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The effect of supplementing an exogenous protease enzyme in diets based on maize and soybean meal on performance and gut health in grower pigs

By

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BSc (Agric) Animal Science

Submitted in partial fulfillment of the requirements for the degree
MSc (Agric) Animal Science: Animal Nutrition

In the Faculty of Natural and Agricultural Sciences

UNIVERSITY OF PRETORIA

2017

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Declaration

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

R Vermeulen

January 2017

Acknowledgements

I would like to express sincerest thanks to the following people:

Dr Christine Jansen van Rensburg for her guidance and mentoring throughout this project. For her role in my professional development and support of my goals and ambitions.

Natasha Snyman from Novus Int for her constant guidance, advice and support throughout the project, especially with feed formulations.

Maja Sakkers and Jacolene Du Toit from Feed First and Dr Hannes Viljoen from ANH for advice with the feed formulations of this trial.

Agricultural Research Council for financial support of my personal studies.

Chantelle Cronje and Judi Rosentrauch as well as the other MSc students from the University of Pretoria for all the late nights and long hours they spent working on the trial. Also a special thanks to Kelly Brannan, Jana Whitehead and Alet de Villiers, for help during slaughter and sampling.

Professor Riana Cockeran from the Department of Immunology, University of Pretoria for her extensive help and guidance with blood analysis.

Mr Roelf Coertze for his help in running and interpreting the statistics of this project.

To my wonderful family for their ongoing support and help during my studies.

Abstract

Protein remains one of the most expensive nutrients to deliver in the pig diet. Theoretical benefits of supplementing protease enzymes include formulating diets with lower crude protein levels, using alternative raw materials with lower digestibility values, eliminating anti-nutritional factors, improving gut health, reduced protein fermentation in the hindgut and decreasing nitrogen excretion in manure and urine. Research studies to substantiate the theoretical advantages of protease enzyme supplementation in pig diets did not show the same repeatability found in broiler trials. The aim of this study was to determine if exogenous protease supplementation (Cibenza DP100) in grower and finisher pig rations would yield any improvement in production, slaughter and gut health parameters.

A completely randomised block design experiment was conducted at the University of Pretoria's experimental farm. One hundred and seventy four male pigs (from Topigs 40 sow line) of 30 kg (+/- 7 kg) were allocated to 4 dietary treatments (positive control; negative control; negative control + 250 mg/kg Cibenza DP100; Negative control + 500 mg/kg Cibenza DP100). The feeding programme of the trial consisted of 3 phases (grower 1, grower 2 and finisher rations). Production parameters (average daily gain, feed intake, feed conversion ratio, body weight and backfat thickness) were measured in each phase. Slaughter parameters (carcass weight, carcass length) and gut health parameters (villi length, crypt depth, ammonia) were quantified for each group of pigs slaughtered at the end of the second grower phase (115 days of age) and the finisher phase (137 days of age).

Significant effects were observed with Cibenza DP100 protease enzyme supplementation after the second grower phase on body weight, body weight gains, feed conversion ratio and reduced backfat thickness. The same trend of increased body weight and body weight gains, reduced feed conversion ratio and reduced backfat thickness was observed when considering the overall grower phase (70-115 days of age). Significant differences were found between the negative control diet and supplemented diets, in warm carcass mass, cold carcass mass and carcass length after the finisher phase. Diets supplemented with 250 mg/kg performed equally well compared to diets supplemented with 500 mg/kg protease enzyme. Data to demonstrate significant effects on gut health parameters were not conclusive.

The results of this trial concluded that protease supplemented at 250 mg/kg to diets of grower and finisher pigs, between 96 and 137 days of age, could reduce backfat thickness, improve feed conversion ratio and average daily gain in growing pigs and improve carcass mass at slaughter in finishing pigs. These enzymes should especially be considered under circumstances where underprocessed soymeal is used or alternative protein sources with lower digestibility are included in the diet.

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List of Abbreviations

AA	Amino acids
ADI	Average daily intake
ADFI	Average daily feed intake
ADG	Average daily gain
ANF	Anti-nutritional factors
AME	Apparent metabolisable energy
ARC	Agricultural Research Council
BFAP	Bureau for Food and Agricultural Production
BW	Body weight
BWG	Body weight gain
CCM	Cold carcass mass
CP	Crude protein
DDGS	Dried distillers grain solubles
FAO	Food and Agricultural Organization
FCR	Feed coversion ratio
FI	Feed intake
GIT	Gastro intestinal tract
HCl	Hydrochloric acid
HPLC	High performance liquid chromotography
IL	Interleukin
NRC	National Research Council
NSP	Non-starch polysaccharide
SAPPO	South African Pork Producers Organisation

SPF	Specific pathogen free
TNF	Tumor necrosis factor
WCM	Warm carcass mass

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Chapter 1

Introduction

Pork is the global protein of choice but comprises only 7% of protein consumed in South Africa (Meyer et al., 2013). The production improvements which have been achieved in the last decade have been attributed to improved efficiency and genetic improvement in the national sow herd, not due to increased pig numbers. Pig farmers in South Africa face a challenging economic climate and the average farm needs at least 500 sows to remain economically viable (Meyer et al., 2013). Feed accounts for around 75% of on farm running costs due to the high prices of raw materials. The second challenging factor for South African pig farmers is that only 20% of abattoirs currently slaughter 98% of all pigs in the country (Meyer et al., 2013). This minimises the bargaining power of the South African pig farmer and forces him to accept lower prices for pig meat. The political climate in South Africa encourages the entrance of emerging farmers into the pig production sector, but it remains challenging due to the massive capital investment involved in a start-up piggery.

In recent years, research in the South African pork industry has been focused on improving gains and growth performance whilst decreasing the cost of feed. Improved feed efficiency and feed conversion ratios have been prioritised (Meyer et al., 2013).

Protein remains one of the most expensive nutrients to deliver in the pig diet (Adeola & Cowieson, 2011). Nutrition research in recent years have focused much on reducing the crude protein content of diets to a minimum requirement whilst ensuring all amino acid requirements are met. Enzyme supplementation has recently gone a long way in improving the digestion efficiency and nutrient contributions of certain raw materials. Soya oilcake remains one of the most popular protein sources used in pig feeds in South Africa, but contains high levels of anti-nutritional factors (Kleyn, 2013). The supplementation of protease enzymes to pig diets has been used to eliminate anti-nutritional factors and improve nutrient efficiency. However, trials evaluating the supplementation of enzymes to pig diets have been concentrated on young piglets as younger animals have lower levels of endogenous enzymes, with an immature gastrointestinal tract, whilst being encouraged to consume creep feed from only a few days of age.

Research efforts on protease enzymes in young pigs are well documented (Caine, 1997; Rantzer *et al.*, 1997; Rooke *et al*., 1997) but there is little evidence in the literature to support the use of protease enzymes in pigs during the grower and finisher phases. The consistency of the results from protease trials has been nowhere near that of phytase.

The objective of this study was to determine the effect of supplementing protease enzymes on performance and gut health in grower and finisher pigs.

The aim of the study was to test the following hypotheses:

- H_0: Supplementation of grower and finisher pig diets containing different quality protein sources with protease enzymes will not improve production parameters, carcass characteristics and gut health parameters
- H_A: Supplementation of grower and finisher pig diets containing different quality protein sources with protease enzymes will improve production parameters, carcass characteristics and gut health parameters

Chapter 2

Literature Review

2.1. Introduction

Pigs were first domesticated from wild boars in the Eastern Asian countries at around 13000BC and were introduced to Europe during the 18-19th centuries (Larson *et al.*, 2007). The early hunters of wild boars used not only the meat, but also the hides, teeth and bones. Today, pig meat remains a global protein source of choice (Meyer, 2013).

The Food and Agricultural Organization of the United Nations (FAO) released food projections in 2010 which indicated that by 2019, the demand for pork would have increased by 24% (Kittawornrat & Zimmerman, 2011). The constant pressure on pig producers to improve production and feed efficiency whilst facing the challenges of scarce natural resources and high feed prices have led to the use of exogenous enzymes to improve production efficiency. Furthermore, pig farmers in South Africa specifically have low bargaining powers on prices received for their products since 98% of all pigs are slaughtered by only 20% of the existing abattoirs (Meyer, 2013).

Supplementation of animal diets with exogenous enzymes to enhance efficiency and performance is not a new concept and earliest published work on this dates back to the 20th century (Hasting, 1946). The modern pig diet has become tremendously sophisticated since the 1960's with the use of enzymes increasing exponentially over the last 20 years (Bedford & Cowieson, 2012). In the early days of pig production a large variety of raw materials would make up the pig diet and it only rarely occurred that one ingredient constituted up to 30% of the diet. In modern pig farming systems, using least cost formulations have allowed us to use as little as three ingredients to constitute 75% of the diet, but these popular ingredients often contain high levels of anti-nutritional factors (Bedford & Schulze, 1998).

A paper released on the future of pig farmers stated that in order to overcome all the challenges of pig farming, one would need to strive to become producers of low cost pork of acceptable quality (Boettiger, 2009). One method to achieve this would be to use low quality feed ingredients, possibly supplemented with enzymes to improve digestion. However, despite the enormous research efforts conducted on the use of exogenous enzymes to improve efficiency, very few publications have led to genuine paradigm shifts in production efficiency or in the consistency or magnitude of feed enzyme efficiency (Cowieson & Bedford, 2009). The issue observed with low return on investment in feed enzyme studies is directly related to the opportunity for an enzyme to elicit a beneficial response which is proportionate to the quantity of undigested

nutrients which exit the intestine, and therefore the quality of the protein sources used, since digestion of any nutrient cannot exceed 100% (Cowieson, 2010).

2.2. Protein digestion

In order to understand how supplementation of protease enzymes might improve protein digestion, one must first understand the natural process of protein digestion. Proteins can be defined as biological compounds consisting of one or more polypeptides folded into a globular or fibrous form, associated with a specific biological function. Other examples of protein types also include simple and conjugated proteins. A polypeptide can be described as a single polymer chain of amino acids bonded by peptide bonds between the carboxyl and amino acid groups (Wang & Fuller, 1989). Protein digestion occurs both in the stomach and in the small intestine through a process of hydrolysis where polypeptides are broken down. These breakdown processes are dependent on both proteases and proteinases.

In order to understand the process of protein digestion, it is firstly important to understand the core structure of protein synthesis, and the concept of the central dogma of biology, shown in Figure 2.1 (Dawson, 2006). The central dogma of biology describes the basic framework of how genetic information flows from a DNA sequence to a protein product.

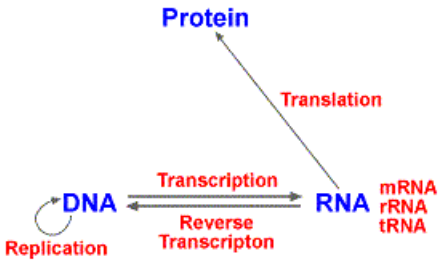

Figure 2.1. The central dogma of biology (Dawson, 2006)

DNA is first replicated by DNA polymerase, after which it undergoes a process of transcription, facilitated by RNA polymerase, yielding mRNA (messenger RNA). Protein is formed by the translation of mRNA facilitated in the ribosome (Suryawan *et al.*, 2008).

Proteins entering the gastrointestinal tract need to be broken down to pass through membranes, in order for it to be utilized by the body. As protein feed enters the stomach, secretin is released in response to the presence of acids and stimulates secretion of fluids and bicarbonate in the stomach. Cholecystokinin is released in response to the presence of ‘feed’ in the stomach and stimulates the release of pancreatic enzymes. Hydrochloric acid (HCl) and pepsin are primarily responsible for gastric digestion which is the first process in protein digestion, occurring in the stomach (Laerke & Hedemann, 2015). Pepsin is produced and released as

inactive precursors (zymogens and proenzymes), with four different types of pepsin found in the GIT, each functional at a different pH. Pepsin is activated when the gastric pH falls to 5 or lower. Gastric digestion is followed by intestinal digestion where feed is mixed with pancreatic and duodenal secretions in the proximal duodenum. During this phase, digestion of protein occurs by pancreatic enzymes rather than pepsin since the pH of the digesta reaches 7 towards the distal ileum. Intraluminal digestion also occurs which can be supplemented by some microbial proteases.

Trypsinogen is released from the pancreas in the inactive form and is converted to trypsin by enterokinase, which is found in the intestinal mucosa. Chymotrypsinogen is then activated by trypsin to chymotrypsin, whereas pro-elastase is also converted to elastase by trypsin, and is the sole enzyme active against elastin (Kunitz & Northtop, 1963). Trypsin and chymotrypsin are responsible for breaking polypeptides down into peptides. The regulatory process of the conversion of trypsinogen to trypsin acts as a safeguard, if trypsinogen should become activated by trypsin prematurely, the enzyme would damage the pancreatic tissues. Once pancreatic secretions reach the small intestine, an explosive development of protease enzymes occurs due to the activation of trypsin (Jensen *et al.*, 1997). Trypsin and chymotrypsin are responsible for digesting protein into peptides, but do not have the ability to digest peptides into amino acids (Lindemann *et al.*, 1986).

Digestive proteolytic enzymes can broadly be classified into proteolytic enzymes found in the gastric juice, pancreatic juice and intestinal mucosa. Enzymes found in each of these sections of the GIT can further be divided into exopeptidases or endopeptidases. Endopeptidases are responsible for breaking peptide bonds within the polypeptide chain whilst exopeptidases break peptide bonds at the end of polypeptide chains.

Proteins are digested to smaller polypeptides by endopeptidases, trypsin, released from the pancreas. Smaller polypeptides are digested to amino acids by exopeptidases, secreted from the pancreas (Butts, 1991). Endopeptidases are normally the exogenous protease enzymes of choice since they are much more efficient in effectively breaking down proteins to smaller polypeptides. The major proteolytic enzymes found in the gastro intestinal tract are classified in Table 2.1 (Bergmann, 2009).

Table 2.1. Major digestive proteolytic enzymes (Bergmann, 2009)

Point of action	Enzyme	Main group
Gastric juice	Pepsin	Exopeptidase
	Trypsin	Endopeptidase
	Chymo-trypsin	Endopeptidase
Pancreatic juice	Elastase	Endopeptidase
	Carboxypeptidase A	Exopeptidase
	Carboxypeptidase B	Exopeptidase
	Aminopeptidase	Exopeptidase
Intestinal mucosa	Dipeptidase	Exopeptidase

2.3. Role of protein in the pig diet

Meeting daily amino acid requirements of the pig without wasting excess protein is economical, environmentally friendly and nutritionally beneficial. Five essential amino acids (lysine, methionine, threonine, tryptophan and valine) can be added to the feed in free form. The non-essential amino acids (isoleucine, leucine and histidine) need to be provided through high protein feed sources. In modern commercial swine diets, lysine is generally the first limiting amino acid with all amino acids present in excess of the first limiting amino acid being of no value to the animal except as an energy source (Han, 2000). The barrel analogy, created by Liebig (Kleyn, 2013) demonstrates that all amino acids should be balanced in proportion to the first limiting amino acid, lysine. If the first limiting amino acid is deficient all other amino acids will be in oversupply. Protein and amino acid balance should be a key focus area in pig nutrition.

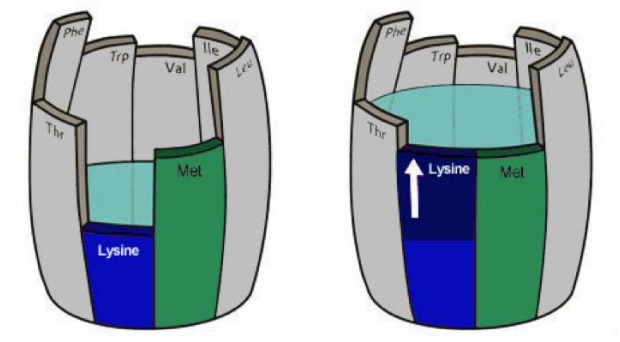

Figure 2.2. Liebig's barrel analogy for amino acid balance (Kleyn, 2013)

High protein diets have long been used in weanling pigs as it was thought to ease the transition from milk to creep feed. However, studies from recent years have revealed that high protein diets may encourage proliferation of pathogenic bacteria in the pig gut, but this challenge has been controlled through the inclusion of antibiotics in the pig feed.

Studies conducted on recently weaned pigs found that decreasing protein from 23% to 18% did not have a negative effect on growth but resulted in firmer faeces, fewer pathogenic bacteria in the gut and an improved ratio of healthy to pathogenic bacteria (Wellock *et al.*, 2008). However, Nyachoti (2006), found that decreasing protein to below 19% resulted in reduced growth and whenever protein was decreased by 4% or more, a deficiency of non-essential amino acids was documented (Nyachoti *et al.*, 2006).

Nutrient requirements change rapidly during the grower phase. Many nutritionists implement more feeding phases in order to accurately supply in the pig's nutrient requirements and to reduce ration costs. Growing pigs also have low feed intake in relation to their high capacity for growth. Le Bellego *et al.* (2001) determined that protein could be decreased by 3% without a negative effect on growth as long as adequate synthetic amino acids were supplemented in the diet. The study by Wellock (2008) also found that clinically healthy pigs fed soya based diets, had a higher incidence of post weaning diarrhea due to a decrease in lactobacilli: coliform bacteria ratio and a higher pH in the GIT.

2.4. Soya oilcake as a source of protein in the pig diet

Soybean meal is the primary source of protein and amino acids in pig and poultry diets globally due to its amino acid digestibility being higher in soya oilcake compared to other oilcake meals. The competitive advantage of the high amino acid digestibility profile of soybean meal is of much lower importance in ruminant rations compared to protein sources such as cotton oilcake at lower cost. Both soybean meal 44% CP and 48% CP have an excellent balance of indispensable amino acids. However, the digestibility of amino acids in soybean meal 48% CP is greater than in 44 % CP (Upadhaya *et al.*, 2016).

