1.34	1.32
Methionine	%	0.55	0.56	0.58
Cysteine	%	0.34	0.35	0.35
Methionine+Cysteine	%	0.89	0.91	0.93
Threonine	%	0.92	0.91	0.88
Arginine	%	1.58	1.62	1.52
Isoleucine	%	1.00	1.01	0.94
Leucine	%	1.97	1.98	1.86
Valine	%	1.12	1.10	1.08
Histidine	%	0.60	0.60	0.56
Phenylalanine	%	1.21	1.22	1.13
Glycine	%	0.94	0.97	0.92
Serine	%	1.13	1.15	1.08
Proline	%	1.39	1.42	1.37
Alanine	%	1.13	1.15	1.09
Asparagine	%	2.36	2.39	2.22
Glutamine	%	4.23	4.30	4.07
CreAMINO® No energy	%	-	592	644
GAA	%	-	568	618

[1]ADF= acid detergent fibre; AME= apparent metabolisable energy; GAA= guanidinoacetic acid; NDF= neutral detergent fibre; 65 kcal /kg = 0.272MJ/ kg feed, 130 kcal /kg = 0.544MJ/kg feed; Analysed values for all amino acids are expressed as total values

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

	Treatments					
	1	**2**	**3**	**4**	**5**	**6**
	Positive Control	***Negative Control (-65 kcal/kg, NC1)***	***NC1 + NSPase***	***NC1) + CreAMINO®***	***Negative Control (-130 kcal/kg, NC2)***	***NC2 + NSPase + CreAMINO®***
Maize ,7.7% CP	65.98	65.90	65.90	65.93	61.87	61.94
Soybean oilcake, 50% CP	23.61	24.75	24.76	24.86	24.17	24.21
Sunflower oilcake (36% CP)	4.00	4.00	4.00	4.00	4.00	4.00
Full fat soya	2.33	-	-	-	-	-
Wheat bran	-	1.80	1.79	1.61	6.53	6.37
Limestone ($CaCO_3$)	0.96	0.98	0.98	0.97	1.01	1.01
Monocalciumphosphate	0.89	0.86	0.86	0.86	0.77	0.77
Sodium bicarbonate	0.56	0.57	0.57	0.57	0.50	0.50
Soybean oil	0.54	-	-	-	-	-
Biolys® (Lysine)	0.25	0.26	0.26	0.26	0.27	0.27
MetAMINO® (Methionie)	0.21	0.21	0.21	0.21	0.21	0.21
Premix Blank Poultry	0.25	0.25	0.25	0.25	0.25	0.25
Salt (NaCl)	0.16	0.15	0.15	0.15	0.15	0.15
Choline Cloride 60%	0.11	0.11	0.11	0.11	0.11	0.11
Salinomycin 60ppm	0.05	0.05	0.05	0.05	0.05	0.05
Zinc Bacitracin 75ppm	0.05	0.05	0.05	0.05	0.05	0.05
ThreAMINO® (Threonine)	0.04	0.04	0.04	0.04	0.05	0.05
Phyzyme XP 10000 TPT 100g/t	0.01	0.01	0.01	0.01	0.01	0.01
Rovabio Advance® 50g	-	-	0.005	-	-	0.005
CreAMINO®	-	-	-	0.06	-	0.06

[1]CP= crude protein, NSPase= nonstarch polysaccharide degrading enzyme; 65 kcal /kg = 0.272MJ/ kg feed, 130 kcal /kg = 0.544MJ/kg feed

Table 3.6 Calculated nutrient concentrations in grower diets (on an as is basis)

		Treatments					
		1	**2**	**3**	**4**	**5**	**6**
		Positive Control	***Negative Control (-65 kcal/kg, NC1)***	***NC1 + NSPase***	***NC1) + CreAMINO®***	***Negative Control (-130 kcal/kg, NC2)***	***NC2 + NSPase + CreAMINO®***
Dry matter	%	89.03	89.00	89.00	89.01	88.97	88.98
Moisture	%	10.97	11.00	11.00	10.99	11.03	11.02
Crude protein	%	20.19	20.21	20.21	20.36	20.35	20.48
Crude fibre	%	2.73	2.81	2.81	2.79	3.16	3.14
Ether extract	%	4.07	3.20	3.19	3.19	3.18	3.18
Ash	%	5.53	5.56	5.56	5.56	5.58	5.58
NDF	%	11.4	11.96	11.96	11.9	13.33	13.27
ADF	%	3.78	3.90	3.90	3.88	4.34	4.33
ADL	%	0.80	0.85	0.85	0.85	0.99	0.98
Starch	%	43.42	43.67	43.67	43.66	41.98	41.99
Sugars	%	4.44	4.51	4.51	4.51	4.69	4.68
NFE	%	56.51	57.23	57.23	57.1	56.69	56.59
Choline	mg/kg	1 600	1 600	1 600	1 600	1 600	1 600
AMEn Poultry	MJ/kg	12.55	12.28	12.28	12.28	12.01	12.01
AMEn Poultry	kcal/kg	3 000	2 935	2 935	2 935	2 870	2 870
Ca	%	0.85	0.85	0.85	0.85	0.85	0.85
P (total)	%	0.76	0.77	0.77	0.77	0.78	0.78
av P Poult Coeff	%	28.0	28.0	28.0	28.0	28.0	28.0
av P Poultry	%	0.49	0.48	0.48	0.48	0.47	0.47
Na	%	0.23	0.23	0.23	0.23	0.21	0.21
Cl	%	0.16	0.16	0.16	0.16	0.16	0.16
S	%	0.19	0.19	0.19	0.19	0.19	0.19
Mg	%	0.15	0.16	0.16	0.16	0.18	0.17
K	%	0.76	0.76	0.76	0.76	0.80	0.79
Electrolyte Balance	mEq/kg	250	250	250	250	250	250
av P Poultry Phytase	%	0.45	0.45	0.45	0.45	0.45	0.45
Lysine	%	1.15	1.15	1.15	1.15	1.15	1.15
Methionine	%	0.53	0.53	0.53	0.53	0.53	0.53
Cysteine	%	0.33	0.33	0.33	0.33	0.34	0.34
Methionine+Cysteine	%	0.86	0.86	0.86	0.86	0.86	0.86
Threonine	%	0.79	0.79	0.79	0.79	0.79	0.79
Tryptophan	%	0.24	0.24	0.24	0.24	0.25	0.25
Arginine	%	1.32	1.32	1.32	1.36	1.33	1.38
Isoleucine	%	0.83	0.83	0.83	0.83	0.82	0.83
Leucine	%	1.69	1.69	1.69	1.69	1.68	1.68
Valine	%	0.94	0.94	0.94	0.94	0.95	0.95
Histidine	%	0.52	0.52	0.52	0.52	0.52	0.52
Phenylalanine	%	0.95	0.95	0.95	0.95	0.95	0.95
Tyrosine	%	0.64	0.64	0.64	0.64	0.64	0.64
Glycine	%	0.80	0.80	0.80	0.80	0.81	0.81
Serine	%	0.92	0.92	0.92	0.92	0.92	0.92
Proline	%	1.13	1.13	1.13	1.14	1.14	1.14
Alanine	%	0.98	0.98	0.98	0.98	0.97	0.97
Asparagine	%	1.87	1.86	1.86	1.86	1.85	1.85
Glutamine	%	3.43	3.43	3.43	3.43	3.46	3.46
EAA	%	9.93	9.92	9.92	9.97	9.94	9.99
NEAA	%	9.13	9.11	9.11	9.12	9.16	9.16
EAA ratio total AA	%	48.00	48.00	48.00	48.00	48.00	48.00
NEAA ratio total AA	%	49.00	49.00	49.00	49.00	49.00	49.00