Soybeans can be fed to pigs as soya oilcake which is a by-product of the oil extraction industry and is the most widely used protein source in animal feeds. Protein content of soya oilcake is very high but it tends to be deficient in methionine (Kleyn, 2013). Full fat soya consists of soybeans which have been cooked by means of an extrusion process and contains up to 20% oil. The manner in which full fat soya is processed significantly influences the energy content as well as the availability of amino acids. The nutrient composition of different types of soybeans as described by the NRC are shown in Table 2.2 whilst the nutrient composition of different types of soybeans as described by the CVB are shown in Table 2.3.

Table 2.2. Nutrient composition (%) of soybeans, soybean meals (SBM) and other soy products (NRC, 2012)

	Full fat soybeans	Dehulled SBM	Non dehulled SBM	Extruded –expelled SBM	Enzyme treated SBM	Fermented SBM	Soy protein concentrate	Soy protein isolate
Dry matter	92.36	89.98	88.79	93.85	92.70	92.88	92.64	93.71
Crude protein	37.56	47.73	43.90	44.56	55.62	54.07	65.20	84.78
Ether extract	20.18	1.52	1.24	5.69	1.82	2.30	1.05	2.76
Carbohydrate	29.73	34.46	37.27	37.90	28.21	29.53	20.28	2.00
Ash	4.89	6.27	6.38	5.70	7.05	6.98	6.11	4.17

Table 2.3. Nutrient composition (%) of soybeans, soybean meals (SBM) and other soy products (CVB, 2007)

	Full fat soybeans	Soybean expeller	Solvent extracted soybean meal
Dry matter	88.50	88.80	87.30
Crude protein	35.10	43.50	46.40
Crude fat	19.20	8.10	1.90
Ash	4.90	6.40	6.50
Crude fibre	5.60	6.40	3.70
Calcium	0.21	0.30	0.27
Phosphorous (total)	0.53	0.65	0.65
Net energy (MJ/kg)	11.98	9.57	8.25
Lysine	1.76	2.36	2.55
Metionine	0.39	0.54	0.58

Cystine	0.38	0.53	0.57
Threonine	1.02	1.4	1.51
Tryptophan	0.36	0.48	0.52
Isoleucine	1.25	1.73	1.87
Arginine	2.22	3.01	3.26
Valine	1.27	1.77	1.91

- All amino acids are in apparent ileal digestible amino acids percentage values

A major drawback on soya oilcake as a protein source in animal nutrition is that it contains high levels of anti-nutritional factors (ANF). Anti-nutritional factors can be described as non-fibrous naturally occurring substances exerting negative effects on performance of animals, with trypsin inhibitors (TI) being the ANF with the most negative effect on animal performance (Qin *et al.,* 1996). Two types of TI, the Bowman Birk and Kunitz trypsin inhibitors, are found in soybean (Huang *et al.,* 2004). In soya meals, TI needs to be deactivated (usually through heat treatment) before it can be fed to monogastric animals. TI bind and inactivate the digestive enzymes, trypsin and chymotrypsin, in the animal small intestine. Trypsin is essential in activating other enzymes in the intestine. As the pancreas produces higher levels of trypsin in order to aid normal digestive function, the pancreas swells and pancreatic hypertrophy can occur. A review of research studies indicate that younger animals are more susceptible to the effect of TI compared to older animals (Ven, 1974). Plant breeders have in recent years achieved the production of low TI soya, which only require half the amount of heat to inactivate TI, compared to normal soybeans (Herkelman *et al.,* 1992).

Soya meal has the highest level of phosphorous of all plant proteins, yet most of the phosphorous forms a complex with phytic acid rendering it unavailable. Carbohydrate components in soya meal include sugars, hulls and non-starch polysaccharides. The dilution effect of these undigested carbohydrates reduces the nutrient density of the feeds (Willis, 2003).

Oligosaccharides, specifically raffinose and stachyose, are short-chained carbohydrates that make up 5-7% of the soya meal and are not digested (Cromwell, 2013) and may cause digestive disturbances in especially recently weaned pigs. These ANF can be removed by special processing resulting in a soy protein concentrate. Apart from the undigested short-chain carbohydrates found in soybean meal, immunogenically active soybean proteins, glycinin and ß-conglycinin, known as antigenic factors, also elicit a negative effect on production, especially in young pigs. These factors are responsible for an inflammatory response in the intestine, resulting in a change in intestinal morphology and depressed absorption when high levels of soya oilcake are included in the diet (Xu, 2014). Due to the high level of osmotically active non- starch oligosaccharides,

galactomannans, raffinose and stachyose in soya oilcake, inclusions exceeding 30% can impair absorption and maldigestion (Ferket & Gernat, 2002).

Soybean meal also contains lectins, which are carbohydrate binding proteins, ubiquitous in nature. These proteins also bind with intestinal cells and interfere with the absorption of nutrients. Saponins, lipoxidases, phytoestrogens and goitrogens are also found in soybean meal but the extent to which they negatively affect digestion in pigs have not been well studied (Vasconcelos & Oliviera, 2004).

In practice, heat treatment to inactivate ANF is carried out during the de-solventising procedures used in the production of soya oil. An incorrect amount of heat is often applied resulting in the soya oilcake being over -or under -processed. Under processing as a result of low or short heat application negatively affects the amino acid digestibility because TI is not completely destroyed. Over processing also negatively impacts on amino acid digestibility because a proportion of amino acids are bound to indigestible compounds or have been destroyed, and are not available to the animal. Excessive heat compromises protein quality resulting in reduced nutrient content of the meal. This is as a result of the Maillard reaction occurring, which causes amino acids to become bound to sugars. Lysine, cysteine, aspartic acid and histidine show the biggest levels of decrease as a results of over processing (Gonzales-Vega, 2011).

It is imperative for all soya products to be processed prior to feeding the monogastric animal in order to have high nutritional value to the animal. Processing conditions have a major effect on the quality of the final soya product. The 3 main types of soybean meals commercially available include full fat soya oilcake, expeller dehulled soya and solvent extracted dehulled soya. Expeller soybean meal usually has a higher level of fibre, whilst sources processed by extrusion usually have higher levels of energy (Kaankuka *et al.*, 1996). The standard process for solvent extracted soya meal involve a series of treatments which includes cracking, dehulling, flaking, extraction and toasting (Willis, 2003).

A study where growing pigs were fed diets comprising of 80% basal and 20% soya oilcake, processed at 102°C for various lengths of time, found that adequately processed soya oilcake had higher apparent digestibility of DM and N and lower levels of ANF (Qin *et al.*, 1996). A certain amount of energy is required to achieve inactivation of ANF either through high temperature exposure for a short time or through lower temperatures used for a longer time. The same study further concluded that animals differed considerably in the way in which they responded to ANF in raw materials and this would have a significant effect on ANF digestibility studies as high levels of variation was found throughout a population.

Quality control through laboratory analysis for various parameters plays a crucial role in the use of soya products as animal feedstuffs. Urease activity (UA) is one of the most widely used quality parameters as an indicator for the degree of underprocessing in soya oilcake. Urease levels, measured by pH rise in ammonia

solution, would be around 2 for raw soybean. Urease test values of 0.05-0.3 indicate acceptable soya oilcake processing in South Africa (Liener, 1981). Urease tests, however, do not measure over processing of soybeans.

Protein solubility in a 2% potassium hydroxide (KOH) solution closely reflects the degree of over processing. Test values of between 70-85% indicate acceptably processed soya oilcake. Activity values of lower than 70% indicate overprocessed soya. The KOH test is often used as complementary to the urease activity test since KOH tests yields more reliable results for overprocessed products whilst the urease activity test indicates underprocessed products effectively (Parsons *et al.*, 1991).

The protein digestibility index (PDI) which measures protein solubility is the simplest of the 3 methods, where values of 45-50% is indicative of good quality soya. The PDI method measures the amount of protein dispersed in water after blending a soya sample with water at high speed (Hsu & Satter, 1995). It is often used supplementary to the KOH and urease tests.

A comparison of all three of the above methods described above concluded that PDI was the most consistent and sensitive indicator of soya quality compared to urease and KOH protein solubility methods (Batal *et al.*, 2000).

Unfortunately, urease activity, PDI and the KOH tests are time and labour intensive and therefore a practical test usually used at the feed mill to determine whether a batch of soya can be accepted is the Phenol Red test, also known as cresol red. This is a useful method since it is extremely simple to do and the results are immediate. Soya oilcake samples are placed in a petri dish in a 2:1 ratio and left for 5 minutes. The phenol red liquid causes underprocessed soya to change its colour to red. The degree of underprocessing can be estimated based on the percentage of sample converted to red. An overwhelmingly yellow sample indicates over processing (Dozier, 2014).

Palliyeguru *et al.* (2011) simulated various degrees of undercooked soya by substituting raw soybeans for optimally cooked full fat soya meal. It was found that the presence of TI reduced protein digestibility and growth whilst increasing feed intakes in broilers. Poor protein digestion also led to decreased gut health as measured by the incidence of necrotic enteritis. Severely overprocessed soya fed to broiler chickens resulted in a significant decrease in energy content/digestibility of the rations, which resulted in lower body weight and reduced feed efficiency (Aburto *et al.*, 1998).

2.5. Protein fermentation in the hindgut

Supplying surplus dietary protein to animals result in undigested protein entering the hindgut and being fermented by the resident microbiota (Jha & Berrocoso, 2012). Fermentation can have a positive or a negative effect on the GIT depending on the substrate (protein or carbohydrate) being fermented. Carbohydrate fermentation results in the production of short chain fatty acids (SCFA) and the utilisation of N as a nutrient source for microbial growth. Potential pathogens are often protein fermenters and therefore more likely to grow in conditions that favour protein fermentation (Williams *et al.,* 2001). Protein fermentation is usually concentrated in the distal colon (Windley, 2012).

Indigestible protein is fermented by decarboxylation, which results in monoamines and polyamines, and deamination resulting in branched chain fatty acids (BCFA) and SCFA, and the production of ammonia, phenols and indoles (Otto *et al.,* 2003). Secondary products such as lactate and succinate and gases such as methane, hydrogen and carbon dioxide are also produced. These end products influence receptor ligands, cell proliferation, cytotoxicity as well as buffering capacity. The end result is tight junction failure and reduced barrier function with inflammation and translocation of pathogens as a consequence. Whilst biogenic amines are required for gut development too high concentrations, produced during protein fermentation, have been shown to cause depressed growth rates and gizzard erosion in broilers (Qaisrani *et al.,* 2015).

The pig microbiota consists mainly of gram positive bacteria when in a healthy state with low levels of protein passing through to the hindgut and becoming available for fermentation. These gram positive bacteria include: *Streptococcus, Lactobacillus, Peptostreptococcus, Clostridium, Eubacterium.* The healthy hindgut with low levels of fermentation have proportionately fewer gram negative bacteria (*Fusobacterium, Selenomonas, Prevotella, Butyrivibrio)* with a high population of lactobacilli in the proximal GIT (Leser *et al.,* 2002). Strategies which have been suggested as means to reduce the amount of protein becoming available for fermentation in the hindgut, resulting in higher concentrations of gram negative bacteria, include, protease enzyme supplementation, the supplementation of pro-and prebiotics (yeast, dietary fibre), increasing levels of Zn and Cu in the diet as well as reducing CP in the diet and only supplementing with synthetic amino acids (Rist *et al.*, 2013). Change in diets to increase carbohydrate fermentation instead of protein fermentation have been shown to improve the production of beneficial *Lactobacillus* species indoles (Otto *et al.,* 2003).Excess protein which passes through to the hindgut and becomes available for fermentation by pathogenic bacteria can launch an immune response as consequence and can result in the release of proinflammatory cytokines into the bloodstream. Proinflammatory cytokines can be used as a measurement of immune response which is activated by protein fermentation. Cytokines are peptide molecules that regulate the immune and inflammatory responses. The effect of proinflammatory cytokines on gut health have been documented in young piglets (Pie *et al.,* 2004). The intestine has a natural physical protection barrier preventing toxins, produced during fermentation, from entering the systemic circulation. If intestinal epithelial cells should sense these toxic substances, they will signal an immune response through the production of proinflammatory cytokines (Jana,

2008). Protein fermentation in the pig hindgut has been linked to increased odour emerging from pig slurry, since production of malodorous compounds such as indolic, phenolic and VFA compounds and ammonia increased proportionately with protein inclusion in the diet (Jha & Berrocoso, 2012). In a study by Otto *et al.* (2003) where protein was decreased in grower pig diets, it was found that the low protein diet resulted in lower NH_3 concentrations in manure, but it did not diminish manure odour offensiveness and had no effect on the concentration of VFA found in the faecal output.

A high level of protein fermentation is associated with high plasma urea levels. Amino acid catabolism by microbes produces ammonia, which diffuses into blood and can be converted to urea by the liver. Urea is excreted in urine or diffused into the caecum where it is converted to microbial nitrogen and used as a substrate for bacterial growth (Kim *et al.*, 2008).

In a recent study, pigs of 70 days old were split into two groups, which were fed either a normal or a low protein diet up to the age of 170 days after which hindgut digesta was collected. No significant differences were found in ADG, FCR and gain between the normal and low protein diets, but a significant decrease in isobutyrate, isovalerate, BCFA and SCFA were found in the small intestines of pigs fed the low protein diet, linked to lower levels of protein fermentation (Zhou *et al.*, 2015). Jeaurond *et al.* (2008) compared a high protein ration to a diet with high levels of fermentable carbohydrates. This study revealed that the higher protein ration was associated with higher ammonia, spermidine and tyramine levels in the colon and lower concentrations of acetic, propionic and butyric acid in the small intestine.

Higher levels of undigested crude protein reaching the hindgut is also associated with increased *E.coli* colonisation in the hindgut, which has been linked to the onset of diarrhea in young pigs (Jha & Berrocoso, 2012). Recently weaned pigs fed diets with increased fermentable carbohydrate content, and to a lesser extent, a decrease in crude protein, resulted in reduced ammonia levels in the hindgut and altered gut microflora with reduced fermentation. No growth response was documented in addition to the effects on gut health (Otto *et al.*, 2003).

2.6. Exogenous protease enzyme supplementation in feed rations

Oversupply of protein in the diet is financially wasteful and damaging to the environment, whilst undersupplying protein results in reduced growth efficiency (Cowieson & Roos, 2013). The digestion of protein is driven by endogenous proteases in a two stage process. The gastric phase occurs in the low pH environment of the stomach where protein is exposed to pepsin, whilst the second phase, during which proteins are digested by trypsin, chymotrypsin and elastase, occurs in the small intestine. Endogenous protease enzymes are secreted in an inactive form and become activated due to a change in pH or enzymes to prevent them from digesting the gut lumen (Twinning, 1994). Proteases are a specific group of enzymes which break down peptide bonds between amino acids of proteins allowing the amino acids to be absorbed and transported via the

bloodstream to the liver, where the amino acids can be utilised by the body. Protein digestion may be compromised for the following reasons:

1) Presence of protease inhibitors in raw materials
2) Damage to intestinal structure and absorptive surface area
3) Rapid transit time through the gastrointestinal tract
4) Insufficient secretion of endogenous protease enzymes

Supplementing exogenous protease enzymes may increase the hydrolysis of proteins with low digestibility, allowing the animal to capitalise on otherwise wasted nutrients (Ghazi *et al.*, 2002). In modern monogastric animal nutrition, serine proteases are most commonly used, isolated from *Bacillus* or *Subtilisin* bacteria (Sultan, 2011). These exogenous proteases function optimally at pH of 9, which is much higher than pH levels encountered in the gut lumen. Protease enzymes are therefore most active in the small intestine and least active in the gastric phase of digestion (Angel & Sorbara, 2014). Current industry practice suggests that exogenous protease enzymes are supplemented in monogastric diets to eliminate anti-nutritional factors (ANF), to supplement naturally occurring endogenous enzymes and to render nutrients more readily available for absorption (Walsh *et al.*, 1993). The early work on protease enzymes was done on fungal and diastatic proteases and aimed to supplement endogenous enzymes while modern work focusses on overcoming anti-nutritional factors in by-product feed ingredients not traditionally used in rations (Thorpe *et al.*, 2001). Protease enzymes can be safely included in the diet because it will improve production without the implementation of a withdrawal period, since exogenous enzymes are broken down in the digestive tract like any other natural protein with no excretion of the enzyme to the environment (Bedford & Apaljalahti, 2001).

2.7. Potential benefits of supplementing exogenous protease enzymes in pig diets

A major challenge in modern pig production during the grower stage is that pigs tend to have low feed intake in relation to their high capacity for growth. As the pig genotype improves with breeding, the modern pig requires more protein for muscle deposition (Dewey, 1993). The goal should be to maximise feed intake during the grower period, in order to ensure adequate protein intake for growth. Feed intake is often constrained further during times of heat stress and by feeding high fibre rations but by adding exogenous protease enzymes, these negative effects can be negated by improving digestibility of proteins in the feedstuff (Fru-Nji *et al.*, 2011).

Several advantages of supplementing an exogenous protease enzyme in the pig diet have been identified: Protease supplementation can eliminate ANF, digest storage proteins and make it available to the animal, improve gut health and reduce nitrogen excretion through reduced protein fermentation in the hindgut. Furthermore, protease supplementation allows for utilisation of raw materials with low digestibility and provides the opportunity to formulate diets with lower crude protein specifications (Jeaurond, 2008; Ziljstra *et al.*, 2010; Van Kempen, 2004; Woyengo, 2014).

2.7.1. Protease enzymes overcome anti-nutritional factors in the diet

High protein cereal raw materials, such as soybean meal are often included in pig diets at up to 25% (Zijlstra *et al.*, 2010). Due to the high inclusion rates of these ANF containing raw materials, the threshold at which digestion is affected by ANF is reached in all phases of pig production (Jansman, 1994). ANF which affect pig production include non-starch polysaccharides in cereal grains and trypsin inhibitors in soybean meal (Bedford & Schulze, 1998). Trypsin inhibitors reduce nutrient utilisation and growth by inhibiting natural endogenous proteases (Torrallardona, 2009). Trypsin inhibitors and lectins have traditionally been deactivated using heat treatments, but the negative effects can be eliminated by the addition of protease enzymes (Bedford, 1996).

Seeds, especially of the legume plant variety, like soya, have high levels of storage proteins which bind to starch and render it unavailable to the animal. Protease enzymes have the potential to break down storage proteins in plants and release energy bound starch (Bedford, 1996). Supplemented exogenous protease enzymes have the ability to digest the kaffirin envelope surrounding starch in sorghum feeds, making the energy available to the pig, resulting in an improved feed conversion ratio which shows a linear relationship to increased protease levels (Codogan, 2010).

2.7.2. Protease supplementation improves pig gut health

Poor protein digestibility is often correlated with a negative growth response due to low availability of amino acids, metabolic cost of nitrogen excretion, changes in gut microflora, immune system stimulation and production of toxic metabolites (Jeaurond, 2008). A complex three way interaction between the host, bacteria and feed occurs in the pig hindgut (Zijlstra *et al.*, 2010). Protein fermentation results in the deamination and caboxylation of amino acids and formation of harmful end products which lead to a decrease in growth and performance (Cone, 2005). Substrates such as amines, indoles, phenols, p-cresols and branched chain fatty acids, favour oxidative stress and cause inflammation and gut lesions which reduces absorption of nutrients as a consequence (Jeaurond, 2008).

Poor protein digestibility causes protein to pass through the stomach and become available as a substrate for bacteria in the hindgut, where the undigested protein is fermented by microbes (Williams *et al.*, 2001). Bacteria such as *E.coli, Clostridium perifringes, Salmonella* and *Campylobacter* in the gut use undigested protein as a substrate for proliferation. Fermentation of protein in the gut also leads to changes in gut morphology such as reduced villi height and smaller surface area available for absorption. Younger pigs are more sensitive to fermentation in the hindgut due to limited endogenous protease for efficient protein breakdown. This challenge is exaggerated by the high protein requirement of young pigs. Parameters which can be measured to determine the extent of protein fermentation in the hindgut include amines such as histidine, putrescine, cadavenine and spermidine, which are produced in the large intestine from arginine, lysine and histidine by bacterial groups such as *Bacteroides, Clostridium, Enterobacterium, Lactobacillus* and

*Streptococcus (*Williams *et al.*, 2001). These amines irritate the gut mucosa, causing high osmotic pressure and diarrhoea (Kim *et al.*, 2008). Anaerobic bacterial degradation of tyrosine and tryptophan in the intestinal tract results in the formation of volatile phenolic and aromatic metabolites of which phenol, p-cresol and indoles are most easily detected and have been correlated to low growth performance (Yokoyama *et al.*, 1982). As a consequence to protein fermentation, the hindgut also contains higher levels of Coliform and Clostridia species which are responsible for gut morphology changes (Jeaurond, 2008).

Protein fermentation results in ammonia release in the hindgut which acts as a base and causes a higher pH level in the hindgut. Average GIT pH levels of healthy pigs are shown in Table 2.4.

Table 2.4. Average pH values in the digestive tract of pigs (Maré, 2013)

Age	Stomach	Small intestine		Caecum	Colon
		Anterior	Posterior		
Neonatal	4.0 - 5.9	6.4 – 6.8	6.3 – 6.7	6.7 – 7.7	6.6 – 7.2
Pre-weaned	3.0 – 4.4	6.0 – 6.9	6.0 – 6.8	6.8 – 7.5	6.5 – 7.4
Weaned	2.6 – 4.9	4.7 – 7.3	6.3 – 7.9	6.1 – 7.7	6.6 – 7.7
Adult	2.3 – 4.5	3.5 – 6.5	6.0 – 6.7	5.8 – 6.4	5.8 – 6.8

Compiled from Smith and Jones (1963), Smith (1965), Boucourt and Ly (1975), Clemens *et al*. (1975), Braude *et al*. (1976), Cranwell *et al*. (1976), Barrow *et al*. (1977), Schulze (1977), Schulze and Bathke (1977) as compiled and provided by Dr L Maré, ARC.

2.7.3. Protease reduces environmental impact of pig production

Protein is the second most costly ingredient in the diet and if not completely hydrolysed, excess protein can end up in manure, in urine as urea or as ammonia gas released from stored pig slurry (Van Kempen, 2004). Manure nitrate can leak into water systems and cause contamination (Jegannathan & Nielsen, 2013). There is tremendous pressure from environmental groups on piggeries to decrease their environmental impact, control foul smells and find more efficient methods to manage pig slurry. To solve the environmental challenges facing the pig production industry, formulations need to be designed to minimise compounds of concern. Phenols, p-cresols and indoles are volatile phenolic and aromatic compounds produced during anaerobic fermentation and released from excreta causing the foul smell of piggeries (Spoelstra, 1977). Protease enzymes serve as a means to control the production of these aromatic compounds. Nitrogen and phosphorous can be decreased in pig slurry by feeding a combination of protease and phytase enzymes (Honeyman, 1993).

2.7.4. Formulation of lower protein specifications in pig diets

Exogenous protease enzymes lack specificity and digest almost all animal and vegetable protein sources (Olukosi *et al.,* 2015). The more indigestible protein present in the diet, the more potential for a protease enzyme to improve digestibility. Pigs are ideal candidates for potentially consuming food products not suitable for the human food market. However, some of these alternative feedstuffs, such as grain legumes like peas and lupins, often contain higher levels of ANF compared to conventional feed ingredients (Jezierny *et al.,* 2010). This creates an area of opportunity to use enzymes such as protease where the goal would be to decrease cost/kg pork produced by using cheaper feed ingredients with low crude protein level and digestibility (Woyengo, 2014).

2.7.5. Protease supplementation decreases the risk of developing pancreatic hypertrophy

In an attempt to overcome the effects of protease inhibitors and undegraded protein in raw legumes, the pancreas is often overstimulated resulting in hypersecretion of pancreatic enzymes. The bowman-birk and kunits trypsin inhibitors, found in soybean meal, cause increased pancreatic enzyme secretion and an enlarged pancreas (Birk, 1985). Pancreatic hypertrophy leads to increased pancreatic hypertrophy-growth inhibition mechanism where growth is inhibited, especially in young animals, brought about by excessive loss of protein secreted by the pancreas. The pancreatic enzymes are very high in sulphur containing amino acids and therefore, the endogenous losses cannot be compensated for in animals fed a soya based diet. Protease enzymes have been shown to eliminate both the bowman-birk and kunits trypsin inhibitor preventing the stimulation of pancreatic hypertrophy (Choct, 2006). Exogenous protease enzymes have also shown promising results when used in animals suffering from exocrine pancreatic insufficiency (Pierzynowski *et al.,* 2012).

Protein in soya and maize based diets have a digestibility of 80% on average (Zanella *et al.,* 1999) allowing opportunity to improve the digestibility of protein using exogenous supplemented enzymes. However, the literature is filled with examples of studies done on proteases where no positive effects were obtained (Chesson, 1993; Ghazi, 2002).

2.8. Effect of protease supplementation in broiler diets

Protease enzyme supplementation has been very successful in the poultry industry, but the value in pigs have been inconclusive (Chesson, 1993; Ghazi, 2002). Although protease can be expected to have a positive effect in broiler diets, Cowieson (2010) has established that the effect varies anywhere between 1 and 12%. Protease in the poultry diet allows for the removal of surplus undigested protein in the gut which reduces flushing kidneys in poultry systems resulting in drier litter and improved air quality (Kleyn, 2013). A study on broiler diets where 12 different batches of soya were fed, found that the growth performance, ileal digestibility and protease effects were completely dependent on the soya batch and its inherent digestibility where protease had a larger effect in diets with low inherent digestibility (Douglas, 2000). In a study where protease enzymes

were fed to male broilers at 3 levels (0, 1 & 2 times the recommended amounts) on a maize and canola meal diet, the enzymes significantly improved body weight gain at all levels and improved FCR when fed at twice the suggested level (Bradley, 2013). Slightly higher endogenous losses were found in broilers fed a soya based diet supplemented with protease compared to non-supplemented diets (Cowieson & Roos, 2013). Ileal digestibility coefficients and apparent metabolisable energy were significantly improved. In a study Barekatain *et al.* (2013), where protease was supplemented in male broilers on a diet based on maize, soybean and meat and bone meal, with various levels of protease supplementation was fed. Although the protease supplementation had no effect on body weight, feed efficiency was improved proportionately with protease supplementation levels. Although broilers on the high protein and high energy positive control diet, slightly outperformed birds on the supplemented negative control diets, an improved effect was observed with increased supplementation of protease enzymes (Barekatain *et al.,* 2013). A keratinolic protease in broiler starter diets based on maize and soybean improved BW at 21 days of age, improved FCR and showed decreased intestinal viscosity when protease was supplemented on a low protein diet (Odetallah *et al.*, 2003). This study highlighted the impact of protease supplementation on a low protein diet where energy was not a limiting factor.

2.9. Effect of protease supplementation in weaned piglets

The majority of growth studies using protease in pigs have been conducted on recently weaned piglets where variable effects on production parameters were found. Caine (1997) found no difference in the crude protein and amino acid digestibility values of soybean meal and protease treated soybean meal, where the two meals were fed to pigs of 20 days old. In a study of Rooke *et al.* (1997) protease treated soybean meal was fed to newly weaned piglets. An increase in growth rate was found but no change in antibody concentrations nor in enzyme concentrations in the small intestine was noted. Upon a histological examination of the gut health no difference was found in crypt depth and villus height in supplemented *vs* unsupplemented diets. Rantzer *et al.* (1997) found that the efficacy of protease was dependent on the age at which it was given to the pigs. If protease was given to young piglets during the first phase after weaning (up to 29 days after weaning), the positive effects of protease on feed intake and gain were negligible. However, protease administered in the second phase after weaning, from 29 days post weaning up to the grower period, more positive results were obtained in feed intake and gain. They concluded that the failure to elicit a response in performance when supplementing protease during the first phase after weaning was due to the exponentially increasing secretion of endogenous protease enzymes in this grower phase, allowing less scope for exogenous supplemented protease enzymes to have an effect. The results of this study was challenged by the opinion of Varley (2001) who proposed that protease enzyme addition would be valuable in post weaning diets because it would eliminate the disadvantages caused by low voluntary feed intake in recently weaned piglets.

2.10. Effect of protease supplementation in grower and finisher pigs

Pierzynowski *et al.* (2009) found that dependency on exocrine enzymes for growth decreased with increasing age, stating that the supplementation will have the biggest impact on weaned pigs but would have a smaller effect in grower-finisher pigs with higher levels of endogenous protease enzymes. A study by O'Shea (2014) found that growing pigs offered protease supplemented diets had a lower average daily feed intake compared to pigs which received no added protease. Pigs on the protease diet also had a lower average daily gain which was associated with lower feed intakes but they did, however, display higher illeal digestibility of nitrogen and decreased feed conversion ratio. One hypothesis on the poor response to protease enzymes in finisher diets was that the extra released nutrients might trigger a feedback mechanism to reduce feed intake, as a result of glucostatic or aminostatic responses or the protease might create an imbalance within the gastrointestinal tract of the pig (Mc Alpine *et al.*, 2012). Both scenarios would combine improved nutrient availability with lower intake, which is often observed in protease supplementation studies. In a protease study on a soya based diet where digesta was collected from ileal cannulas, pigs supplemented with protease enzymes had increased endogenous amino acid losses and lower apparent ileal digestibility compared to unsupplemented pigs (Cowieson & Roos, 2013). Another important finding of this study was that protease did not improve the digestibility of the essential amino acids as much as it improved digestibility of non-essential amino acids. In another protease supplementation study on grower-finisher pigs fed a protease supplemented maize soybean diet, no effect was observed on digestibility of dry matter, crude protein or energy (Thacker, 2005).

2.11. Effect of protease supplementation as part of an enzyme cocktail

Phytase appears to be the only exogenous enzyme which consistently performs up to expectation in pig diets (Harper *et al.*, 1997; Cromwell, 2013). Feeding a phytase enzyme decreases endogenous losses as a result of its ability to facilitate gastric and small intestine digestibility and is currently considered a standard inclusion in all monogastric diets. Additional feeding of a nonstarch polysaccharide degrading enzyme (NSPase) will further recover 15% of the remaining undigested fraction (Sultan *et al.*, 2011). Thus, when phytase is used in addition to NSPase the amino acid matrix of NSPase needs to be adjusted downwards to take into account the decrease in the undigested fraction recovered by the phytase. Sultan (2011) suggested that the combination of phytase, xylanase and protease did not result in any improvement in nitrogen digestibility. Yin *et al.* (2001) found a lack of beneficial effects on performance in growing pigs attributed to protease when protease was supplemented in conjunction with carbohydrase in a barley diet. They concluded that this was because carbohydrase increased digestibility of feed sources which resulted in less substrate available for the protease enzyme to elicit an effect. In another study by Barekatain *et al.* (2013) where xylanase and protease were supplemented together and separately in diets based on dried distillers grain soluble (DDGS) it was found that when supplementing xylanase and protease separately, xylanase improved digestibility of non-starch polysaccharides whilst protease improved amino acid digestibility. However, when xylanase and protease were

supplemented together, the response of birds to xylanase supplementation on concentrations of arabinose, xylose and total insoluble NSP was compromised. A significant lack of synergy between these two enzymes were concluded (Barekatain *et al.,* 2013). In a study where a β-glucanase and a protease enzyme were fed in combination to grower finishing pigs on a basal diet of maize and soybean meal, a decrease in apparent and standardised digestibility of methionine, alanine and serine was found. The enzyme combination did however improve the faecal digestibility of dry matter (Ji *et al.,* 2008). *In vitro* studies showed that protease present in an enzyme preparation may digest other enzymes and decrease the efficacy of the enzyme preparation (Saleh *et al.,* 2004).

2.12. Conclusion

Protein remains one of the most expensive ingredients to deliver in the pig diet. Methods to improve protein utilisation have received increased attention over the past decade, with the use of protease enzymes showing some potential for improving the efficiency of protein delivery in the diet. Protease supplementation should allow the nutritionist to formulate diets with lower crude protein levels, decreasing diet costs as well as negative effects associated with high protein diets such as protein fermentation and nitrogen excretion into the environment. It should also allow the nutritionist to include by-products in feeds which often contain high concentrations of anti-nutritional factors and permit the use of raw materials with inherently low digestibility. Protease eliminates ANF in the diet and can improve gut health by preventing proliferation of pathogenic bacteria and decreasing the incidence of hindgut fermentation. These benefits are becoming increasingly important as a means to decrease the environmental impact of piggeries.

A review of the literature on the use of protease in grower pig diets comes up short on studies to substantiate that the use of protease is beneficial in the grower pig diet. Researchers have hypothesised two main reasons for why protease sometimes fails to show positive results. The first is that protease supplementation is often correlated to a decreased feed intake. This could be due to negative feedback from increased nutrients and amino acids made available by the enzyme (O'Shea, 2014). The second reason can be related to the inherent digestibility of formulated nutrients which is essential to the magnitude and consistency of enzyme effects (Cowieson, 2010)**.** If the digestibility of the diet being fed is not inherently low enough, no effect will be seen when adding enzymes. Throughout the literature it has become clear that protease enzymes demonstrated a positive effect when added to a low protein diet, where energy was not limiting. Studies where protease was fed as part of an enzyme cocktail concluded a lack of synergy and some authors ascribed results to the fact that other enzymes decreased the substrate available for protease enzymes to elicit an effect.

Protease has many theoretical benefits, however results from current trials are inconsistent and more research is required to demonstrate how we can harness the benefits of protease enzyme supplementation in pig diets. Current knowledge is especially limited in the effects of protease on growing and finishing pigs, with the bulk of literature studies focusing on the recently weaned piglet. This study aims to investigate the effects

of supplementing protease enzymes in a diet containing underprocessed soybean meal on production, slaughter and gut health parameters in growing and finishing pigs.

The study also aimed to determine if any benefits could be derived by supplementation of protease enzymes to pigs in the grower and finisher phases (70-137 days of age). Protease was supplemented at two different levels (250 mg/kg and 500 mg/kg respectively) to determine if the level of supplementation had any significant effect on the production, slaughter and gut health parameters.

The study consisted of four treatments; a positive control, negative control, a negative control diet supplemented with 250 mg/kg Cibenza DP100 protease enzymes and a negative control diet supplemented with 500 mg/kg Cibenza DP100.

The hypothesis of this study was that protease supplementation will have a positive effect on production parameters, slaughter parameters and gut health parameters in pigs during the grower and finisher phases.

Chapter 3

Materials and methods

3.1. Trial design

In order to determine the effect of protease enzymes on grower and finisher pigs, a study was conducted using a completely randomised block design. Three feeding phases were used to feed 174 male pigs over a 10 week period. The first phase consisted of a grower 1 diet which was fed from 70 to 95 days of age. The grower 2 phase was fed from 96 to 114 days of age and finisher diets were fed from 115 to 137 days of age. Energy, protein and lysine levels of each feeding phase were adjusted to the requirement of the average animal for growth and maintenance during that specific phase, according to the average South African standard pig diet, considering minimum specification laid out by Act 36 of 1947. All parameters in the trial were measured per phase with a weighing date at the start of the trial and at the transition between each phase. Production parameters, slaughter parameters and gut health parameters were measured for each phase. Facilities included 58 pens in an enclosed housing environment with extraction fans for temperature control.

The four treatments used in the trial are described in Table 3.1. Pigs in Treatment 1 were fed a positive control diet, while pigs in Treatment 2 were fed a negative control diet. The diet for Treatment 3 consisted of the negative control diet supplemented with 250 mg/kg Cibenza DP100 whilst the negative control supplemented with 500 mg/kg Cibenza DP100 made up Treatment 4. The negative control was formulated to be marginally deficient in energy and protein, calculated using the Novus Cibenza DP100 calculator. It was also formulated to have lower amino acid levels, compared to the positive control. Due to the number of pens available in the house, pens per treatment could not be divided up equally and 15 pens were assigned for Treatment 1 and 2 and 14 pens for Treatment 3 and 4 in an unbalanced experimental design.