ADF= acid detergent fibre; ADL= acid detergent lignin; av P= available phosphorus; AMEn= apparent metabolisable energy corrected for nitrogen; EAA= essential amino acids; NDF= neutral detergent fibre; NEAA=

non-essential amino acids; NFE= nitrogen free extract; NSPase= nonstarch polysaccharide degrading enzyme; all amino acids are formulated on total values; 65 kcal /kg = 0.272MJ/ kg feed, 130 kcal /kg = 0.544MJ/kg feed

Table 3.7 Analysed nutrient concentrations in the grower diets (on an as is basis)

		Treatments		
		1	**4**	**6**
		Positive Control	**NC1 + CreAMINO®**	**NC2 + NSPase + CreAMINO®**
Dry matter	%	88.35	88.4	88.82
Crude protein	%	19.63	20.19	20.56
Crude fibre	%	2.80	3.69	3.64
Ether extract	%	4.60	3.44	2.91
Ash	%	5.80	4.89	4.71
NDF	%	10.10	11.76	12.92
ADF	%	3.90	4.07	4.45
Starch	%	42.00	43.60	41.20
Sugars	%	3.10	3.96	3.98
AME Poultry	MJ/kg	12.02	12.08	11.56
AME Poultry	kcal/kg	2869.93	2884.78	2759.98
Calcium (Ca)	%	-	0.57	0.63
Phosphorus (P)	%	0.58	0.49	0.52
Sodium (Na)	%	-	0.19	0.19
Magnesium (Mg)	%	-	0.17	0.19
Potassium (K)	%	-	0.82	0.86
Lysine	%	1.13	1.14	1.15
Methionine	%	0.53	0.49	0.51
Cysteine	%	0.31	0.31	0.32
Methionine+Cysteine	%	0.83	0.80	0.82
Threonine	%	0.77	0.77	0.79
Arginine	%	1.30	1.32	1.35
Isoleucine	%	0.82	0.83	0.84
Leucine	%	1.67	1.71	1.72
Valine	%	0.92	0.93	0.94
Histidine	%	0.49	0.50	0.51
Phenylalanine	%	0.99	1.02	1.02
Glycine	%	0.80	0.81	0.83
Serine	%	0.94	0.96	0.97
Proline	%	1.21	1.24	1.24
Alanine	%	0.98	1.00	1.02
Asparagine	%	1.91	1.94	1.95
Glutamine	%	3.51	3.59	3.66
CreAMINO® No energy	%	-	632	541
GAA	%	-	607	519

[1]ADF= acid detergent fibre; AME= apparent metabolisable energy; GAA= guanidinoacetic acid; NDF= neutral detergent fibre; Analysed values for all amino acids are expressed as total values; 65 kcal /kg = 0.272MJ/ kg feed, 130 kcal /kg = 0.544MJ/kg feed

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

	Treatments					
	1	**2**	**3**	**4**	**5**	**6**
	Positive Control	**-65 kcal/kg**	**-65 kcal/kg + NSPase**	**-65 kcal/kg + CreAMINO®**	**-130 kcal/kg**	**-130 kcal/kg + NSPase + CreAMINO®**
Maize ,7.7% CP	68.11	69.34	69.34	69.34	67.28	67.28
Soya oilcake, 50% CP	23.88	23.54	23.55	23.65	20.38	20.50
Sunflower oilcake (36 % CP)	4.00	4.25	4.23	4.07	9.50	9.32
Soya oil	1.13	-	-	-	-	-
Limestone ($CaCO_3$)	0.89	0.90	0.90	0.90	0.91	0.91
Monocalciumphosphate	0.78	0.77	0.77	0.77	0.68	0.69
Salt (NaCl)	0.28	0.28	0.28	0.28	0.27	0.27
Sodium bicarbonate	0.21	0.21	0.21	0.21	0.22	0.22
Premix Blank Poultry	0.20	0.20	0.20	0.20	0.20	0.20
MetAMINO® (Methionine)	0.16	0.16	0.16	0.16	0.14	0.14
Biolys® (Lysine)	0.15	0.16	0.16	0.15	0.22	0.22
Choline Cloride 60%	0.09	0.09	0.09	0.09	0.08	0.08
Salinomycin 60ppm	0.05	0.05	0.05	0.05	0.05	0.05
Zinc Bacitracin 75ppm	0.05	0.05	0.05	0.05	0.05	0.05
Phyzyme XP 10000 TPT 100g/t	0.01	0.01	0.01	0.01	0.01	0.01
Rovabio Advance® 50g	-	-	0.005	-	-	0.005
CreAMINO®	-	-	-	0.06	-	0.06

CP= crude protein; NSPase= nonstarch polysaccharide degrading enzyme, 65 kcal /kg = 0.272MJ/ kg feed, 130 kcal /kg = 0.544MJ/kg feed

Table 3.9 Calculated nutrient concentrations in finisher diets (on an as is basis)