Table 3.1. Control and treatment diets used in the trial to evaluate the effect of protease on grower pigs

Treatment	Nutrient and energy density	Cibenza DP100	Replicates
T1: Positive control [1]	Positive Control	0	15
T2: Negative control [2]	Negative Control	0	15
T3: Negative control + 250 mg/kg protease [3]	Negative Control	250 mg/kg	14
T4: Negative control + 500 mg/kg protease [4]	Negative Control	500 mg/kg	14

[1] Positive control formulated to higher energy, protein and amino acid levels compared to other diets

[2] Negative control formulated to lower energy, protein and amino acid levels compared to the positive control

[3] Negative control supplemented with 250 mg/kg protease enzyme (Cibenza DP100, Novus Int)

[4] Negative control supplemented with 500 mg/kg protease enzyme (CibenzaDP100, Novus Int)

3.2. Experimental animals

One hundred and seventy four male pigs from the Topigs 40 genetic line, were purchased from GH Braak Piggery. The pigs were randomly selected on the farm at 10 weeks (70 days) of age and were delivered to the University of Pretoria's experimental farm in Hatfield, Pretoria, South Africa.

The pigs were unloaded and placed in holding pens with no access to food and water to prevent high water intakes from influencing pig weights. Pigs were processed which consisted of tagging, weighing and measuring of backfat thickness using the Renco backfat meter probe. After processing, a 13 kg variation in pig mass was observed. Pigs were divided in light, medium and heavy groups and an equal amount of piglets from each group were randomly allocated to each of the three blocks in the house, in an effort to minimise within-block variation. Three pigs were placed in each pen. The pigs were of similar size and weight to limit bullying and dominance. Pens containing smaller and larger pigs were evenly spread out over treatments.

Upon arrival all animals were in good health and injury free. Animal processing was conducted as fast and accurately as possible to limit stress experienced by the animals.

3.3. Health management

The animals originated from a high health unit with specific pathogen free (SPF) housing conditions. No prophylactic treatments against any diseases were given.

The pig house was clear of any pigs for one year prior to the start of the trial. The pig house was washed out with an antiseptic solution (Virkon S, Du Pont), 3 weeks and again 1 week prior to the arrival of the experimental animals.

For the duration of the trial strict hygiene measures were implemented to maintain a high level of biosecurity. All people working with the pigs were required to shower upon entry and before exiting the pig house using antiseptic soap. Overalls and gumboots were provided to workers and visitors and remained inside the pig house for the duration of the trial. The overalls were washed after use with washing machines located inside the pig house. A footbath containing Virkon (Du Pont) antiseptic solution was placed at the entrance of the pig house and used as a boot dip.

Pens had partially slatted floors. The house was equipped with a flushing system to remove manure which collected under the slats. Manure which did not fall through the slats was mucked out daily and floors

were fully washed with a high pressure hose once a week. A fly control program was implemented using fly poison.

During the grower 1 and 2 phases, three pigs showed symptoms of illness. All three pigs were lethargic, but were eating and drinking. They were treated with Advocin, Zoetis antibiotic (Danofloxacin mesylate) at a dosage of 1.5 mL per day for 3 days. Another pig developed a joint infection and had difficulty walking, with periodic body shivers. This pig was treated with 7.5 mL tetracycline (Terralon, Zoetis) for 5 days. All medications were prescribed by the consulting veterinarian and were deemed safe to inject, since they were administered in a safe time frame prior to slaughter.

3.4. Ethics approval

This project was approved for commencement by the Animal Ethics Committee of the University of Pretoria on 24 November 2014 under the project code, EC090-14.

3.5. Housing and environmental management

Animals were housed in an enclosed pig house with glass windows on both sides of the house. The house was fitted with 2 extraction fans on the northern side of the building. All windows were open at a maximum and the fans were run 24 hours per day to ensure adequate removal of heat and gas from the house. A natural lighting program was followed throughout the trial. Ambient temperatures and humidity were recorded daily.

The house consisted of 58 pens of 3.5 m^2 each, with a capacity to hold three pigs during the grower phase and two pigs during the finisher phase. Three pigs were housed per pen for a total of 43 days after which one pig per pen was randomly selected and slaughtered at 115 days of age. The two remaining pigs were housed for a further 22 days up to 137 days of age.

Each pen had partially slatted concrete floors, one nipple drinker and one feed trough. Feed and water was available *ad libitum*. Metal chains were hung from the pens of the animals to provide environmental enrichment to the pigs and were available throughout the trial.

3.6. Feed ration

A feed formulation program (Format International least cost feed formulations software) was used to formulate the pig diets for the three phases (grower 1, grower 2 and finisher). Four treatments were fed during each phase which included a positive, a negative control, and 2 diets with similar nutrient specifications to the negative control but supplemented with either 250 mg/kg Cibenza DP100 (Treatment 3) or 500 mg/kg Cibenza DP100 (Treatment 4), respectively. The maize and soya oilcake were chemically analysed for nutrient concentrations before formulation. The Novus Int. Cibenza calculator was used to determine the matrix contribution for energy and protein of 500 mg/kg Cibenza DP100 enzyme. The negative control was formulated to contain less energy and protein equaled to this matrix value. The Cibenza DP100 calculator takes

into account the levels of raw materials included in the diet as an estimate of the substrate available for the enzyme action and consequently the potential to improve digestibility of the diet.

Proximate analysis of raw materials prior to formulation:

Representative samples of two main raw material ingredients, maize and soybean meal were collected and analysed prior to feed formulation in order to formulate the diets based on the exact nutrient profiles of the raw materials. Maize samples were taken from one batch but two types of soybean meals were sampled, an underprocessed soybean meal which had a urease level of 1.7 and an optimally processed soybean meal which had a urease level of 0.3. The proximate analysis was conducted at Nutrilab, University of Pretoria, according to the Association of Analytical Chemists (AOAC International 2000) official methods of analysis. Additionally, a Near-Infrared spectroscopy (NIR) analysis was conducted on the maize and underprocessed soybean meal for comparative purposes. The NIR analyses were completed through Adisseo (Antony, France) on a calibrated NIR apparatus. Furthermore, both the underprocessed and optimally processed soya samples were analysed for amino acid concentrations. The optimally processed soya samples were analysed at the Agricultural Research Council in South Africa using the HPLC method and the underprocessed soya samples were analysed at CVAS (Cumberland Valley analytical services) in the United States, also using the HPLC method (Appendix 1).

The aim in the trial was to achieve an average urease level of 0.65 in order to test the effect of the enzyme on a slightly underprocessed soymeal included in the ration. The optimally processed soya and the underprocessed soya were mixed in a ratio of 25% underprocessed soya and 75% optimally processed soya in order to achieve a 0.65 urease level.

Diets had similar dietary fibre and fat levels to reduce its effects on microbial activities in the gut. The formulated diet contained no antibiotics or any other growth promotors or coccidiostats.

A phytase enzyme, Axtra Phy 10 000TPT (Du Pont-Delaware, United States) was included in the pig ration at 50 g/kg to provide 500 FTU/kg. The phytase enzyme's matrix values were included in the formulation according to manufacturer's recommendations. A premix containing vitamins, minerals and choline was added at 2.5 g/kg.

Table 3.2. Nutrient contributions of the phytase matrix (FTU)

Nutrient	500 FTU Matrix	Nutritional Contribution
Phosphorous	2863	0,143
Available Phosphorous	2800	0,140
Calcium	2188	0,109
NE (MJ/kg)	2100	0,105
Crude Protein	4868	0,243
Lys	301	0,015
Met	92	0,005
Cys	203	0,010
Met+Cys	294	0,015
Thr	473	0,024
Ile	406	0,020
Leu	804	0,040
Trp	124	0,006
Val	423	0,021
Na	605	0,030

The raw material inclusion levels and calculated nutrients for the formulated diets fed in each trial phase are shown in Table 3.3.

Table 3.3. Raw material inclusion and calculated nutrient levels for the positive and negative control in each dietary phase

Ingredients (%)	Grower 1 Pos control	Grower 1 Neg control	Grower 2 Pos Control	Grower 2 Neg Control	Finisher Pos control	Finisher Neg control
Yellow Maize	64,10	64,07	65,53	66,10	64,17	64,86
Soya Oilcake (46.5%CP)	16,50	15,10	11,60	10,50	7,60	6,70
Wheat Bran	16,60	18,00	20,20	20,40	25,60	25,30
Limestone (Fine)	1,100	1,100	1,180	1,520	1,250	1,750
Salt (Fine)	0,620	0,620	0,620	0,617	0,612	0,613
Mono Dicalcium Phosphate (WS>70%)	0,468	0,474	0,200	0,212	0,059	0,074
Lysine HCL (78%)	0,281	0,298	0,328	0,343	0,352	0,365
Methionine (MHA 84%)	0,007	0,002	0,000	0,000	0,002	0,000
Threonine (98%)	0,087	0,080	0,086	0,079	0,095	0,087
Axtra Phy 10000 TPT	0,005	0,005	0,005	0,005	0,005	0,005
Pig grower Premix + choline chloride	0,250	0,250	0,250	0,250	0,250	0,250
Calculated Nutrient Concentrations						
NE Pigs (MJ/kg)	9,86	9,83	9,84	9,82	9,67	9,66
DE Pigs (MJ/kg)	13,50	13,42	13,30	13,23	13,00	12,93
Crude Protein	15,69	15,26	14,11	13,67	13,01	12,60
Calcium	0,630	0,630	0,602	0,715	0,599	0,766
Phosphorous	0,680	0,680	0,629	0,627	0,620	0,616

Available phosphorous	0,295	0,300	0,244	0,244	0,219	0,219
Sodium	0,250	0,250	0,250	0,250	0,250	0,250
Total Lysine	0,875	0,853	0,783	0,762	0,711	0,691
Total Methionine	0,263	0,254	0,237	0,232	0,225	0,217
Total Methionine + Cysteine	0,549	0,535	0,504	0,492	0,479	0,466
Total Threonine	0,658	0,634	0,591	0,566	0,551	0,528
Total Tryptophan	0,176	0,171	0,156	0,150	0,143	0,137
Total Isoleucine	0,615	0,592	0,531	0,510	0,469	0,450
Total Valine	0,741	0,720	0,664	0,642	0,609	0,589
Ileal Dig. Lysine	0,817	0,793	0,725	0,705	0,647	0,630
Ileal Dig. Lysine +Cysteine	0,482	0,466	0,429	0,416	0,394	0,381
Ileal Dig. Threonine	0,531	0,509	0,472	0,450	0,434	0,414
Ileal Dig. Tryptophan	0,241	0,236	0,224	0,220	0,209	0,206
Ileal Dig. Isoleucine	0,475	0,457	0,412	0,397	0,363	0,349
Ileal Dig. Valine	0,563	0,546	0,500	0,485	0,453	0,438

Feeds were formulated in a manner that the diets were still comparable to commercial diets used in the South African pig industry.

Mixing of the feed:

Feeds were mixed at Pennville Animal Feeds using a Fountain blender. To avoid cross contamination of the enzyme, the negative control was mixed as a basal blend, after which the basal feed mixture was split up and the premix containing different levels of protease enzyme (0, 250 and 500 mg/kg, respectively) was then blended with the basal mixture. The mixer was flushed between the mixing of each batch. Each mixture was blended for 6 minutes. Samples of each diet were taken from the feed bags as they were filled. These samples were mixed and then sub-sampled so that a 1 kg sample was obtained for analysis.

3.7. Chemical analysis of feed samples

A representative sample was taken from each of the 12 final feeds (4 treatments and 3 feed phases) and were analysed according to the proximate analysis system for their nutritional content at Nutrilab (Department of Animal and Wildlife Science, University of Pretoria). The system used at Nutrilab determines seven fractions of nutrients identified in the feed including dry matter, ash, crude protein, ether extract lipid content, crude fibre, calcium and total phosphorous.

Chemical analysis was done according to the following methods:

Dry matter of feed and ash were analysed according to AOAC's official method of analysis (AOAC, 2000, Official method of analysis 942.05). Moisture determination was done according to the method followed by AOAC's official method of analysis (AOAC, 2000, Official method of Analysis 943.01). Crude fibre was determined following the AOAC's method of analysis (AOAC, 2000, Official method of Analysis 962.09) using the Fibre-Tech apparatus, as was crude fat, using the ether extract method (AOAC, 2000, Official method of Analysis 920.39). The Leco FP-428 (Leco Corporation, 3000 Lakeview Avenue, St. Joseph, MI 49085-2396) was used to analyse the nitrogen content of the feed and the method used was according to the AOAC's official method of analysis (AOAC, 2000, Official method of Analysis 988.05), with the Dumas method used to calculate total crude protein content. The calcium and phosphorus concentrations in feed were determined using the AOAC's official method of analysis, calcium (AOAC, 2000, Official method of Analysis 935.13), phosphorus (AOAC, 2000, Official method of Analysis 965.17).

3.8. Slaughter of pigs

The effect of the protease enzyme on slaughter parameters at the end of the second grower phase and at the end of the finisher phase were evaluated. One pig per pen was randomly selected for slaughter at the end of the second grower phase (115 days of age), with the remaining two pigs in each pen slaughtered at the end of the finisher phase (137 days of age). For slaughter, pigs were transported to Eskort abattoir, Heidelberg, in the afternoon, fasted overnight and slaughtered early the following morning. Pigs were stunned and rendered unconscious, then killed by exsanguination. A consulting veterinarian to Eskort abattoir was present with each slaughter and approved all carcasses used for sampling.

Upon arrival of the pigs to be slaughtered at 137 days of age, one pig appeared to suffer and was immediately slaughtered. The GIT of the pig was confiscated by the attending veterinarian and could not be obtained for sampling and evaluation of the gut parameters.

During the process of slaughter, 13 carcasses lost ear tags and could not be identified and had to be removed from the slaughter parameter results.

3.9. Sampling and data collection

Measurements were taken for production parameters, carcass parameters and gut health parameters at the end of each phase (grower 1, grower 2 and finisher).

3.9.1. Production parameters

3.9.1.1. Feed intake (FI)

Feed intake was determined per phase (ie. at the end of grower 1, grower 2 and finisher period). Pigs had *ad libitum* access to feed and feed was constantly replenished to ensure that pigs were never without feed. All feed was weighed and recorded before being placed into the feeder. At the end of each feeding phase the remaining feed was weighed back and subtracted from the total amount fed. Negligible wastage of feed was noted during the trial, by scraping up all feed on the floor around feeding troughs on a daily basis and weighing the spent feed. The feed intake was determined per pen of 3 pigs for the grower phase 1 and 2 and per pen for the finisher phase. Feed intake per pig was determined by dividing the amount of feed consumed per pen by the number of pigs in that pen. Pigs remained on the grower 2 phase feed until 115 days of age when one pig from each pen was slaughtered. The two remaining pigs immediately received the finisher diet.

3.9.1.2. Body weight, body weight gain and average daily gain

Pigs were weighed at the end of grower 1 (95 days of age), grower 2 (115 days of age) and finisher phases (137 days of age). Total gain in body weight was determined per phase by subtracting the initial body weight from the final body weight. Average daily gain (ADG) was calculated by dividing the total body weight gained by the number of days in the feeding phase. The gain and ADG were calculated per pen and then divided by the number of pigs per pen to obtain the ADG per pig.

3.9.1.3. Feed conversion ratio (FCR)

Feed conversion ratio was calculated per phase by dividing the total feed per pen per phase by the total body weight gain for the phase.

3.9.1.4. Backfat thickness

Backfat was measured at the start of the trial and at the end of each phase in the trial (grower 1, grower 2 and finisher). Backfat thickness was measured as an indicator of percentage lean meat in live pigs using ultrasound (Renco Lean Meater series 12, Minneapolis-USA). The measurement was taken at the P2 position, located vertically above the 10^{th} rib at 2.5cm from the spine. A lubricating gel was used to ensure sufficient contact between the Renco backfat meter and the pig's skin for accurate readings.

3.9.2. Carcass parameters

Post grower and post finisher slaughters were conducted at Eskort slaughter house in Heidelberg, South Africa. All pigs were slaughtered within one hour and measurements were taken immediately post slaughter.

3.9.2.1. Carcass classification

Carcasses were graded according to the PORCUS classification system which is an industry standard method used in South Africa to grade carcasses and influences the price paid for the carcass.

Table 3.4. South African carcass classification system

Classes for pork carcasses	Estimated % lean meat in carcass
Class P	70 and more
Class O	68-69
Class R	66-67
Class C	64-65
Class U	62-63
Class S	61 and less

3.9.2.2. Lean meat percentage

Lean meat percentage was determined post slaughter using the Hennessey grading probe (Hennessey grading systems Ltd, New Zealand), an optoelectronic meat grading probe, based on reflectance spectroscopy. The Hennessey grading probe was chosen for use based on its accuracy and practicality (Fortin *et al.*, 1984). The Hennessey grading probe is also a standard tool in the South African pig market as a means of calculating lean meat percentage. These measurements were taken between the 2nd and the 3rd last rib, 45 mm from the mid-back line whilst the carcass was hanging.

3.9.2.3. Carcass fat content

Carcass fat content as a percentage of carcass composition was measured immediately post slaughter by using the Hennessey grading probe (Hennessey grading systems Ltd, New Zealand), whilst the carcass was hanging. The Hennessey grading probe uses reflectance spectroscopy to record between 10 and 2000 measurements per second. The carcass fat percentage was measured at 65 mm from the backbone over the last rib.

3.9.2.4. Carcass mass

Each carcass was weighed immediately post slaughter from a hanging scale, with the head still intact but the GIT removed, to provide warm carcass mass. Cold carcass mass was taken after a 24 hour period of carcasses in the chill room at 6°C.

3.9.2.5. Carcass length

Carcass length was measured in a straight line from the junction of the first rib and sternum to the symphysis pubis (Braude *et al.*, 1957). This measurement was taken immediately post slaughter before pigs were moved into chilling rooms.