		Treatments					
		1	**2**	**3**	**4**	**5**	**6**
		Positive Control	***Negative Control (-65kcal/kg, NC1)***	***NC1 + NSPase***	***NC1 + CreAMINO®***	***Negative Control (-130 kcal/kg, NC2)***	***NC2 + NSPase + CreAMINO®***
Dry matter	%	89.04	88.9	88.9	88.91	88.81	88.82
Moisture	%	10.96	11.1	11.1	11.09	11.19	11.18
Crude protein	%	19.51	19.53	19.53	19.65	19.72	19.84
Crude fibre	%	2.65	2.71	2.71	2.68	3.55	3.52
Ether extract	%	4.34	3.26	3.26	3.26	3.21	3.21
Ash	%	5.00	5.00	5.00	5.00	5.07	5.07
NDF	%	11.42	11.62	11.61	11.57	12.78	12.73
ADF	%	3.69	3.76	3.76	3.73	4.69	4.65
ADL	%	0.79	0.82	0.81	0.80	1.15	1.14
Starch	%	44.76	45.56	45.56	45.56	44.19	44.19
Sugars	%	4.33	4.34	4.34	4.34	4.30	4.30
NFE	%	57.54	58.4	58.4	58.31	57.27	57.19
Choline	mg/kg	1 500	1 500	1 500	1 500	1 500	1 500
AMEn Poultry	MJ/kg	12.76	12.49	12.49	12.49	12.22	12.22
AMEn Poultry	kcal/kg	3050	2985	2985	2985	2920	2920
Ca	%	0.80	0.80	0.80	0.80	0.80	0.80
P (total)	%	0.73	0.73	0.73	0.73	0.74	0.74
av P Poult Coeff	%	28.0	28.0	28.0	28.0	28.0	28.0
av P Poultry	%	0.46	0.46	0.46	0.46	0.45	0.45
Sodium(Na)	%	0.18	0.18	0.18	0.18	0.18	0.18
Chloride(Cl)	%	0.23	0.23	0.23	0.23	0.23	0.23
Sulphur(S)	%	0.18	0.18	0.18	0.18	0.19	0.19
Magnesium(Mg)	%	0.15	0.15	0.15	0.15	0.17	0.17
Potassium(K)	%	0.73	0.73	0.73	0.73	0.75	0.75
Electrolyte Balance	mEq/kg	201	201	201	201	206	205
av P Poultry Coeff Ph	%	63.0	64.0	64.0	64.0	64.0	64.0
av P Poultry Phytase	%	0.42	0.42	0.42	0.42	0.42	0.42
Lysine	%	1.05	1.05	1.05	1.05	1.05	1.05
Methionine	%	0.47	0.47	0.47	0.47	0.47	0.47
Cysteine	%	0.32	0.33	0.33	0.33	0.33	0.33
Methionine+Cysteine	%	0.80	0.80	0.80	0.80	0.80	0.80
Threonine	%	0.73	0.73	0.73	0.73	0.73	0.73
Tryptophan	%	0.23	0.23	0.23	0.23	0.23	0.23
Arginine	%	1.27	1.27	1.27	1.32	1.3	1.35
Isoleucine	%	0.80	0.80	0.80	0.80	0.80	0.80
Leucine	%	1.66	1.66	1.66	1.66	1.64	1.64
Valine	%	0.92	0.92	0.92	0.92	0.93	0.93
Histidine	%	0.50	0.50	0.50	0.50	0.50	0.50
Phenylalanine	%	0.93	0.93	0.93	0.93	0.92	0.92
Tyrosine	%	0.62	0.62	0.62	0.62	0.61	0.61
Glycine	%	0.77	0.78	0.78	0.78	0.82	0.82
Serine	%	0.89	0.89	0.89	0.89	0.89	0.89
Proline	%	1.11	1.12	1.12	1.12	1.11	1.11
Alanine	%	0.96	0.96	0.96	0.96	0.97	0.96
Asparagine	%	1.80	1.80	1.80	1.80	1.78	1.78
Glutamine	%	3.33	3.34	3.34	3.34	3.40	3.40
EAA	%	9.51	9.51	9.51	9.56	9.52	9.57
NEAA	%	8.87	8.88	8.88	8.88	8.96	8.96
EAA ratio total AA	%	47.0	48.0	48.0	48.0	48.0	48.0
NEAA ratio total AA	%	49.0	49.0	49.0	49.0	50.0	49.0

ADF= acid detergent fibre; ADL= acid detergent lignin; av P= available phosphorus; AMEn= apparent metabolisable energy corrected for nitrogen; EAA= essential amino acids; NDF= neutral detergent fibre; NEAA=

non-essential amino acids; NFE= nitrogen free extract; NSPase= nonstarch polysaccharide degrading enzyme; All amino acids are formulated on total values; 65 kcal /kg = 0.272MJ/ kg feed, 130 kcal /kg = 0.544MJ/kg feed

Table 3.10 Analysed nutrient concentrations on the finisher diets (on an as is basis)

		Treatments		
		1	**4**	**6**
		Positive Control	***NC1+ CreAMINO®***	***NC2 + NSPase + CreAMINO®***
Dry matter	%	88.18	87.92	87.77
Crude protein	%	17.77	17.69	17.38
Crude fibre	%	3.30	4.05	4.28
Ether extract	%	4.70	3.04	3.61
Ash	%	5.40	4.42	4.62
NDF	%	11.20	12.13	12.76
ADF	%	4.60	5.00	5.16
Starch	%	43.20	44.80	45.70
Sugars	%	2.90	4.13	3.20
AME Poultry	MJ/kg	11.94	11.77	11.96
AME Poultry	kcal/kg	2851.15	2812.25	2855.90
Calcium (Ca)	%	-	0.64	0.59
Phosphorus (P)	%	0.52	0.46	0.43
Sodium (Na)	%	-	0.18	0.17
Magnesium (Mg)	%	-	0.17	0.18
Potassium (K)	%	-	0.72	0.72
Lysine	%	1.00	0.94	0.90
Methionine	%	0.41	0.44	0.43
Cysteine	%	0.27	0.27	0.28
Methionine+Cysteine	%	0.69	0.71	0.70
Threonine	%	0.68	0.65	0.63
Arginine	%	1.21	1.17	1.15
Isoleucine	%	0.76	0.73	0.70
Leucine	%	1.62	1.58	1.52
Valine	%	0.86	0.84	0.82
Histidine	%	0.46	0.46	0.44
Phenylalanine	%	0.93	0.91	0.88
Glycine	%	0.75	0.73	0.73
Serine	%	0.90	0.86	0.82
Proline	%	1.14	1.12	1.10
Alanine	%	0.95	0.93	0.90
Asparagine	%	1.77	1.68	1.60
Glutamine	%	3.31	3.20	3.14
CreAMINO®	%	-	760	726
GAA	%	-	730	697

ADF= acid detergent fibre; AME= apparent metabolisable energy; GAA= guanidinoacetic acid; NDF= neutral detergent fibre; Analysed values for all amino acids are expressed as total values; 65 kcal /kg = 0.272MJ/ kg feed, 130 kcal /kg = 0.544MJ/kg feed

3.3 Analysis of experimental diets

The proximate analyses of the experimental diets was performed by the Evonik Health and Nutrition Laboratory (Hanau, Germany) to quantitatively determine the macronutrients in feed that had been ground using a 1 mm sieve. This method of analysis partitions the feed components into six categories which are moisture (or dry matter, DM), ash, crude protein (CP), ether extract (EE), crude fibre (CF) and nitrogen-free extract (NFE).

For DM analysis, the ISO 6496 (1999) and VDLUFA Methodenbuch Bd. III, 2.1/3.1 official methods of analysis were followed wherein the ground feed samples was weighed first and then dried at 103 ˚C for 4 hours. The weight loss (dry weight) of the sample was then determined and the moisture content calculated as the difference between the wet and dry weights. After this, the samples were then heated to 550 ˚C for approximately 4 hours to remove all the carbon (i.e. all the organic matter) from the samples. The weight loss of the feed samples was then calculated after the 4 hours from the dry matter to crude ash in order to determine the organic matter fraction following the AOAC 942.05 (2000) as well as ISO 5984 (2002) methods for crude ash determination.

Crude protein content of the feed samples was calculated from nitrogen readings measured from combustion of the samples using Dumas method, also following AOAC 990-03 (2000) guidelines. The resulting Nitrogen fraction was then multiplied by 6.25 (protein conversion factor) to give an approximate protein content of each sample.

The fat or ether extract content of the samples was continuously extracted with petroleum ether solvent using to the Soxhlet instrument. The remaining residue after evaporation of the solvent is the crude fat fraction following the ISO 6492 (1999) and AOAC 920.39 (2000) methods.

The ISO 6865 (2000) and AOAC 973.18 (2010) methods of fibre analysis were followed for determination of crude fibre (CF) which include dissolving the samples in defined concentrations of an alkali and acid detergent. The insoluble remnant was defined as the CF fraction. The nitrogen-free extract was calculated

by subtracting CP, EE, and CF fractions from the total dry matter. Starch was determined by a polymetric measurement according to Ewers following VDLUFA methodology book (III, 7.2).

The feed samples were analysed for mineral content (calcium, sodium, phosphorus, potassium and magnesium) using Advances ICP-OES method of analysis based on VDLUFA Methodenbuch Bd. III, 10. Finally, the feed samples were analysed for total and free amino acid contents using AMINOLab technology from Evonik Industries (Hanau, Germany) according to the AOAC Official Methods 994.12 (1995) and 999.13 (2000) of amino acid determination in animal feed.

The metabolisable energy content, derived from WPSA (1989), of each diet was then calculated from the resulting proximate analysis values as:

AME [MJ/Kg DM] = ((15.51 × CP [% DM]) + (34.31 × EE [% DM]) + (16.69 × STARCH [% DM]) + (13.01 × 0.95 × SUGAR [% DM])) / 100
*Where the sugar content was determined according to VDLUFA Methodenbuch Bd. III, 7.1

An ion chromatograph with gradient pump and a variable wavelength detector was used to analyse CreAMINO® content of ground and homogenised pelleted feed samples. The homogenised pellet samples were extracted with water sonification and an aliquot was then filtrated through a 0.45 µm filter membrane. The solution was analysed immediately thereafter. To quantify the subsequent percentage of GAA in the feed sample, the analysed value of CreAMINO® was multiplied by 0.96 (this was done on the basis that CreAMINO® contains 96% GAA).

3.4 Measurement of performance parameters

The initial feed weight was recorded one day prior to placing the birds and the body weights of the birds per pen were measured on the day of placement. Feed intake, body weight (BW) and feed conversion ratio (FCR) were measured weekly. Mortalities and cullings were monitored and recorded daily and used to correct feed conversion ratios for mortalities during the period. Feed intake (FI) was calculated weekly as the weight of the feed offered minus the weight of residual feed in the feeder and bin. The cumulative FI was the sum of feed the birds ate over a certain period of time. The FCR was calculated as feed intake divided by weight gain. This was measured weekly and also over a cumulative period. Mortalities were collected twice daily, weighed and an autopsy done to determine the probable cause of death. Dead birds were subsequently stored in a freezer and disposed of accordingly at the end of the trial.

3.5 Measurement of carcass portion yield and tibia strength

On day 34, individual body weights per pen were measured and birds that weighed closest to the pen average were selected for slaughter on day 35. On day 35, two birds per pen were slaughtered at the abattoir located at the Hillcrest Experimental Farm, University of Pretoria. The birds were electrically stunned before decapitation to minimise pain. They were then hoisted upside down to drain the blood and scaled in a scalder that contained hot water (70 ˚C) for approximately 30 seconds to loosen the feathers. The carcasses were then plucked inside a de-feathering machine. Care was taken not to leave the birds too long in the de-feathering machine for longer than 30 seconds in order to avoid damage of the carcass. Afterwards, the birds were eviscerated and the empty carcass as well as the abdominal fat pad weighed. Carcasses were portioned into thighs, drumsticks, wings, and breast meat and weighed. The breast meat was weighed before and after deboning so as to determine breast meat yield. The dressing percentage as a measure of the amount of meat or product produced was also determined and calculated as:

$$\text{Dressing \%} = \frac{Empty\ Carcass\ Weight}{Live\ BW} \times 100$$

For determination of bone strength, two tibia (left and right) samples were collected per pen from the birds that were used to measure carcass parameters, de-fleshed and kept inside zip-lock bags. The bones were stored in the freezer overnight at a temperature of about -20°C and defrosted for at least 12 hours before breaking them on the 5 kN Lloyd Tensile Strength at the Civil Engineering laboratory located at the University of Pretoria main campus in Hatfield. The machine was set to move at a speed of 2 mm/minute to apply pressure on the bone, with a distance of 20-90 cm between each bone. The point at which the bone broke was taken as the maximum strength required breaking the bone in two.

3.6 Statistical analysis

All data were analyzed as a two-way ANOVA using the General Linear Model procedure of SAS (SAS Institute, 2004) over repeated measures of variance analysis. The data were analyzed using pen means with procedures appropriate for a randomized complete block design. The data were then presented as mean values with pooled standard error of means (SEM) estimates, and a significance level of $\alpha = 0.05$ was set. Differences among treatment means were evaluated using the least significance procedure (Carmer and Walker, 1985) and overall, treatment effects with a probability of $P < 0.05$ were assumed to be statistically significant. Dietary supplemental CreAMINO® and Rovabio Advance® concentrations were independent variables in this model, whereas the weekly (or cumulative) FCR, FI and BWG, weekly BW, bone strength as well as day 34 data were regarded as dependent variables.

Chapter 4

Results

4.1 Performance parameters

4.1.1 Body weight

The broilers in the Positive Control group performed consistently better than those from the Negative Control (-65 kcal/kg, NC1) group from 14 days until the end of the trial. Supplementation of NSPase to the NC1 diet did not improve the growth of the birds at any period. When CreAMINO® was added to the NC1 diet, however, broiler growth did improve. Although BWs for the CreAMINO® group were not significantly (P>0.05) better than the NC1 group, broiler growth was still not significantly lower than the Positive Control either.

The second Negative Control (-130 kcal/kg, NC2) group performed significantly (P>0.05) worse than the broilers that received both the Positive Control and NC1 diets throughout the trial, with the exception of day 28 where NC1 and NC2 did not differ significantly (P>0.05). When NSPases and CreAMINO® were added to the NC2 diet, broiler performance was not significantly (P>0.05) improved. Only on day 14, the broilers that received the feed additives in the NC2 diet achieved slightly higher BW compared to those without the feed additives.