3.9.2.6. Carcass compactness

Carcass compactness (kg/cm^3) was calculated by using the following formula:

$$\text{Carcass compactness} = \frac{cold\ carcass\ mass}{carcass\ length}$$

3.9.2.7. Dressing percentage

Dressing percentage was calculated for each pig using the following formula:

$$\text{Dressing \%} = \frac{warm\ carcass\ mass}{liveweight} \times 100$$

3.9.3. Gut health parameters

3.9.3.1. Faecal score

Faeces in pens was scored weekly as an indication of gut health of the pigs and the level of fermentation in the hindgut. Pens were scored based on the consistency and texture of the faeces in the pens. Only fresh excreta was ranked using the following scale: 1 = normal hard faeces; 2 = slightly soft faeces; 3 = soft, partially formed faeces; 4 = loose, semi-liquid faeces; and 5 = watery, mucous-like faeces. The scoring was done by technicians who were unaware of the dietary treatments of the pigs.

3.9.3.2. pH values of digesta samples post slaughter

The pH of digesta from the duodenum, jejunum, ileum, caecum and colon was measured using a temperature sensitive pH meter (Hanna- HI98121). A new probe was used for the trial to ensure accuracy. The pH meter was calibrated after each group of carcasses measure, using the pH 4 and pH 7 standards. Carcasses were transported from the abattoir to the laboratory and pH was measured upon arrival at the laboratory facility, immediately post slaughter. Carcasses were placed in the cooler before processing to minimise fermentation in the gut.

3.9.3.3. Ammonia concentrations of digesta post slaughter

Digesta samples were taken from the duodenum, jejunum, ileum, caecum and colon of every pig slaughtered at 115 days of age. These samples were used to determine ammonia nitrogen concentration in the digesta as an indication of excess protein in the diet. The method was used as described by Broderick & Kang (1980).

The digesta samples were centrifuged at 1000 x G for 10 minutes to allow a liquid to separate from the solid portions. The fluid section was removed and 0.005 ml of samples, standards, and controls were pipetted into test tubes containing 0.1 N HCl. 2.5 ml Phenol red reagent was added to each test tube and mixed. A further 2 ml hypochlorite reagent was added and samples were mixed again. Tubes were then placed in a water bath at 95°C for 5 minutes in order to allow a change in colour. Tubes were then removed and cooled in ice water for 5-7 minutes until a temperature of 25-30°C was reached. Tubes were read on the spectrophotometer (Seletech-SP8001) at 630 nm. A formula was used to determine the ammonia nitrogen concentration in the digesta liquid. The method used was described for rumen fluid ammonia but was used due to the lack of a standardised method for digesta sampled from pigs. Due to the solid consistency of digesta from the caecum and colon this method could not be practically used to determine the ammonia levels in the digesta since the amount of liquid which could be obtained after centrifuging was too small to be detected by the spectrophotometer.

3.9.3.4. Intestinal epithelial morphology

Intestinal morphology samples were collected to determine villi length and crypt depth of the jejunum, as an indication of gut health. Since Cibenza DP100 is an alkaline protein it is most active in the lower small intestine (Odetallah *et al.*, 2003). The enzyme is least active in the duodenum and more active in the jejunum and ileum, therefore the jejunum was selected as a point for measuring villi lengths where the effect of the enzyme could be quantified. Jejunum samples were collected from each pig post slaughter on both 115 and 137 days of age, and preserved in a 10% buffered formalin mixture. Jejunum samples were stained on HE slides at the Department of Pathology, Faculty of Veterinary science, University of Pretoria, Onderstepoort, South Africa.

Stained samples were analysed using a Zeiss (Schott) Discovery V20 Stereomicroscope (EMS-2) at the Microscopy Unit of the University of Pretoria. Images were captured on the stereomicroscope at a 13x enlargement and were scaled to 500 µm. Villi length and crypt depth were measured using ImageTool V3 Analysis Software. The length and depth of ten villi and crypts, respectively, were measured for each image sample and the average of the ten measurements were recorded for each sample. The villi length: crypt depth ratio was further calculated, for the grower and finisher periods respectively.

3.9.3.5. Blood collection

Blood was collected from one pig per pen at 137 days of age in order to determine the level of proinflammatory cytokines released as a result of protein fermentation in the hindgut. Blood was collected in tubes at slaughter from the jugular vein. The blood tubes were centrifuged to obtain serum samples. Serum was used in antigen-capture enzyme-linked immunosorbent assay (ELISA) to detect the presence of pro-inflammatory cytokines, specifically Interleukin 1β, Interleukin 6 and Tumor necrosis Factor Alpha (TNF-α) to indicate an immune response to infection.

Pig ELISA kits (Abcam.com, United Kingdom) were used for IL1β, IL6 and TNF α, respectively. These proinflammatory cytokines were selected based on the availability of kits specifically designed for use in pigs, as well as the ease of measurement.

The test kits consisted of 96-well plates coated with antibodies against each of the cytokines, respectively. Standards were prepared, and standards and samples were pipetted into the wells. The cytokine present in the sample bound to the wells by the immobilised antibodies. The wells were then washed and biotinylated anti-Pig antibody against each of the cytokines was added. After washing away unbound biotinylated antibodies, HRP-conjugated streptavidin was pipetted into the wells. After incubation, the wells were washed again and a tetramethylbenzidine (TMB) substrate solution was added to the wells. After the addition of the TMB substrate solution, the plates were incubated at 28°C for 10 minutes. Colour developed in proportion to the amount of cytokines bound. A stop solution was then added, which changed the colour from blue to yellow. The intensity of the colour was measured at 450nm using the Bio-Tek PowerWaveX to obtain absorbance values. Process is described in Table 3.1.

After obtaining the absorbance values, the mean value of the background standards was subtracted from each absorbance value. A standard curve was plotted on a log-log graph using the absorbance value and concentration values of the standards, with standard concentration on the X axis and absorbance on the Y axis. A straight line that fitted the data best was drawn through the standard points using GraphPad. The standard curve was used to determine the concentration values for each sample in pg/mL.

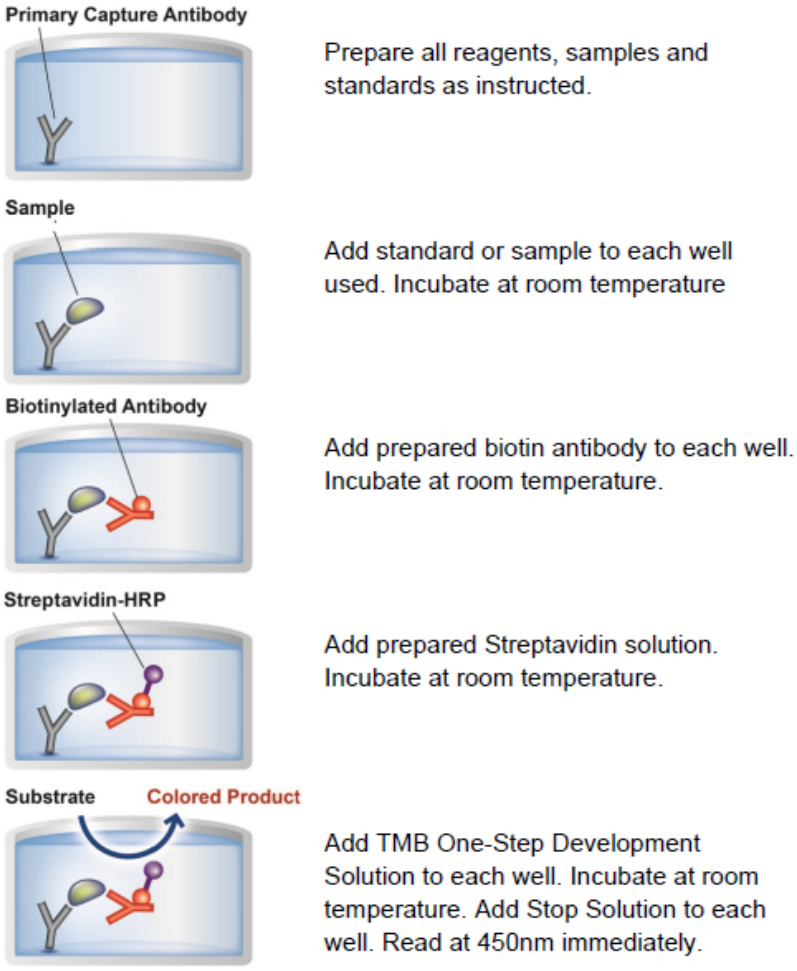

Figure 3.1. A presentation of the ELISA method used for the quantification of cytokines in the serum of pigs (www.abcam.com/pig-il-6-interleukin-6-elisa-kit-ab100755.html)

3.10. Statistical analysis

An unbalanced experimental design was used in this trial assigning 15 pens to treatment 1 and treatment 2 whilst assigning only 14 pens to treatment 3 and 4. Data was analysed statistically as a completely randomised block design with the GLM model (Statistical Analysis System, SAS, 2015) for the average effect over time. Repeated Measures Analysis of variance with the GLM model was used for repeated period measures. Means and standard errors were calculated and significance of difference ($P<0.05$) between means using the LS means test.

Standard chi-square tests were used for the frequency data and the data were analysed with the frequency model of SAS.

The linear model used is described by the following equation:

$Y_{ij} = \mu + T_i + B_j + E_{ij}$

Y_{ij} = variable studied during the period

μ = overall mean of the population

T_i = effect of the i^{th} treatment

B_j = effect of the j^{th} block

E_{ij} = error associated with each Y

For body weights and gains, starting body weight was used as a covariate, to account for the variation in starting body weights. Starting body weight did not differ between treatments at the start of the trial, however body weights at the end of the grower 2 phase differed significantly and were used as covariate for finisher phase data.

LSMEANS was used to analyse the data, with $P \leq 0.05$ being a significant difference and a tendency to be significant being recorded between $P > 0.05$ and $P \leq 0.10$.

Chapter 4

Results

4.1. Environmental conditions

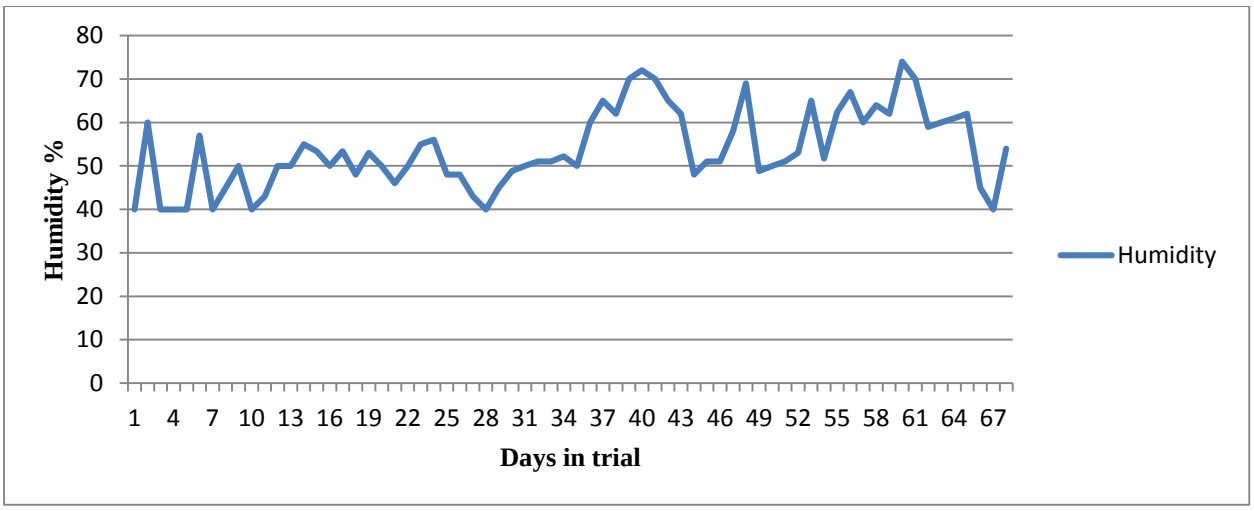

Figure 4.1. Humidity inside the pig house during the trial period

Humidity was found to be an extremely variable parameter but linked to precipitation outside the house (16°C- 33.5°C)(Figure 4.1). Humidity was also observed to be higher for short periods on days that the pig house was washed out. A slight downward trend can be seen in the daily temperature levels over the trial period (Figure 4.2). This was an expected result as the trial ran from mid- summer to end of the summer period.

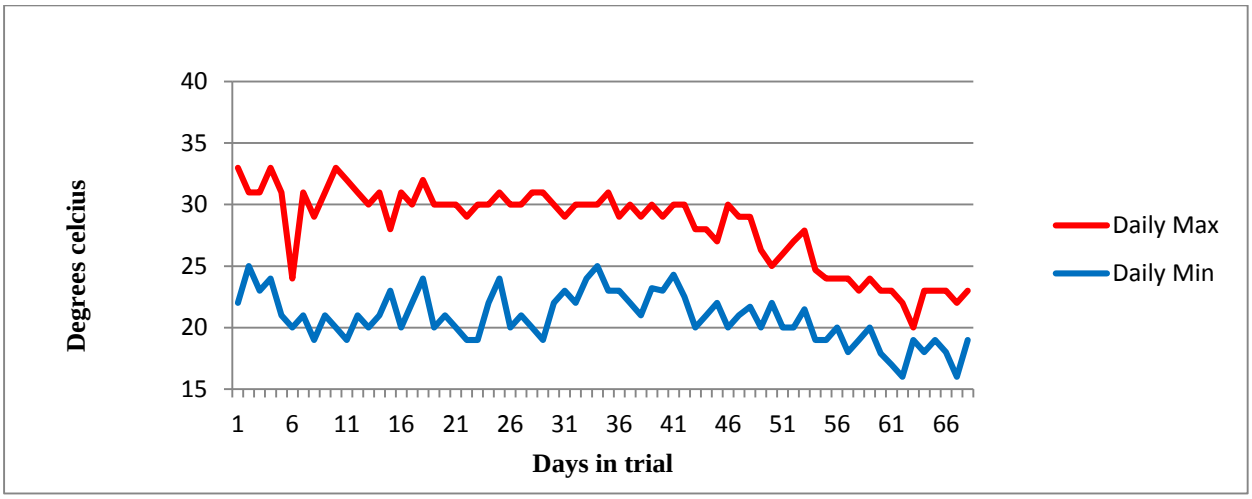

Figure 4.2. Minimum and maximum temperatures inside the pig house during the trial period

4.2. Feed ration

A proximate analysis, as well as starch and the urease test on soya oilcake, was done on the main raw materials contained in the diet to determine the nutrient levels of the raw materials, and the nutrient contributions from raw materials towards the final feed. The nutrient concentrations of the raw materials are shown in Table 4.1.

Table 4.1. Analysed nutrient concentration of the main raw materials used in the trial (As Is basis)

Nutrient %			
Parameter	**Maize**	**Soya Oilcake -Under Processed**	**Soya oilcake-Optimally Processed**
Dry Matter	88.94	87.7	90.2
Ash	1.03	6.0	5.7
Crude Protein	7.87	44.3	45.7
Fat	3.60	1.36	1.7
Crude Fibre	2.85	5.08	3
Urease	N/A	1.76	0.25
Starch	69.3	3.2	3.5

In order to achieve an underprocessed urease value of 0.6 for soya oilcake meal used in the trial, two batches of soya oilcake were blended in a ratio of 75% optimally processed soya oilcake to 25% underprocessed soya oilcake in order to achieve an average urease value of 0.6. The amino acid profile of each of the batches were measured and listed in Table 4.2.

Table 4.2. Amino acid profiles of two batches of soya oilcake (underprocessed and optimally processed)

%	Optimally processed soya oilcake	Underprocessed soya oilcake	Blend of 75 optimal: 25% underprocessed soya
Lys	3.14	2.84	3.07
Met	0.98	0.63	0.90
Thr	1.66	1.69	1.67
Val	1.86	2.16	1.94
Gly	1.58	1.86	1.65
Iso	1.7	2.03	1.78
Leu	3.28	3.4	3.31
Glu	6.4	7.62	6.71
Tyr	2.99	1.64	2.65
Phe	2.05	2.27	2.11
Ser	1.78	1.84	1.80
Pro	1.56	2.19	1.72
Ala	1.66	1.88	1.72
His	1.83	1.25	1.69
Asp	3.72	4.95	4.03
Arg	4.18	3.26	3.95
Dig. Lys	2.79	2.53	2.73
Dig. Met	0.89	0.57	0.81
Dig. Ala	1.44	1.64	1.49
Dig. Tyr	2.66	1.46	2.36
Dig. Thr	1.44	1.47	1.45
Dig Trp	5.82	6.93	6.09
Dig. Gly	1.37	1.62	1.43

Dig. Ser	1.58	1.64	1.60
Dig. Val	1.64	1.9	1.71
Dig. Iso	1.51	1.81	1.60
Dig. Asp	3.27	4.36	3.54
Dig. Pro	1.51	2.12	1.66
Dig. Leu	2.85	2.96	2.88
Dig. Phe	1.82	2.02	1.87
Dig. His	1.67	1.14	1.54
Dig. Arg	3.97	3.097	3.75

*Total amino acid values were determined using HPLC method (Cumberland Valley Analytical Services, USA)

Final feeds were analysed for dry matter, ash, starch, crude protein, fat and fibre and results for each phase and treatment are shown in Table 4.3.