Table 4.1 Average weekly body weight (g) of broilers that received diets containing various energy levels and feed additives[1]

Treatment	Days of age					
	0	**7**	**14**	**21**	**28**	**34**
1. Positive Control	39.5	183[a]	480[a]	1005[a]	1683[a]	2344[a]
2. Negative Control (-65 kcal/kg, NC1)	39.2	179[ab]	465[bc]	970[b]	1625[bc]	2291[b]
3. NC1+ NSPase	39.5	178[bc]	467[bc]	976[ab]	1636[bc]	2273[bc]
4. NC1 + CreAMINO®	39.2	179[ab]	479[ab]	988[ab]	1657[ac]	2298[ab]
5. Negative Control (-130 kcal/kg, NC2)	39.1	170[d]	444[d]	934[c]	1602[be]	2225[cd]
6. NC2 + NSPase + CreAMINO®	39.3	174[cd]	458[c]	935[c]	1586[de]	2216[d]
± Standard Error of the mean (SEM)	0.178	1.65	4.90	11.05	14.24	18.99
R Square	0.431	0.546	0.448	0.443	0.396	0.457

[a-e] Means within a column with common superscripts do not differ significantly (P>0.05)
[1]NC1= Negative Control 1 containing 65 kcal/kg (0.272 MJ/kg) less AME than the Positive Control, NC2 = Negative Control 2 containing 130 kcal/kg (0.544 MJ/kg) less AME than the Positive Control, NSPase = nonstarch polysaccharide degrading enzyme (Rovabio Advance®)

4.1.2 Feed intake

Feed intake did not differ between the NC1 diet with 65 kcal (0.272 MJ) less metabolisable energy per kilogram of feed and the Positive Control diet (P>0.05). The supplementation of the feed additives to the NC1 diet did not affect feed intake either. Birds that received the Negative Control diet with 130 kcal (0.544 MJ) lower metabolisable energy per kilogram of feed, consumed significantly more feed during the first week of production, less feed during the second week but essentially similar amounts of feed thereafter compared to the other treatment groups. The addition of feed additives to the NC2 diet did not affect feed intake of the broilers at any time (Table 4.2).

Table 4.2 Average weekly feed intake (g/bird) of broilers receiving diets containing various metabolisable energy levels and feed additives

Treatment	Days of age				
	0-7	**7-14**	**14-21**	**21-28**	**28-34**
1. Positive Control	121[c]	358[a]	723[a]	1014	1240[a]
2. Negative Control (-65 kcal/kg, NC1)	123[bc]	348[ab]	721[a]	1020	1281[ab]
3. NC1+ NSPase	126[abc]	349[ab]	717[a]	1015	1253[b]
4. NC1 + CreAMINO®	123[bc]	355[ab]	722[a]	1030	1288[ab]
5. Negative Control (-130 kcal/kg, NC2)	130[a]	346[b]	697[ab]	1014	1256[ab]
6. NC2 + NSPase + CreAMINO®	129[ab]	345[b]	689[b]	1009	1244[ab]
± Standard Error of the mean (SEM)	2.45	3.69	9.76	8.98	16.5
R Square	0.316	0.283	0.289	0.270	0.390

[a-c] Means within a column with common superscripts do not differ significantly (P>0.05)
[1]NC1= Negative Control 1 containing 65 kcal/kg (0.272 MJ/kg) less AME than the Positive Control, NC2 = Negative Control 2 containing 130 kcal/kg (0.544 MJ/kg) less AME than the Positive Control, NSPase = nonstarch polysaccharide degrading enzyme (Rovabio Advance®)

Cumulative feed intake (Table 4.3) did not differ between the Positive Control and the NC1 diet, containing 65 kcal (0.272 MJ) less metabolisable energy per kilogram of feed. The supplementation of feed additives to NC1 did not affect cumulative feed intake either. Lowering the AME content of the diet with 130 (0.544 MJ) kcal per kilogram (NC2) increased the amount of feed consumed by the broilers. However, when the feed additives were added to NC2 there was a significant, drop in cumulative feed intake during the third and fourth weeks of the study, compared to the Positive Control.

Table 4.3 Average cumulative feed intake (g/bird) of broilers over determined periods of days

Treatment	Cumulative feed intake				
	0-7	**0-14**	**0-21**	**0-28**	**0-34**
1. Positive Control	121^{c}	479	1211^{a}	2242^{a}	3514^{ab}
2. Negative Control (-65 kcal/kg, NC1)	123^{bc}	471	1194^{ab}	2229^{ab}	3588^{ac}
3. NC1+ NSPase	126^{abc}	476	1200^{a}	2218^{ab}	3495^{bc}
4. NC1 + CreAMINO®	123^{bc}	478	1206^{a}	2247^{a}	3604^{a}
5. Negative Control (-130 kcal/kg, NC2)	130^{a}	477	1184^{ab}	2206^{ab}	3477^{c}
6. NC2 + NSPase + CreAMINO®	129^{ab}	475	1164^{b}	2185^{b}	3465^{c}
± Standard Error of the mean (SEM)	2.45	4.46	12.3	19.5	36.1
Root Square	0.316	0.241	0.254	0.212	0.349

[a-c] Means within a column with common superscripts do not differ significantly (P>0.05)
[1]NC1= Negative Control 1 containing 65 kcal/kg (0.272 MJ/kg) less AME than the Positive Control, NC2 = Negative Control 2 containing 130 kcal/kg (0.544 MJ/kg) less AME than the Positive Control, NSPase = nonstarch polysaccharide degrading enzyme (Rovabio Advance®)

4.1.3 Feed conversion ratio

Weekly feed conversion ratios of the broilers are shown in Table 4.4. The broilers fed the NC1 diet with 65 kcal (0.272 MJ) less metabolisable energy per kilogram of feed than the Positive Control had significantly worse (higher) FCR from the third week of production onwards, and neither NSPases nor CreAMINO® supplementation significantly improved the FCR. A further reduction of AME to 130 kcal (0.544 MJ) per kilogram of feed (NC2) lower than the Positive Control, did not worsen the feed efficiency any more than that noted for the NC1 group, except for during the second week. Addition of the feed additives to the NC2 diet improved FCR only during the second week of production.

Table 4.4 Weekly feed conversion ratios (g/g/bird) of broilers receiving diets containing various metabolisable energy levels and feed additives

Treatment	Days of age				
	0-7	**7-14**	**14-21**	**21-28**	**28-34**
1. Positive Control	0.844^{c}	1.19^{bc}	1.38^{c}	1.51^{b}	1.86^{b}
2. Negative Control (-65 kcal/kg, NC1)	0.881^{bc}	1.22^{b}	1.43^{ab}	1.54^{ab}	2.02^{a}
3. NC1+ NSPase	0.908^{b}	1.21^{b}	1.41^{bc}	1.54^{ab}	1.97^{a}
4. NC1 + CreAMINO®	0.880^{bc}	1.18^{c}	1.42^{ab}	1.54^{ab}	1.96^{a}
5. Negative Control (-130 kcal/kg, NC2)	0.993^{a}	1.26^{a}	1.43^{ab}	1.52^{b}	2.03^{a}
6. NC2 + NSPase + CreAMINO®	0.958^{a}	1.22^{b}	1.45^{a}	1.55^{a}	1.99^{a}
± Standard Error of the mean (SEM)	0.0176	0.0101	0.0103	0.0104	0.0324
R Square	0.472	0.507	0.403	0.202	0.258

[a-c]Means within a column with common superscripts do not differ significantly (P>0.05)
[1]NC1= Negative Control 1 containing 65 kcal/kg (0.272 MJ/kg) less AME than the Positive Control, NC2 = Negative Control 2 containing 130 kcal/kg (0.544 MJ/kg) less AME than the Positive Control, NSPase = nonstarch

polysaccharide degrading enzyme (Rovabio Advance®). Feed conversion ratio was calculated as the weight of feed consumed per kilogram weight gain corrected for the number of birds.

Reducing the AME content of the Positive Control diet with 65 kcal/kg (0.272 MJ) per kilogram of feed (NC1) had a negative effect on the FCR for the entire rearing period of 34 days (Table 4.5). The supplementation of CreAMINO® to the NC1 diet slightly improved FCR during the second and third weeks of production only. Whereas NSPase supplementation showed a positive effect during the third week of production. A further reduction of AME (-130kcal/kg; NC2) had an even worse effect on FCR over the entire period (0-34 days). Neither of the feed additives could significantly improve the FCR.

Table 4.5 The cumulative feed conversion ratios (g/g/bird) of broilers receiving diets containing various metabolisable energy levels and feed additives

	Days of age				
Treatment	**0-7**	**0-14**	**0-21**	**0-28**	**0-34**
1. Positive Control	0.844^{c}	1.08^{d}	1.28^{b}	1.35^{c}	1.50^{d}
2. Negative Control (-65 kcal/kg, NC1)	0.881^{bc}	1.11^{cd}	1.28^{b}	1.39^{ab}	1.55^{bc}
3. NC1+ NSPase	0.908^{b}	1.11^{cd}	1.27^{b}	1.38^{b}	1.55^{bc}
4. NC1 + CreAMINO®	0.880^{bc}	1.09^{d}	1.27^{b}	1.38^{b}	1.54^{c}
5. Negative Control (-130 kcal/kg, NC2)	0.993^{a}	1.17^{a}	1.32^{a}	1.40^{a}	1.58^{a}
6. NC2 + NSPase + CreAMINO®	0.958^{a}	1.13^{b}	1.30^{a}	1.40^{a}	1.57^{ab}
± Standard Error of the mean (SEM)	0.0176	0.00921	0.00660	0.00540	0.00848
R Square	0.472	0.555	0.533	0.491	0.485

$^{a-d}$ Means within a column without a common superscript significantly differ (P>0.05)

[1]NC1= negative control one with minus 65 kcal/kg (0.272 MJ/kg) energy, NC2 = negative control two at minus 130 kcal/kg (0.544 MJ/kg) energy, NSPase = nonstarch polysaccharide degrading enzyme. Feed conversion ratio was calculated as the weight of feed consumed per kilogram weight gain corrected for the number of birds

4.1.4 Mortality

The total mortality rate of the broilers in this study was 4.3%, however, this was evenly distributed throughout the house and between the treatments. There were no observed significant differences between the treatments with regard to mortalities.

4.2 Tibia bone strength

The effects of CreAMINO® and Rovabio Advance® (NSP degrading enzymes) on tibia bone strength of broilers fed treatment diets from day one to slaughter age are summarised in Table 4.6. From the data presented, the reduction in feed energy had reduced tibia breaking strength. Supplementation of the NC1 diet with NSPases restored the tibia breaking strength.

Table 4.6 The effect of CreAMINO® and nonstarch polysaccharide enzymes on tibia bone strength of broilers receiving diets containing various metabolisable energy

Treatment	Maximum Load (kN)
1. Positive Control	0.254[a]
2. Negative Control (-65 kcal/kg, NC1)	0.207[b]
3. NC1+ NSPase	0.237[ab]
4. NC1 + CreAMINO®	0.208[b]
5. Negative Control (-130 kcal/kg, NC2)	0.218[b]
6. NC2 + NSPase + CreAMINO®	0.212[b]
± Standard Error	0.0127
Root Square	0.275

[ab]Means within a column without a common superscript significantly differ (P>0.05)
NC1= negative control one with minus 65 kcal/kg (0.272 MJ/kg) energy, NC2 = negative control two at minus 130 kcal/kg (0.544 MJ/kg) energy NSPase = nonstarch polysaccharide degrading enzyme, kN= amount of force applied to break each bone measured in kilo-Newton.

4.3 Carcass and portion yield

The effects of CreAMINO® and Rovabio Advance® (NSP enzymes) on carcass yield and portions are shown on Table 4.7. When AME of the Positive Control group was dropped with 65 kcal (0.272 MJ) per kilogram of feed (NC1), no significant effect was noted for any of the carcass parameters. The addition of the NSPases to the NC1 diet improved the dressing percentage compared to the Positive Control group, however, the magnitude of this effect was not statistically significant (P>0.05). The addition of the feed additives had no significant effect on the carcass parameters either. The addition of the feed additives had no significant effect (P>0.05) on the carcass parameters overall.

The abdominal fat on the carcasses of birds that received the NC2 (-130 kcal/kg feed) diet was significantly less than that of the NC1 group, while adding the feed additives increased the abdominal fat produced significantly (P<0.05) compared to the group that did not received supplementation. No other treatment effects were noted for the NC2 groups.

Table 4.7 The effect of feed additives in diets containing reduced metabolisable energy on carcass parameters expressed as a percentage of live body weight

Treatment	Live body weight (kg)	Dressing Percentage (%)	Abdominal fat (%)	Breast meat (%)	Deboned breast (%)	Thighs (%)	Drumsticks (%)	Wings (%)
1. Positive Control	2.35^{a}	76.2^{b}	1.35^{ab}	25.58	21.86	12.45	9.71	7.43
2. Negative Control (-65 kcal/kg, NC1)	2.29^{ab}	77.1^{ab}	1.51^{a}	26.55	22.67	12.41	9.66	7.46
3. NC1+ NSPase	2.28^{bc}	77.3^{a}	1.34^{ab}	25.76	22.07	12.41	9.84	7.48
4. NC1 + CreAMINO®	2.30^{ab}	76.9^{ab}	1.38^{a}	26.36	22.77	12.06	9.72	7.53
5. Negative Control (-130 kcal/kg, NC2)	2.23^{cd}	76.9^{ab}	1.18^{b}	26.45	22.75	12.40	9.68	7.36
6. NC2 + NSPase + CreAMINO®	2.21^{d}	76.7^{ab}	1.38^{a}	26.55	22.79	12.66	9.77	7.60
±Standard Error	0.0201	0.399	0.0641	0.376	0.346	0.236	0.138	0.0829
Root Square	0.469	0.429	0.193	0.206	0.252	0.253	0.259	0.489

NC1= negative control one with minus 65 kcal/kg (0.272 MJ/kg) energy, NC2 = negative control two at minus 130 kcal/kg (0.544 MJ/kg) energy, NSPase = nonstarch polysaccharide degrading enzyme.
$^{a-c}$ Means within a column without a common superscript significantly differ ($P<0.05$).