Table 4.3. Proximate analysis of the final feed products on "As Is" basis

Nutrient %	G1T1	G1T2	G1T3	G1T4	G2T1	G2T2	G2T3	G2T4	F1T1	F1T2	F1T3	F1T4
Dry matter	88.300	89.072	88.373	88.692	88.795	88.897	88.978	88.928	88.706	88.609	88.509	88.595
Ash	5.625	5.351	5.126	4.868	4.617	5.190	5.049	5.049	4.523	5.396	5.121	5.066
Starch	49.750	50.227	49.821	49.660	53.710	52.689	52.658	49.472	48.902	53.961	50.128	49.714
CP	15.811	15.200	15.380	15.215	14.265	13.027	13.349	13.784	13.460	12.522	12.833	13.067
Fat	2.148	2.295	1.822	1.897	2.245	2.497	3.342	2.548	3.251	2.803	3.371	3.199
Fibre	4.298	4.396	4.541	4.144	4.404	5.045	4.945	4.805	6.247	6.440	5.200	5.539

G1T1:Grower1 Treatment 1, G1T2: Grower 1, Treatment 2, G1T3: Grower 1, Treatment 3, G1T4: Grower 1, Treatment 4, G2T1: Grower 2, Treatment 1, G2T2: Grower 2, Treatment 2, G2T3: Grower 2, Treatment 3, G2T4: Grower 2, Treatment 4, F1T1: Finisher, Treatment 1, F1T2: Finisher, Treatment 2, F1T3: Finisher, Treatment 3, F1T4: Finisher, Treatment 4

Phytase enzyme analysis:

The diet was formulated to contain expected levels of 500 FTU/kg at an inclusion of 50 mg/kg in the final feed. Representative samples of the feed were also analysed for phytase enzyme activity at Chemunique Int. with results shown in Table 4.4.

Table 4.4. Analysed levels of phytase (FTU) per treatment

Feed	Phytase recovery FTU/kg #
Grower 1/ Treat 1	1342
Grower 1/ Treat 2	1180
Grower 1/ Treat 3	858
Grower 1/ Treat 4	1135
Grower 2/ Treat 1	1137
Grower 2/ Treat 2	973
Grower 2/ Treat 3	1054
Grower 2/ Treat 4	896
Finisher/ Treat 1	911
Finisher / Treat 2	819
Finisher / Treat 3	1206
Finisher/ Treat 4	1048

AxtraPhy 10 000TPT (Du Pont-Danisco) was added to all diets at 50mg/kg to result in a phytase concentration of 500 FTU/kg.

4.3. Production parameters

4.3.1. Feed intake

Feed intake was consistently monitored to determine if the enzyme addition had any effect on the amount of feed consumed by the pig. No significant differences (P>0.05) in feed intake were found between treatments for the grower 1 (70-95 days of age) period, although numerically, pigs that were fed diets supplemented with 250 mg/kg Cibenza DP had slightly lower feed intakes compared to the other treatments. During the grower 2 period (96-115 days of age), the feed intake of the negative control tended to be significantly lower than that of the positive control (P=0.097). No significant effect on feed intake was observed for the entire grower phase, which included the grower 1 and grower 2 phases. The results of feed intake during the grower phase can be seen in Table 4.5.

Table 4.5. Mean feed intake (kg) per pig per day during the grower period (grower 1 and grower 2) (± standard error)

	Mean feed intake (kg)		
Treatment	Grower 1 (70-95 days of age)	Grower 2 (96-115 days of age)	Grower 1+2 (70-115 days of age)
T1: Positive control	1.96 ± 0.05	2.81 ± 0.07	2.31 ± 0.05
T2: Negative control	1.93 ± 0.05	2.63 ± 0.07	2.22 ± 0.05
T3: 250 mg/kg Cibenza DP100	1.85 ± 0.05	2.69 ± 0.07	2.20 ± 0.05
T4: 500 mg/kg Cibenza DP100	1.95 ± 0.05	2.73 ± 0.07	2.28 ± 0.05

No significant differences (P>0.05) for feed intake were found between treatments for the finisher period (116-137 days of age). Numerically, the pigs from the negative control seemed to have slightly lower feed intake. The feed intakes during the finisher period can be seen in Table 4.6.

Table 4.6. Mean feed intake (kg) per pig per day during the finisher period (standard error)

Mean feed intake (kg)	
Treatment	Finisher period (116-137 days of age)
T1: Positive control	3.48 ± 0.07
T2: Negative control	3.36 ± 0.07
T3: 250 mg/kg Cibenza DP100	3.49 ± 0.08
T4: 500 mg/kg Cibenza DP100	3.48 ± 0.08

4.3.2. Body weight

Pigs were weighed at the start of the trial (70 days of age) and thereafter at the end of the grower 1 (95 days of age), grower 2 (115 days of age) and finisher phases (137 days of age). Pig weights at the start of the trial (70 days of age) did not differ significantly between treatments. Body weights at the end of the grower 1 phase (95 days of age) did not differ significantly (P>0.05) between treatments. At the end of the grower 2 phase (115 days of age) pigs from the positive control group were significantly heavier than pigs from the negative control group (P<0.05). Pigs from the 250 mg/kg Cibenza DP100 supplemented group tended to be heavier compared to pigs from the positive control group (P=0.083). Starting body weights and body weights throughout the grower period are shown Table 4.7.

Table 4.7. Mean body weight (kg) of pigs at the start of the trial (70 days of age) and the end of the two grower phases (standard error)

Body weight (kg)			
Treatment	Start of the trial (70 days of age)	Grower 1 (70-95 days of age)	Grower 2 (96-115 days of age
T1: Positive control	30.6 ± 0.62	53.3 ± 0.43	73.3^{a} ± 0.75
T2: Negative control	30.6 ± 0.62	53.1 ± 0.43	70.8^{b} ± 0.75
T3: 250 mg/kg Cibenza DP100	30.9 ± 0.64	52.4 ± 0.46	71.3^{ab} ± 0.81
T4: 500 mg/kg Cibenza DP100	31.1 ± 0.64	53.2 ± 0.44	72.1^{ab} ± 0.78

^{ab} Column means with different superscripts differ significantly (P≤0.05)

Body weights of pigs did not differ significantly (P>0.05) between treatments at the end of the finisher period (137 days of age) shown in Table 4.7. and finisher body weights shown in Table 4.8.

Table 4.8. Body weights at the end of the finisher period (Day 116 to 137) with standard error

	Body weight (kg)
Treatment	Finisher (137 days of age)
T1: Positive control	97.7 ± 0.70
T2: Negative control	97.2 ± 0.70
T3: 250 mg/kg Cibenza DP100	98.2 ± 0.74
T4: 500 mg/kg Cibenza DP100	97.8 ± 0.72

4.3.3. Total body weight gain

Mean body weight gains for each phase, and for the overall grower phase (grower 1 + 2), are shown in Table 4.9. and 4.10.

No significant differences in total body weight gains between treatments during the grower 1 period (70 to 95 days of age) (P>0.05) were observed. A significant difference was observed for the grower 2 period (96-115 days of age) between total body weight gain of the positive and the negative control treatments (P<0.05). No significant differences were observed between the Cibenza DP100 supplemented diets compared to both the negative and positive control diets. Body weight gain over the overall grower phase (grower 1 and 2) was significantly more for the positive control group compared to the negative control as well as the group supplemented with Cibenza DP100 at 250 mg/kg. No significant differences were observed between the positive control and the group supplemented with 500 mg/kg Cibenza DP100. The total gains between treatments over the grower phase are shown in Table 4.9.

Table 4.9. Mean total body weight gain (kg) of pigs for the grower (70-95 days of age), grower 2 (96-115days of age), and overall grower phase (70-115 days of age) (± standard error)

Body weight gain and total body weight gain over the grower 1 and 2 phases (kg)			
Treatment	Grower 1 (70-95 days of age)	Grower 2 (96-115 days of age)	Grower 1 +2 (70-115 days of age)
T1: Positive control	22.4 ± 0.42	19.9[a] ± 0.53	42.3[a] ± 0.76
T2: Negative control	22.2 ± 0.42	17.7[b] ± 0.53	39.8[b] ± 0.76
T3: 250 mg/kg Cibenza DP100	21.5 ± 0.45	18.9[ab] ± 0.56	40.1[b] ± 0.78
T4: 500 mg/kg Cibenza DP100	22.4 ± 0.43	18.9[ab] ± 0.54	41.2[ab] ± 0.78

[ab] Column means with different superscripts differ significantly (P≤0.05)

No significant differences (P>0.05) were found between treatments for body weight gains over the finisher period (116-137 days of age) which can be seen in Table 4.10.

Table 4.10. Mean total body weight gain (kg) of pigs for the finisher phase (116-137 days of age) (standard error)

Body weight gain (kg)	
Treatment	Finisher phase (116-137 days of age)
T1: Positive control	27.2 ± 0.83
T2: Negative control	26.1 ± 0.83
T3: 250 mg/kg Cibenza DP100	27.6 ±0.88
T4: 500 mg/kg Cibenza DP100	27.2 ± 0.85

4.3.4. Average daily gain

Average daily gain (ADG) was calculated using the average gain in weight over a period divided by the number of days in the period.

No significant differences (P<0.05) were observed between treatments for the pigs in the grower 1 phase (70-95 days of age). During the grower 2 phase (96-115 days of age) significant differences in ADG (P<0.05)

between pigs from the negative and the positive control groups were demonstrated. The same trend between the positive and negative control diets was noted in data for the total grower period (grower 1 and 2). A significant difference in ADG was noted between pigs from the positive control and those that received Cibenza DP100 supplemented at 250 mg/kg in the overall grower phase (grower 1 + 2). Differences between treatments of the entire grower phase were highly significant (P<0.01) for pigs of both the positive control compared to the pigs fed the negative control diet as well as pigs fed the positive control compared to the diet supplemented with 250 mg/kg enzyme. The ADG values for the grower 1, grower 2 and overall grower phase can be seen in Table 4.11.

Table 4.11. Mean ADG for pigs in the grower 1 phase (70-95 days of age), grower 2 phase (96-115 days of age) and overall grower phase (70-115 days of age) (standard error)

Average daily gain (kg)			
Treatment	Grower 1 (70-95 days of age)	Grower 2 (96-115 days of age)	Grower 1& 2 (116-137 days of age)
T1: Positive control	0.897 ± 0.02	1.106[a] ± 0.03	0.997[a] ± 0.02
T2: Negative control	0.889 ± 0.02	0.981[b] ± 0.03	0.929[b] ± 0.02
T3: 250 mg/kg Cibenza DP100	0.857 ± 0.02	1.035[ab] ± 0.03	0.931[b] ± 0.02
T4: 500 mg/kg Cibenza DP100	0.894 ± 0.02	1.051[ab] ± 0.03	0.963[ab] ± 0.02

[ab] Column means with different superscripts differ significantly (P≤0.05)

No significant differences (P<0.05) were found between treatments for the finisher period (D116-137). Values for ADG during the finisher period can be seen in Table 4.12.

As the grower period ended with three pigs per pen whilst the finisher period ended with two pigs per pen, ADG for the overall trial period (day 70-137 of age) could not be calculated.

Table 4.12. Mean ADG (kg) for pigs during the finisher phase (116-137 days of age) (standard error)

Average daily gain (kg)	
Treatment	Finisher phase (116-137 days of age)
T1: Positive control	1.23 ± 0.03
T2: Negative control	1.21 ± 0.03
T3: 250 mg/kg Cibenza DP100	1.26 ± 0.03
T4: 500 mg/kg Cibenza DP100	1.24 ± 0.03

4.3.5. Feed conversion ratio

Feed conversion ratio (FCR) was calculated by dividing the feed intake by average daily gain to determine the efficiency with which the animal was converting food into mass as shown in Table 4.13. and Table 4.14.

No significant differences ($P>0.05$) were observed for the grower 1 period (70-95 days of age). Highly significant differences ($P<0.01$) were observed between pigs in the positive and the negative control during the grower 2 phase (96-115 days of age). Furthermore, significant differences ($P<0.05$) were observed between the pigs fed the negative control and pigs fed the Cibenza DP100 diet, supplemented at 250 mg/kg ($P<0.05$) and also between the negative control and Cibenza DP100 diet supplemented at 500 mg/kg. Data considered for the entire grower period (grower 1 & 2) showed only significant differences ($P<0.05$) between the positive and negative control. The FCR values over the grower 1, grower 2 and grower 1+2 phases can be seen in Table 4.13.

Table 4.13. Mean FCR of pigs in the different treatments during the grower 1 (70-95 days of age), grower 2 (96-115 days of age) and overall grower phase (70-137 days of age) (standard error)

	Feed conversion ratio		
Treatment	Grower 1 (70-95 days of age)	Grower 2 (96-115 days of age)	Grower 1 & 2 (70-115 days of age)
T1: Positive control	2.17 ± 0.04	2.51^{b} ± 0.05	2.32^{b} ± 0.03
T2: Negative control	2.18 ± 0.04	2.73^{a} ± 0.05	2.40^{a} ± 0.03
T3: 250 mg/kg Cibenza DP100	2.16 ± 0.04	2.60^{b} ± 0.05	2.37^{ab} ± 0.03
T4: 500 mg/kg Cibenza DP100	2.17 ± 0.04	2.58^{b} ± 0.05	2.36^{ab} ± 0.03

^{ab} Column means with different superscripts differ significantly (P≤0.05)

No significant differences were observed between the FCR of the various treatments of the finisher period. Values can be seen in Table 4.14.

Table 4.14. Mean FCR of pigs in the different treatments during the finisher phase (116-137 days of age) (standard error)

	Feed conversion ratio
Treatment	Finisher (116-137 days of age)
T1: Positive control	2.80 ± 0.06
T2: Negative control	2.85 ± 0.06
T3: 250 mg/kg Cibenza DP100	2.81 ± 0.07
T4: 500 mg/kg Cibenza DP100	2.85 ± 0.07

4.3.6. Backfat thickness

Backfat thickness was measured at the start of the trial and at the end of each phase using the Renco backfat meter probe. No significant differences (P<0.05) were found between treatments at the start of the trial (70 days of age) or at the end of the grower 1 (70-95 days of age) period. Significant differences (P>0.05) were observed at the end of the grower 2 phase (96-115 days of age) with the backfat of the positive control being significantly thicker than that of pigs from both the negative control and those that received 500 mg/kg Cibenza DP100. Backfat tended to be lower (P=0.08) in pigs that received diets supplemented with 500 mg/kg Cibenza

DP100 compared to diets supplemented with 250 mg/kg Cibenza DP100. Backfat values over the grower phases can be observed in Table 4.15.

Table 4.15. Mean backfat thickness (mm) at the start of the trial (70 days of age), for the grower 1 phase (70-95 days of age), grower 2 phase (96-115 days of age) (standard error)

	Mean backfat thickenss P[2] (mm)		
Treatment	Start of the trial (70 days of age) P[2] (mm)	Grower 1 (95 days of age) P[2] (mm)	Grower 2 (115 days of age)
T1: Positive control	4.69 ± 0.10	6.43 ± 0.10	7.57[a] ± 0.20
T2: Negative control	4.56 ± 0.10	6.37 ± 0.10	6.93[b] ± 0.20
T3: 250 mg/kg Cibenza DP100	4.53 ± 0.11	6.29 ± 0.10	7.37[ab] ± 0.20
T4: 500 mg/kg Cibenza DP100	4.60 ± 0.11	6.34 ± 0.10	6.86[b] ± 0.20

[ab] Column means with different superscripts differ significantly (P≤0.05)

No significant differences (P>0.05) were found between backfat thickness of the pigs in the finisher period but differences between the negative control and Cibenza DP100 supplemented at 250 mg/kg tended to be significant (P=0.08).

Table 4.16. Mean backfat thickness values (mm) at the end of the finisher phase (137 days of age) (standard error)

Mean backfat thickenss P[2] (mm)	
Treatment	Finisher phase (116-137 days of age) P[2] (mm)
T1: Positive control	8.15 ± 0.24
T2: Negative control	7.91 ± 0.24
T3: 250 mg/kg Cibenza DP100	8.54 ± 0.25
T4: 500 mg/kg Cibenza DP100	8.00 ± 0.25

4.4. Carcass quality parameters measured at slaughter

One pig per pen was slaughtered at the end of the grower phase (115 days of age) and the remaining two pigs in each pen were slaughtered at the end of the finisher phase (137 days of age). Carcass quality was evaluated at slaughter for each treatment by measuring lean meat percentage, carcass fat, warm and cold carcass mass, carcass length and carcass compactness.

4.4.1. Carcass classification at slaughter

Carcasses were classified according to the standard South African PORCUS classification system. Average grade of carcasses per treatment are shown in Table 4.17. and Table 4.18.

Table 4.17. Number of carcasses per treatment of pigs slaughtered at 115 days of age that received a classification score of P (> 70% lean meat) and O (68-69% lean meat)

	Number of carcasses per classification	
Treatment	P graded carcasses	O graded carcasses
T1: Positive control (n =15)	12	3
T2: Negative control (n =15)	13	2
T3: 250 mg/kg Cibenza DP100 (n=14)	13	1
T4: 500 mg/kg Cibenza DP100 (n=14)	11	3

No significant differences were found between treatments for carcass grades of pigs slaughtered at 115 days of age. Numerically, pigs supplemented with 250 mg/kg Cibenza DP 100 had the lowest count of ‘O’ scoring carcasses. Carcass grading for pigs slaughtered at the end of the finisher phase (137 days of age) are shown in Table 4.18.

Table 4.18. Number of carcasses per treatment of pigs slaughtered at 137 days of age that received a classification score of P (> 70% lean meat) and O (68-69% lean meat)

Number of carcasses per classification		
Treatment	P graded carcasses	O graded carcasses
T1: Positive control (n=24)	20	4
T2: Negative control (n=28)	20	8
T3: 250 mg/kg Cibenza DP100 (n=25)	16	9
T4: 500 mg/kg Cibenza DP100 (n=24)	18	6

No significant differences were observed in carcass grades between treatments, after slaughter at 137 days of age. After the finisher phase, carcasses from the positive control had the lowest number of carcasses with an O score.

4.4.2. Lean meat percentage of carcasses at slaughter

Lean meat percentage of carcasses was measured immediately post slaughter using a Hennessey grading probe to determine the amount of lean meat on each carcass. Significant differences were not observed between treatments at slaughter at the end of the grower phase or at the end of the finisher phase. Values of average lean meat percentage can be seen in Table 4.19.