Chapter 5

Discussion

Energy is the most expensive nutrient in the diet, therefore any savings in this area would be of great benefit in poultry production systems. Feed additives have been used by nutritionists to not only improve the performance and health of animals, but to effectively reduce feed costs and environmental pollution by lowering excretion of undigested nutrients (such as nitrogen and phosphorus). Rovabio Advance® (a multiple enzyme cocktail comprised of xylanases, ß-glucanases, pectinases, cellulases, proteases and arabinofuranosidases) and CreAMINO® alone or in combination, were evaluated in this study for their potential to lower feed costs and total costs of broiler production while improving broiler performance. It was hypothesised that CreAMINO® may provide secondary energy on the metabolic level, whereas Rovabio Advance® would provide energy compensation by improving digestibility of feed ingredients, thus liberating excess nutrients entrapped in the plant cell wall structure.

Nutritionists are faced with the task to ensure that when feed additives such as enzymes are used, the nature and scale of response is well understood and accounted for in the design of the diets offered to animals, so as to give advantage to the cost saving potential and efficacy of the feed additives (Cowieson *et al*., 2006; Segobola, 2016). There are generally two approaches for supplementing feed additives to the diets of animals in order to elicit a response. The first approach as discussed by Segobola (2016) is one in which the standard diet formulation is manipulated by reducing the nutritional levels of certain nutrients such as energy or phosphorus, and then adding feed additives to restore the nutritional value of the standard diet. The nutritionists will thus allocate a matrix value to the feed additive included. The second approach is through the addition of feed additives to standard rations without tampering with any nutrient level. The cost saving may not be observed and additional costs related to addition of the feed additives would then have to be justified, thus making this approach unpopular. The first approach is commonly used in broiler production systems without adversely affecting bird performance (Kutlu *et al*., 2019). According to Abudabos *et al*. (2014) the high cost of supplemental energy necessitates optimisation of dietary ME, especially during the finisher phase where feed intake will be highest.

The first method was applied in this study, whereby the energy concentration of the standard diet was manipulated or reduced to provide three different energy levels using matrix values following the Aviagen (2017) recommended energy values for Ross 308 broiler diets. The energy level of the Positive Control was that of a standard commercial diet (11.70 MJ/kg, 11.97 MJ/kg, and 12.02 MJ/kg for the starter, grower and finisher diets, respectively) formulated to meet the daily requirements of Ross 308 broilers. The second

energy level was reduced with 2% or 65 kcal/kg (0.272 MJ/kg) and finally the third level was reduced with 4% or 130 kcal/kg (0.544 MJ/kg). The level of energy reduction was to determine whether, firstly, CreAMINO® and/or Rovabio Advance® could compensate for a deficiency of 65 kcal/kg metabolisable energy in the diet. It was also hypothesised that the broilers receiving the nutrient deficient diets would be able to perform the same as those receiving the standard commercial energy diet (Positive Control group). This reduced energy content was required to allow for the feed additives to show a measurable effect on performance.

The analysed nutrient levels of the treatment diets were somewhat different from the calculate/formulated values in the sense that some nutrients were generally lower (starch and CP) while others were higher (fat & CF) for the same energy groups. This affected the metabolisable energy calculations as the equation used included CP, EE, starch and sugar concentrations. It is clear that any error made with the analysis of any of these nutrients would affect the accuracy of the calculated ME values.

Mousavi *et al.* (2013) found no significant response of broilers in terms of body weight gain due to ME content even when CreAMINO was supplemented. Heger *et al*. (2014) observed a significant effect of CreAMINO at higher levels of AME only in the finisher phase. For the current study, a 65 kcal/kg (0.272 MJ/kg) reduction in ME resulted in significantly lower BW from day 14 to the end of the trial. This indicates that energy levels of the Negative Control 1 (NC1) diets were indeed lower than that of the Positive Control diet throughout the trial. This finding is in contrast with the calculated ME values of the trial diets, based on analysed values for CP, EE, starch and sugar. The analysed ME value for Treatment 4 (-65 kcal/kg + CreAMINO®) for the grower diets, for example, was higher than the ME of the Positive Control. The accuracy of the results for some nutrient levels received from the laboratory was questioned. Inclusion of CreAMINO® in the -65 kcal/kg (0.272 MJ/kg) diet improved the BW from day 14 until the end of the trial. This finding supports the hypothesis that CreAMINO® does have an energy sparing effect.

It is generally accepted that dietary energy content has an impact on the intake of all other nutrients as broilers regulate their feed intake based on the first limiting nutrient in the diet (Mousavi *et al*., 2013; Abudabos *et al*., 2014). Mousavi *et al*. (2013) found that a 10% decrease in AME resulted in an increase in feed consumption only from 11-22 days of age. Heger *et al*. (2014) and Metwally *et al.* (2015) observed similar trends when they reduced energy concentrations in their broiler diets. However, this was contradictory to the study by Abudabos *et al*. (2014) who observed no significant influence of ME level on feed intake of broilers. Mousavi *et al*. (2013) observed an in direct correlation between feed intake and energy in response to reduction in dietary ME during the finisher phase. This inconsistent behaviour of broilers in response to dietary ME level questions whether the modern broiler chicken has the ability to

adjust voluntary feed intake when fed diets with varying energy content, or whether broilers consume feed to a certain capacity regardless of dietary ME content (Mousavi *et al*., 2013). Cho and Kim (2013) also observed no significant difference in feed intake in broilers fed high versus low ME and concluded that feed intake of modern broilers is not dependent on dietary energy concentration. This lack of response in feed intake of broilers to varying dietary energy levels was confirmed by the findings of the current study where no effect of AME on intake was noted.

The observed differences in FCR between the broilers that received the Positive Control and reduced energy diets were expected seeing that body weight, but not feed intake, was negatively affected by a lower AME concentration. The addition of either the feed additives to the reduced energy diets improved the FCR of the broilers.

The reduction of dietary energy resulted a negative effect on tibia strength with a slight improvement in tibia strength in the broilers that received NSPase in the NC1 (-65 kcal/kg) diet. CreAMINO®, however, did not improve the tibia strength in the broilers. In their studies, Francesch & Geraert. (2009) and Yang *et al*. (2016) did not find a reduction in bone mineralisation density as a result of diluting the energy concentration of broilers supplemented with exogenous NSPase enzymes in their diets. However, no significant effect of energy reduction on tibia parameters. There is limited published research on the effect of reduced ME on tibia breaking strength. Heger *et al*. (2014) and Metwally *et al*. (2015) did not observed of CreAMINO® supplementation on carcass portions. This was in agreement with studies by Mousavi *et al*. (2013) and Abudabos *et al*. (2014) who found no significant differences between carcass portions in response to dietary metabolisable energy level and GAA supplementation. In the current study, there were also no significant differences between treatments for carcass parameters observed.

The second feed additive tested in this study was an exogenous enzyme cocktail produced by *Talaromyces versatilis* species for the degradation of NSPs in the diet, which would express its effect on broiler growth performance by improving feed digestibility. Therefore, an indication of the response of broiler chickens to enzyme supplementation is usually presented as weight gain (Olukosi *et al*., 2008). In fact, Govil *et al*. (2017) observed a significant improvement in weight gain of broilers receiving a reduced energy diet (100 kcal/kg) when supplemented with multicarbohydrase enzymes compared to their counterparts. The authors stipulated that the increased total tract CP and starch digestibilities observed in broilers fed maize-based diets could be the reason for the improved body weight gain. This was in agreement with the study of Rios *et al*. (2017) who also found that the supplementation of exogenous *Talaromyces versatilis* enzymes lead to an improvement in FCR due to increased energy and amino acid digestibility in broilers receiving reduced AME diets. Govil *et al*. (2017) also found that the supplemented broilers produced higher carcass yield in

terms of dressing percentage, expressed as a percentage of live BW. In contrast, Vieira *et al*. (2006) and West *et al*. (2007) found no significant response in terms of growth performance in broilers receiving reduced energy diets supplemented with exogenous carbohydrase enzymes. Vieira *et al*. (2006) also observed a significant reduction in broiler BW at 21 days of production in the supplemented group compared to the control and other treatment groups and hypothesised that this reduction in BW was indicative of some sort of toxicity due to the supplemented feed additives. West *et al*. (2007) concluded that the nutrient levels in their test diets might have been above the requirements of the broilers in their study, rendering the results on the supplemented exogenous enzyme complex somewhat inconclusive. Nadeem *et al*. (2005) observed a more pronounced effect of NSPase supplementation on feed intake in broilers receiving reduced energy diets but only in the starter phase. However, similar to the studies of Vieira *et al*. (2006), West *et al*. (2007) and Amerah *et al*. (2017), there were no significant effects arising from enzyme supplementation on body weight gain, feed intake and carcass parameters. The authors reasoned that traditional broiler diets are comprised of different cereals with different types of NSPs that cannot be properly digested by broilers, which could explain why exogenous enzyme supplementation does not always improve broiler performance. This was in agreement with the findings of this current study as there were no observed significant effects of exogenous enzyme supplementation on broiler BWG, FCR, feed intake and carcass parameters. It is possible that the diets in this study contained relatively low levels of NSPs and therefore insufficient substrate for the enzymes to act upon thus rendering the results of this study inconclusive. However, it was interesting to note that the supplementation of exogenous *Talaromyces versatilis* enzymes numerally improved tibia bone strength and warrants further study.

For the third part of this study ME was lowered by 130 kcal/kg (0.544 MJ/kg) and the efficiency of combining GAA and exogenous carbohydrase enzymes was evaluated for possible additivity or synergism between the feed additives. If CreAMINO® and Rovabio Advance® were to have an additive effect, it would be expected that the sum of the effect attributed by each test additive individually should not be different from the effect attributed to the combined use of the CreAMINO® and Rovabio Advance®. The 4% energy reduction (NC2) resulted in an even further drop in production by the broilers receiving this treatment feed compared to the Positive Control and NC1. The inclusion of both feed additives improved BW only on day 14 compared to the NC2 diet. However, no significant effect was noted compared to the NC2 group for the other weeks. Overall, there were no significant differences between any of the performance parameters of birds receiving the NC2 diet with and without the combined feed additives.

Chapter 6

Conclusion

Energy is the most expensive nutrient in the diet therefore any savings in this regard will be beneficial to broiler production systems. The implementation of feed additives such as guanidinoacetic acid and exogenous carbohydrase enzymes provides nutritionists with a worthy and cost effective tool to optimize broiler production in such a way to accommodate their rapid growth, without adverse effects on health or the environment. In this study, it was expected that the efficacy of guanidinoacetic acid or exogenous carbohydrase enzymes, supplied independently or in combination, would compensate for reduced dietary ME content and improve the growth response of broilers to that of the positive control group by providing compensatory energy for tissue growth and development and/or by improving nutrient digestibility through the breakdown of NSPs. This was on the general accession that the test diets were formulated to contain low concentrations of ME by increasing the oil content of the positive control diet and increasing the inclusion of more fibrous and cheaper raw material ingredients in the test diets and thus, it was expected that ME would be the first limiting nutrient. In general, the expected drop in growth performance of the broilers receiving the reduced energy treatment groups was observed. The birds that received CreAMINO® treatment diet responded positively to supplementation showing that this feed additive has the potential to improve broiler performance through its effect on body weight gain and improved FCR by compensating for reduced dietary ME via GAA serving as a secondary source of energy for ATP. This positive response to guanidinoacetic acid supplementation provides a beneficial means to the farmer by giving them an opportunity to produce more meat at a lower feed cost thereby having a positive effect on profit.

The lack of response in growth performance with regards to NSPase enzyme supplementation could be attributed to the low availability of substrate for enzyme degradation in maize-soybean diets as they contain very low quantities of pectins and other low molecular-weight substances that impede feed digestion. However, it is also probable that the effect of the both feed additives tested in this study was inconclusive because the nutrient levels of the experimental diets were high enough to meet the broiler bird's requirements, leaving no space for the feed additives to exert their effect. It is for this reason that nutritionists should rather not add enzymes (or other feed additives) 'on top' of the specification but rather formulate them into the feed, by reducing the energy level of the diet and assigning a matrix value for them. The field of using feed additives such as guanidinoacetic acid and exogenous NSPase enzymes in broiler feed holds much promise as numerous previous research trials on individual supplementation of the feed additives has resulted in improvement in BW, FCR, breast meat yield and feed digestibility.

In this study, the findings showed that CreAMINO® may add at least 65 kcal/kg (0.272 MJ/kg) in broiler diets, but no benefit could be shown for the NSPase that was included in the diets.

Critical Review and Recommendations

The following observations were made during the study which may have affected the final results:

1. The study was conducted soon after the country faced the Avian Influenza (H5N8) challenge that hit in 2017. This outbreak affected most of the parent stock leading to shortage of broilers. The first flock after the outbreak was used in this study and this may have had an impact on the quality of the flock, resulting in a relative high mortality rate of 4.3%.
2. Enzyme recovery was not conducted to verify the mixing efficiency of the enzyme complex (Rovabio Advance®) in the treatment diets. This analysis is an essential element when carrying out trials with enzymes. The problem with this analysis is that there are a limited number of laboratories conducting such a test resulting in long turnaround times.
3. The experimental feed was not analyzed for proximate analysis prior to commencement of the trial to verify whether the test feed was in line with the nutrient formulations with regard to the energy required to elicit a response from the feed additives that were to be tested. The feed samples were collected and sent to a laboratory in Germany for analyses shortly after production but unfortunately the results were received long after completion of the broiler performance trial. The major drawback with this was that it could not be confirmed in due time whether the energy in the diets were in line with the formulated (intended) values. The calculated ME values of the treatment diets (based on analysed values for CP, EE, starch, and sugars) did not show the intended reductions compared to the Positive Control. Based on the reduced growth of the broilers throughout the trial that received these diets it was suggested that feed analyses were inaccurate. It would have been beneficial to test the formulated diets prior to conducting the experiment and to also determine the expected enzyme recovery to determine the mixing efficiency/activity of the Rovabio Advance®. It is unfortunate that no retention samples were kept so as to verify the results of the proximate analysis.
4. It would be interesting if a follow-up study is done to test the additivity (if any) between CreAMINO® and Rovabio Advance® as this was to date the first study performed combining the effects of these two feed additives.

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