Table 4.19. Lean meat percentage of carcasses from pigs slaughtered at 115 days of age and at 137 days of age (standard error)

Lean meat percentage of muscle (%)		
Treatment	Age at slaughter	
	115 days of age	137 days of age
T1: Positive control	69.9 ± 0.17	69.9 ± 0.18
T2: Negative control	70.3 ± 0.17	69.7 ± 0.16
T3: 250 mg/kg Cibenza DP100	70.2 ± 0.17	69.6 ± 0.18
T4: 500 mg/kg Cibenza DP100	70.1 ± 0.17	69.8 ± 0.17

4.4.3. Carcass fat percentage at slaughter

Carcass fat percentage was measured immediately post slaughter. No significant differences were observed between average fat content of the carcass at slaughter at the end of the grower and the finisher periods. A tendency for significance was observed between the negative and the positive control for the first slaughter at 115 days of age (P=0.08). The mean fat percentages of carcasses for both slaughters at 115 days of age and at 137 days of age are shown in Table 4.20.

Table 4.20. Mean fat content (%) per treatment at slaughter (standard error)

Percentage fat (%)		
Treatment	Age at slaughter	
	115 days of age	137 days of age
T1: Positive control	10.3 ± 0.36	11.2 ± 0.39
T2: Negative control	9.4 ± 0.36	11.3 ± 0.35
T3: 250 mg/kg Cibenza DP100	10.0 ± 0.38	12.0 ± 0.39
T4: 500 mg/kg Cibenza DP100	10.1 ± 0.38	11.0 ± 0.38

4.4.4. Warm carcass mass

Carcasses were weighed immediately post slaughter from a hanging scale to obtain warm carcass mass. No significant differences were found between carcasses of the various treatments at the end of the grower phase (115 days of age). Numerically, carcass mass was lowest for the negative control and highest in the pigs fed rations supplemented with 500 mg/kg Cibenza DP100. Carcasses at 137 days of age were significantly lighter for the negative control as compared to the group supplemented with 250 mg/kg Cibenza DP100. Mean warm carcass mass for pigs slaughtered at different ages is shown in Table 4.21.

Table 4.21. Mean warm carcass mass for each treatment at different ages of slaughter (standard error)

Mean warm carcass mass at slaughter (%)		
Treatment	Age at slaughter	
	115 days of age (kg)	137 days of age (kg)
T1: Positive control	53.5 ± 1.40	73.0[ab] ± 1.50
T2: Negative control	51.9 ± 1.40	70.2[b] ± 1.35
T3: 250 mg/kg Cibenza DP100	53.7 ± 1.44	74.1[a] ± 1.50
T4: 500 mg/kg Cibenza DP100	54.8 ± 1.44	71.6[ab] ± 1.44

[ab] Column means with different superscripts differ significantly (P≤0.05)

4.4.5. Cold carcass mass

Carcasses were placed in a cooler room immediately post slaughter and carcasses were weighed 24 hours later. Cold carcass mass of pigs slaughtered at 115 days of age did not differ significantly between treatments. Cold carcass mass of pigs slaughtered at 137 days of age was significantly different between the negative control and Cibenza DP100 diet supplemented at 250 mg/kg. Cold carcass mass can be observed in Table 4.22.

Table 4.22. Mean cold carcass mass (kg) for each treatment at different ages of slaughter (standard error)

Mean cold carcass mass at slaughter (%)		
Treatment	Age at slaughter	
	115 days of age kg	137 days of age kg
T1: Positive control	51.5 ± 1.37	70.6[ab] ± 1.48
T2: Negative control	49.9 ± 1.37	68.0[b] ± 1.33
T3: 250 mg/kg Cibenza DP100	51.7 ± 1.42	71.7[a] ± 1.47
T4: 500 mg/kg Cibenza DP100	52.8 ± 1.42	69.3[ab] ± 1.42

[ab] Column means with different superscripts differ significantly (P≤0.05)

4.4.6. Carcass length

Carcass lengths were measured immediately post slaughter. The carcasses of pigs from the negative control were significantly shorter than carcasses from the positive control group or pigs supplemented with 500 mg/kg Cibenza DP100, when slaughtered at 137 days of age. Values can be seen in Table 4.23.

Table 4.23. Mean carcass length (cm) for each treatment at different ages of slaughter (standard error)

Mean length (cm) of carcasses		
Treatment	Age at slaughter	
	115 days of age (cm)	137 days of age (cm)
T1: Positive control	77.1 ± 0.60	87.7[a] ± 0.64
T2: Negative control	77.5 ± 0.60	85.2[b] ± 0.58
T3: 250 mg/kg Cibenza DP100	77.8 ± 0.62	86.2[ab] ± 0.64
T4: 500 mg/kg Cibenza DP100	78.7 ± 0.62	87.0[a] ± 0.62

[ab] Column means with different superscripts differ significantly (P≤0.05)

4.4.7. Carcass compactness

As shown in Table 4.24, no significant differences were found between treatments at slaughter at the end of the grower or the finisher period. Numerically, the negative control had the lowest compactness at the end of the grower and the finisher period.

Table 4.24. Average carcass compactness for carcasses slaughtered at different ages (standard error)

Average carcass compactness		
Treatment	Age at slaughter	
	115 days of age	137 days of age
T1: Positive control	0.668 ± 0.02	0.806 ± 0.02
T2: Negative control	0.644 ± 0.02	0.798 ± 0.02
T3: 250 mg/kg Cibenza DP100	0.665 ± 0.02	0.829 ± 0.02
T4: 500 mg/kg Cibenza DP100	0.671 ± 0.02	0.801 ± 0.02

4.4.8. Dressing percentage

As shown in Table 4.25, no significant differences were found in dressing percentages between pigs of the different treatment, at the end of the grower and the finisher phase in Table 4.25.

Table 4.25. Dressing percentage of carcasses at different ages of slaughter (standard error)

Average dressing percentage		
Treatment	Age at slaughter	
	115 days of age	137 days of age
T1: Positive control	73.7 ± 1.51	73.6 ± 1.69
T2: Negative control	74.4 ± 1.51	74.8 ± 1.76
T3: 250 mg/kg Cibenza DP100	76.8 ± 1.68	75.1 ± 1.76
T4: 500 mg/kg Cibenza DP100	74.3 ± 1.61	75.4 ± 1.86

4.5. Gut health parameters

Gut health was quantified by measuring faecal score, digesta pH, ammonia, villi lengths and crypt depth as indicators of gut health.

4.5.1. Faecal scores

Pens were given weekly scores for appearance of faeces according to a faecal scoring scale, placing pens in a healthy or poor health class. No statistically significant differences were observed in faecal scores between any treatments during the grower 1 period (70-95 days of age), but pigs that were supplemented with Cibenza DP 100 had numerically higher counts of healthy pen scores compared to pens which were not supplemented. Faecal scores for the 1st grower phase (70-95 days of age) are shown in Table 4.26. data for the 2nd grower phase is shown in Table 4.26 and faecal scores for the finisher phase are shown in Table 4.27. No statistical significance was found between treatments in any phase of the trial.

Table 4.26. Mean number of healthy and poor scoring pens per treatment, based on faecal scores, in the grower 1 phase (70-95 days of age) (standard error)

Mean number of healthy and poor scoring pens		
Treatments	Healthy faecal score	Poor faecal score
T1: Positive control	7	8
T2: Negative control	7	8
T3: 250 mg/kg Cibenza DP100	10	4
T4: 500 mg/kg Cibenza DP100	9	5

Table 4.27. Mean number of healthy and poor scoring pens per treatment, based on faecal scores, for the grower 2 phase (96-115 days of age) (standard error)

Mean number of healthy and poor scoring pens		
Treatments	Healthy faecal score	Poor faecal score
T1: Positive control	11	4
T2: Negative control	10	5
T3: 250 mg/kg Cibenza DP100	13	1
T4: 500 mg/kg Cibenza DP100	14	3

Table 4.28. Mean number of healthy and poor scoring pens per treatment, based on faecal scores, for the finisher phase (116-137 days of age) (standard error)

Mean number of healthy and poor scoring pens		
Treatments	Healthy faecal score	Poor faecal score
T1: Positive control	11	4
T2: Negative control	11	4
T3: 250 mg/kg Cibenza DP100	8	6
T4: 500 mg/kg Cibenza DP100	12	2

4.5.2. Post slaughter pH in different sections of the gastrointestinal tract

Immediately post slaughter, a pH measurement was taken for the duodenum, jejunum, ileum, caecum and colon. No significant differences were observed between pH of digesta in the duodenum at slaughter on 115 days of age or at slaughter on 137 days of age. Mean pH values measured in the duodenum are shown in Table 4.29.

Table 4.29. Mean duodenal pH values of pigs in each treatment for slaughter at the end of the grower phase (115 days of age) and at the end of the finisher phase (137 days of age) (standard error)

Mean duodenal pH values		
Treatment	Age of slaughter	
	115 days of age	137 days of age
T1: Positive control	6.07 ± 0.07	6.05 ± 0.07
T2: Negative control	5.98 ± 0.07	5.99 ± 0.07
T3: 250 mg/kg Cibenza DP100	6.08 ± 0.07	5.98 ± 0.07
T4: 500 mg/kg Cibenza DP100	6.05 ± 0.07	6.03 ± 0.07

No significant differences were found between mean jejunal pH values measured at slaughter at the end of the grower phase (115 days of age) or at the end of the finisher phase (137 days of age). Mean jejunal pH values measured are shown in Table 4.30.

Table 4.30. Mean jejunal pH values of pigs in each treatment for slaughter at the end of the grower phase (115 days of age) and at the end of the finisher phase (137 days of age) (standard error)

Mean jejunal pH values		
	Treatment	Age at slaughter
	115 days of age	137 days of age
T1: Positive control	6.12 ± 0.73	6.20 ± 0.79
T2: Negative control	5.82 ± 0.73	6.12 ± 0.73
T3: 250 mg/kg Cibenza DP100	6.07 ± 0.76	6.17 ± 0.76
T4: 500 mg/kg Cibenza DP100	6.16 ± 0.76	6.16 ± 0.76

No significant differences were found between mean ileal pH values measured at slaughter at the end of the grower phase (115 days of age). Ileal pH values measured at the end of the finisher phase (137 days of age) demonstrated significant differences between the positive and the negative control. Mean ileal pH values measured are shown in Table 4.31.

Table 4.31. Mean ileal pH values of pigs in each treatment for slaughter at the end of the grower phase (115 days of age) and at the end of the finisher phase (137 days of age) (standard error)

Mean ileal pH values		
Treatment	Age at slaughter	
	115 days of age	137 days of age
T1: Positive control	6.10 ± 0.07	6.27^{a} ± 0.07
T2: Negative control	6.21 ± 0.07	6.03^{b} ± 0.07
T3: 250 mg/kg Cibenza DP100	6.13 ± 0.07	6.17^{ab} ± 0.07
T4: 500 mg/kg Cibenza DP100	6.15 ± 0.07	6.21^{ab} ± 0.07

[ab] Column means with different superscripts differ significantly (P≤0.05)

No significant differences were found between mean caecal pH values measured at slaughter at the end of the grower phase (115 days of age) or at the end of the finisher phase (137 days of age). Mean caecal pH values measured are shown in Table 4.32.

Table 4.32. Mean caecal pH values of pigs in each treatment for slaughter at the end of the grower phase (115 days of age) and at the end of the finisher phase (137 days of age) (standard error)

	Mean caecal pH values	
Treatment	Age at slaughter	
	115 days of age	137 days of age
T1: Positive control	5.20 ± 0.08	5.50 ± 0.08
T2: Negative control	5.21 ± 0.08	5.39 ± 0.08
T3: 250 mg/kg Cibenza DP100	5.23 ± 0.08	5.44 ± 0.08
T4: 500 mg/kg Cibenza DP100	5.27 ± 0.08	5.39 ± 0.08

No significant differences were found between mean colon pH values measured at slaughter at the end of the grower phase (115 days of age) or at the end of the finisher phase (137 days of age). Mean colon pH values measured in the colon are shown in Table 4.33.

Table 4.33. Mean colon pH values of pigs in each treatment for slaughter at the end of the grower phase (115 days of age) and at the end of the finisher phase (137 days of age) (standard error)

	Mean colon pH values	
Treatment	Age at slaughter	
	115 days of age	137 days of age
T1: Positive control	5.99 ± 0.07	6.16 ± 0.08
T2: Negative control	6.02 ± 0.07	6.04 ± 0.07
T3: 250 mg/kg Cibenza DP100	6.02 ± 0.07	6.05 ± 0.07
T4: 500 mg/kg Cibenza DP100	6.05 ± 0.07	6.16 ± 0.07

4.5.3. Post slaughter ammonia concentrations in gastrointestinal tract digesta

Ammonia nitrogen levels in the digesta were determined as an indicator of gut health. No significant differences were observed in ammonia concentrations measured in the duodenum, jejunum or ileum at slaughter on 115 days of age. Values are shown in Table 4.34.

Table 4.34. Mean ammonia concentrations measured in the duodenum, jejunum and ileum at slaughter at the end of the grower phase (115 days of age)

	NH_3 concentration		
Treatment	Duodenum	Jejunum	Ileum
T1: Positive control	6.17 ± 0.88	8.01 ± 0.85	11.29 ± 1.61
T2: Negative control	5.04 ± 0.95	6.29 ± 0.85	10.50 ± 1.66
T3: 250 mg/kg Cibenza DP100	4.62 ± 0.91	6.68 ± 0.82	10.81 ± 1.66
T4: 500 mg/kg Cibenza DP100	5.36 ± 0.95	7.43 ± 0.93	8.33 ± 1.66

4.6. Jejunal villi length and crypt depth

Villi length and crypt depth of the jejunum were measured as an indicator of gut health.At 115 days of age pigs from the positive control group had significantly shorter jejunal villi than pigs from any of the other treatments.However, at slaughter at the end of the finisher phase (137 days of age) the jejunal villi of the positive control pigs were numerically, but not significantly, longer than those of the other groups. Mean jejunal villi length are shown in Table 4.35.

Table 4.35. Mean jejunal villi lengths (µm) for pigs slaughter at the end of the grower phase (115 days of age) and at the end of the finisher phase (137 days of age) (standard error)

	Villi length (µm)	
Treatment	Grower phase	Finisher phase
T1: Positive control	588.9[b] ± 19.27	621.9 ± 18.13
T2: Negative control	651.3[a] ± 19.27	608.9 ± 16.84
T3: 250 mg/kg Cibenza DP100	654.3[a] ± 19.27	574.4 ± 18.05
T4: 500 mg/kg Cibenza DP100	656.9[a] ± 20.87	614.9 ± 17.39

[ab] Column means with different superscripts differ significantly (P≤0.05)

No significant differences were found between mean jejunal crypt depths for pigs slaughtered at the end of the grower phase (115 days of age), neither at the end of the finisher phase (137 days of age). Crypt depth values are shown in Table 4.36.

Table 4.36. Mean jejunal crypt depth for pigs slaughter at the end of the grower phase (115 days of age) and at the end of the finisher phase (137 days of age) (standard error)

	Crypt depth (µm)	
Treatment	Grower phase	Finisher phase
T1: Positive control	321.1 ± 8.67	309.1 ± 9.10
T2: Negative control	319.0 ± 8.40	297.8 ± 8.45
T3: 250 mg/kg Cibenza DP100	304.0 ± 9.00	291.0 ± 8.73
T4: 500 mg/kg Cibenza DP100	310.6 ± 8.67	311.0 ± 8.73

At 115 days of age pigs from the positive control group had significantly lower villi length to crypt depth ratios compared to pigs from any of the other treatments. Pigs slaughtered at the end of the finisher phase (137 days of age) showed no significant differences between any of the treatments. This followed a similar pattern as seen for villi length data. Mean jejunal villi lengths: crypt depth ratios are shown in Table 4.37.

Table 4.37. Mean jejunal villi lengths: crypt depth ratios for pigs slaughter at the end of the grower phase (115 days of age) and at the end of the finisher phase (137 days of age) (standard error)

Villi length: Crypt depth ratio		
Treatment	Grower phase	Finisher phase
T1: Positive control	1.86[b] ± 0.08	2.03 ± 0.09
T2: Negative control	2.06[a] ± 0.08	2.08 ± 0.09
T3: 250 mg/kg Cibenza DP100	2.15[a] ± 0.08	2.01 ± 0.09
T4: 500 mg/kg Cibenza DP100	2.14[a] ± 0.08	1.99 ± 0.09

[ab] Column means with different superscripts differ significantly (P≤0.05)

4.7. Blood parameters

Serum samples were collected at slaughter at the end of the finisher phase (137 days of age) and tested for the presence of proinflammatory cytokines Interleukin 1β (IL1β), Interleukin 6 (IL6) and Tumor Necrosis Factor α (TNFα) using ELISA test kits (enzyme linked immuno absorbent assay).

No significant differences (P>0.05) were found between treatments for Interleukin 1-Beta or for Interleukin 6. Significant differences (P<0.05) were found between the negative control and Cibenza DP100 supplemented at 500 mg/kg for Tumor Necrosis Factor. Values for Interleukin 1, 6 and Tumor Necrosis factor can be seen in Table 4.38.

Table 4.38. Mean cytokine concentrations found in blood serum collected at slaughter at the end of the finisher phase (137 days of age)

Mean cytokine concentration in blood			
Treatment	IL-1-Beta	IL-6	TNF
T1: Positive control	95.5 ± 45.05	333.5 ± 107.03	95.8[ab] ± 30.59
T2: Negative control	148.4 ± 45.05	336.9 ±107.03	63.9[b] ± 30.59
T3: 250 mg/kg Cibenza DP100	222.4 ± 46.53	284.6 ± 110.55	106.7[ab] ± 31.59
T4: 500 mg/kg Cibenza DP100	192.2 ± 46.53	151.4 ± 110.55	163.1[a] ± 31.59

[ab] Column means with different superscripts differ significantly (P≤0.05)

Chapter 5

Discussion

The benefits of supplementing protease enzymes to animal diets are well documented with good supporting studies in broiler chickens and weaned pigs (Chesson, 1993; Rooke *et al*., 1997; Ghazi *et al.*, 2002). These benefits include increased digestibility of previously unavailable proteins, elimination of anti-nutritional factors, such as the Bowman Birk and Kunitz trypsin inhibitors (Bedford, 1996), decreased nitrogen release into the environment (Van Kempen, 2004) and improved gut health, linked to reduced hindgut fermentation (Close *et al*., 2000). However, the literature comes up short on studies to substantiate the benefits of protease enzyme supplementation in pigs during the grower and finisher phases, with these pigs having more mature gastro intestinal tracts and developed endogenous enzyme systems.

5.1. Ration evaluation

For the negative control and feeds supplemented with protease, one batch of basal feed was mixed and split with premixes for each treatment mixed in different batch sections thereafter. The nutrient concentrations of these feeds should be very similar. Small variations in the nutrient content were noted after chemical analysis, but these variations could be attributed to analytical error and within laboratory variation. The positive control diets had higher analysed protein levels in all three feeding phases compared to the formulated CP value, which might explain the better performance seen with the positive control diets. The differences observed between the formulated and analysed protein were less than 1% (0.2-0.5%) which falls into acceptable within batch variation.

Energy levels of both the positive and negative control feeds were formulated to be lower than recommended by the NRC (2012) for growing pigs. However, amino acid concentrations in all feeds were higher than recommended by NRC (2012).

The effects of protease enzymes are dependent on the nutrient substrates available in the raw material. The more undigested proteins there are in the diet, the higher the potential for the protease enzymes to improve nutrient digestibility (Cowieson *et al.*, 2010). Two different batches of soya oilcake, one underprocessed with a urease value of 1.7 and an optimally processed soya oilcake batch with a urease value of 0.3 were blended in a 25% under processed: 75% optimally processed ratio to achieve a final urease level of 0.65. In South Africa, incorrect processing of soymeal is often reported by industry nutritionists. Over- and especially, under processed batches of soya oilcake are often observed in the feed industry.

5.2. Production parameters

In this trial, protease supplementation had no effect on the amount of feed consumed by the pigs during any phase of production. It was expected that an increased feed intake would be observed in pigs on the negative control diet in order to meet nutrient requirements. This failure to increase feed intake caused these pigs to be significantly lighter than the positive control pigs at 115 days of age. The ADG for the negative control pigs during the second grower phase was also significantly lower than that of the positive control pigs.

However, the body weights of pigs that received the protease supplemented diet did not differ from the pigs fed the positive control diet. Total body weight gain and ADG were significantly improved with protease supplemented at 500 mg/kg over the entire grower period. These results were in contrast to McAlpine *et al.* (2012) and also O'Shea *et al*. (2014) who found that protease supplemented to grower and finisher pigs resulted in reduced ADG. A possible explanation for this could be nutrient density and raw material quality of the different diets.

Feed conversion ratio was measured as an indication of efficiency of the pig to convert feed to body weight gain. Pigs on the negative control diet showed less efficient conversion of feed to body mass. The supplementation of protease to this diet, however, prevented the decrease in efficiency of feed utilisation during the second part of the grower phase. This was a significant finding, since the supplementation of protease enzymes, even at 250 mg/kg was able to achieve the same FCR as pigs that received the diet which had a higher protein concentration. O'Doherty (1999) reported similar improvements in feed conversion ratio for grower pigs in a trial where grower and finisher pigs were fed protease supplemented diets. FCR is one of the most important profitability traits in the South African pig industry because of high cost of feed and these results could therefore make a valuable contribution towards the industry.

Backfat thickness was an important measurement as an indication of lean pig growth, since the modern consumer is highly aware of the health benefits of leaner meat. At the end of the grower 2 phase (96-115 days of age), pigs from the positive control had the highest backfat level, which could be attributed to their diet with higher energy and protein. The protein and energy requirements were possibly exceeded during the second grower phase which resulted in a higher degree of backfat deposition at an earlier onset compared to the negative control diets. Backfat deposition was lowest in pigs that received the rations supplemented with 500 mg/kg Cibenza DP100 enzymes in the grower 2 phase. This could be an indication that protease resulted in more efficient protein utilisation allowing successful deposition of protein muscle over a longer period of time and lower levels of excess energy available for fat deposition.

Supplementation of protease enzymes to pigs had no significant effect on any of the production parameters (feed intake, body weight, body weight gains, ADG, FCR, back fat or faecal score) during the first grower phase (70-95 days of age) and during the finisher phase (115-137 days of age). Possibly, no responses

were observed during these phases due to an oversupply of nutrients and there was no scope for the enzyme to improve on protein utilisation.

5.3. Carcass parameters

Carcass parameters were evaluated as an indication of protease effect. Warm carcass mass, cold carcass mass, carcass classification, lean meat percentage, carcass fat, carcass length, carcass compactness and dressing percentage were measured as indicators of the effect of protease supplementation on pig carcass quality.

After slaughter, carcasses were classified according to the South African PORCUS classification system. No significant differences were found between classes assigned to carcasses at the first (115 days of age) or second (134 days of age) round of slaughter. Lean meat percentage data also demonstrated no significant differences between any treatments at slaughter. Protease supplementation had no significant effect on carcass classification as all carcasses were classified as either a P or an O. This could possibly be due to the age of the pigs at slaughter, since pigs slaughtered at 140 days of age and under, usually show P and O scores. Although protease supplementation decreased backfat deposition as measured with ultrasound at the P2 position, no effect on within carcass fat and lean meat percentage were noted after slaughtered. There was a tendency for significance in fat percentage between the positive and negative control (P=0.08) which might reflect higher available energy levels for pigs in the positive control group.

Warm and cold carcass mass are recorded by all slaughter houses in South Africa and are used as parameters on which payment to the farmer is calculated. Protease supplementation had no effect on carcass weights of pigs slaughtered at the end of the grower phase whilst pigs slaughtered at the end of the finisher phase (137 days of age) were positively affected by protease supplementation. Protease supplemented at 250 mg/kg improved carcass mass beyond the positive control and therefore profitability per carcass. Protease supplementation at 500 mg/kg resulted in carcass masses equal to those of pigs that were in the positive control treatment group. Protease enzymes supplemented at 250 mg/kg therefore delivered a higher return on investment whilst supplementing protease at a higher inclusion level showed diminishing returns. Protease supplementation possibly increased available protein for muscle deposition and improved carcass muscle mass. Protease only benefitted carcass weights when fed from the grower to the finisher phase and had no effect on pigs slaughtered at 115 days of age, at the end of the grower phase.

Carcass length was measured as it has the potential to directly affect the profit margin of the processor. Carcass length was increased by the supplementation of protease enzymes in the diets of pigs at both inclusions rates. However, carcasses from pigs that received 500 mg/kg protease enzymes had longer carcass lengths compared to those pigs that were supplemented with 250 mg/kg protease. The resulting increase of carcass length and carcass weights after pigs received feeds supplemented with protease enzymes could be related to more protein being available for protein deposition and therefore muscle growth.

Protease enzyme supplementation had no effect on carcass compactness or dressing percentages of carcasses at any supplementation level. The results of this study indicated potential for protease enzyme supplementation to significantly improve carcass weights and lengths if supplemented to pigs during the finisher phase. A review of the literature came up short on studies that measured the effect of protease supplementation to pig diets on carcass quality.

Apparent and standardised ileal digestibility of protein and amino acids are often studied in protease enzyme trials (Barekatain, 2013; Cowieson and Roos, 2013; O'Shea, 2014). This could be an important parameter for future studies to further quantify the effect of protease enzyme supplementation on pigs during the grower and finisher phases.

5.4. Gut health parameters

Faecal scores were measured as an indication of gut health, since a healthy gut would result in more consistent solid faeces compared to a gut with shorter villi length and higher levels of protein fermentation and higher incidences of diarrhoea (Kim *et al.*, 2008). No significant differences were noted between faecal scores of the pigs from the different treatments. Faecal scoring is extremely difficult to standardise since the person assigning the score has to rely on personal judgement. Faecal scoring could also be influenced by time passed since the pen was last cleaned. The factors were minimised as far as possible by having the same person conducting the faecal scoring tests and scoring the pens before cleaning. Although no statistically significant differences were observed, numerically, more pens with healthy scores were noted for pigs on supplemented diets compared to non-supplemented diets. Further investigation in future trials with higher pen numbers might describe this positive effect.

Digesta pH was measured as soon as possible post slaughter. Every effort was made to cool samples as quickly as possible after slaughter to slow down fermentation which could potentially influence gut pH values. However, some fermentation could possibly have occurred during time lapsed between processing of first to the last samples. This should be taken into consideration. More effective protein digestion, lower ammonia levels, fewer counts of harmful bacteria and reduced hindgut fermentation would result in lower pH levels (Williams *et al.*, 2001). Protease was expected to lower excess protein available for fermentation and thus ammonia production in the hindgut (Jeaurond, 2008). A higher pH would have been associated with a higher level of ammonia production (Yokoyama *et al.*, 1982). A healthy gut with low protein fermentation, colonised by healthy bacteria such as Lactobacillus, would have a lower pH due to lactic acid production in the gut (Yin, 2001). Gut pH was measured at the duodenum, jejunum, ileum, caecum and colon, but significant differences were only observed for values taken in the ileum for pigs slaughtered after the finisher phase (137 days of age). The positive control had higher pH levels which would be associated with higher ammonia production, coupled to higher protein fermentation in the hindgut. Protease also had no significant effect on gut ammonia concentrations.Villi lengths measured using ImageTool were comparable to that measured in same aged pigs

by Song *et al.*(2014), Li *et al.* (2012) and Redlich *et al.*, (1997). Villi lengths are negatively affected by protein fermentation, which is associated with higher protein containing diets. This trend was noted in the trial, where the positive control with higher levels of protein, demonstrated a lower villi length and also lower villi length to crypt depth ratios. The villi length to crypt depth ratios for the negative and supplemented diets were not significantly different, which could indicate that protease supplemented diets result in lower fermentation levels in the hindgut, comparable to that found for a lower protein diet.

A lack of response in gut health could be explained by the age of the pigs since mature pigs, with well-developed immune defences were not prone to scouring. Mature pigs also have set gut linings which are well developed. Pigs were kept in a low stress environment with little exposure to pathogens in the trial environment. The effect of Cibenza DP100 on gut health might be more accurately tested when used on pigs under stressed commercial environments.

The effect in this trial on villi length might have been due to a dietary effect with or without an enzyme effect. However, some significant differences in villi length and villi length to crypt depth ratio could indicate potential of protease on gut health and further studies might be warranted.

The aim of this trial was to quantify the effect of protease enzymes at two different levels of supplementation. Since the diets supplemented with 250 mg/kg were formulated on the 500 mg/kg matrix of the enzyme this treatment might have been slightly overestimated, but still performed well when compared to the treatment supplemented with 500 mg/kg. No incidence of significant differences between the two levels of supplementation was observed for the grower 1 phase of the trial or for the finisher phase of the trial.

5.5. Blood parameters

Blood cytokine levels were measured as an indicator of inflammatory response to protein fermentation in the hindgut. Interleukin 1, interleukin 6 and TNF were chosen based on availability of specialised pig ELIZA kits available on these cytokines (Abcam Int). The selection of these 3 cytokines also allowed the selection of cytokines from the group which enhances immune response (TNF) as well as the group which favours antibody responses (IL). Cytokines are released in animals undergoing an immune response which could possibly have been caused by protein fermentation in the hindgut (Pie *et al*., 2004).

Protease supplementation had a significant effect on TNF. Significant differences were found between pigs from the negative control and the protease group supplemented at 500 mg/kg. TNF levels for the negative control were less than half the levels of the protease supplemented diet. Low cytokine levels in the negative control were expected due to the lack of excess protein which could become available for protein fermentation. The high cytokine levels demonstrated for the pigs from the protease supplemented group was unexpected as one would expect to have reduced cytokine activity as protein digestion and utilisation improve and protein fermentation in the hindgut decreases.

Hotamisigil *et al.* (1995) found that people with obesity suffer chronic low grade inflammation and higher levels of TNF, IL1 and IL-6 were noted. Adipose tissue macrophages respond to increased fat cell mass by stimulating secretion of proinflammatory cytokines IL1-β /IL6 and TNF which in turn stimulate inflammatory pathway signalling. Data of this type has not been published in pigs yet. In this study, no correlation was observed between fat percentage and cytokine concentrations but pigs in this study were also not overly fat. Interleukin 6 cytokines are produced by adipocytes. In this study, pigs supplemented with protease at 500 mg/kg had the lowest percentages of carcass fat at slaughter which correlated with the lower levels of IL6 in these pigs, although differences were not significant compared to other treatments. IL-6 has both a pro-inflammatory and anti-inflammatory functions. Part of the anti-inflammatory action of IL-6 is the inhibition of TNF and IL-1beta (Hotamisligil *et al.*, 1995). We would therefore expect lower IL-6 concentrations to correlate with higher TNF and IL-1 beta concentrations, as observed in both protease supplemented diets.

Variation within cytokine measurements were extremely high with a standard error of 45.05 for IL-1, 107.03 for IL6 and 30.59 for TNF which was the only cytokine test where significance was observed. Due to the large number of samples which had undetected levels of cytokines found in blood samples and the high degree of variation, no significant conclusion could be drawn from these results and further investigation should be launched into determining the effect of protease enzymes on immune function in the gut.

Chapter 6

Conclusion

Studies to substantiate the efficacy of protease enzymes in broiler and weanling pigs are well documented, but there is a lack of studies which substantiates its use in grower and finisher pig diets (Chesson, 1993; Ghazi, 2002). The potential for protease to have a significant effect in rations is larger when protein sources have a lower digestibility (Douglas, 2000).

In this study, the effect of protease supplementation on grower and finisher pigs were quantified for production, carcass quality and gut health. Protease supplementation significantly improved feed conversion ratio, average daily gain and reduced backfat thickness during the second grower phase (96-115 days of age). Supplementing protease enzymes at 500 mg/kg did not improve production parameters significantly more compared to 250 mg/kg and therefore one could conclude that supplementing protease enzymes at 250 mg/kg offered a higher return on investment.

Protease supplementation demonstrated significant effects on carcass quality in pigs slaughtered at the end of the finisher phase. Protease supplemented pigs had significantly improved warm carcass mass with a similar trend observed for cold carcass mass. Since there were no significant differences in live body weight prior to the slaughter but significant differences in carcass mass, the difference was attributed to the size of the GIT. Protease supplemented diets were thus associated with a smaller GIT which was possibly more efficient and had reduced levels of fermentation. However, this study did not deliver significant evidence to show that protease enzyme supplementation had a significant effect on gut health parameters in grower and finisher pig diets.

Protease enzymes should be supplemented at 250 mg/kg to diets of grower and finisher pigs, between 96 and 137 days of age, to reduce backfat thickness and improve feed conversion ratio and average daily gain in growing pigs and to improve carcass mass at slaughter in finishing pigs. These enzymes should especially be considered under circumstances where underprocessed soymeal is used or alternative protein sources with lower digestibility are included in the diet.

Chapter 7

Critical review and recommendations

In this trial, protease supplementation had no significant effect on gut health parameters. Pigs were kept in a trial facility and were exposed to fewer physiological challenges compared to pigs in commercial group housing conditions which might have reduced scope for the enzyme supplementation to positively affect gut health. Reduced gut size compared to carcass weight in protease supplemented pigs suggested that protease supplementation should have improved gut health and future studies should further investigate these findings. The effect of protease enzymes on gut health parameters might be best researched in pigs under stressed conditions where a challenge is placed on the pigs' ability to perform. This should be considered for future research trials.

No significant conclusions could be drawn from cytokine blood studies and extremely high variability was observed within cytokine concentrations. Larger population groups should be considered for future studies in order to draw accurate conclusions.

References

Aburto, A., Vazquez, M. & Dale, N., 1998. Strategies for utilizing over processed soybean meal: II. Lysine supplementation. *The Journal of Applied Poultry Research.* 7, 196-201.

Adeola, O. & Cowieson, A., 2011. Board invited review: Opportunities and challenges in using exogenous enzymes to improve non-ruminant animal production. *Journal of Animal Science*. 89, 3189-3218.

Angel, R. & Sorbara, J. O. B., 2014. Why is it important to understand substrates if we are to optimise exogenous enzyme efficacy. *Poultry Science Journal.* 93, 2375-2379.

AOAC, 2000. Official method of analysis 935.13 (17th Edition) Volume I. Association of Official AnalyticalChemists, Inc., Maryland, USA.

Barekatain, M. R., Antipatis, C., Choct, M. & Liji, P. A., 2013. Interaction between proteases and xylanase in broiler chicken diets containing sorghum distiller dried grains with solubles. *Animal Feed Science and Technology*. 182, 71-81.

Batal, A. B., Douglas, M. W., Engram, A.E. & Parsons, C. M., 2000. Protein dispersibility index as an indicator of adequately processed soybean meal. *Poultry Science Journal.* 79, 1592-1596.

Bedford, M. R. & Apaljalahti, J., 2001. Microbial interactions in response to exogenous enzyme utilization, Wallingford, Oxfordshire, CABI.

Bedford. M. R. & Cowieson. A. J., 2012. Exogenous enzymes and their effects on intestinal microbiology. *Animal Feed Science and Technology*. 173, 76-85.

Bedford, M. R. & Partridge, G. G., 1996. Enzymes in animal nutrition. CABI Publishing, Wallingford, U.K.

Bedford, M. R. & Schulze, H., 1998. Exogenous enzymes for pigs and poultry. *Nutrition Research Reviews.* 11, 91-114.

Bergmann, M., 2009. A classification of proteolytic enzymes. *Advances in enzymology*. New York. 49-67.

Birk, Y., 1985. The Bowman-Birk inhibitor: Trypsin and Chymotrypsin inhibitor from soybeans. *Internaitonal Journal of Peptide and Protein Research,* 25, 113-131.

Boettiger, H. N., 2000. Challenges facing pig farmers. *South African Journal of Animal Science.* 1, 28-32.

Bradley, C. D., 2013. Effects of using canola meal as a protein source. PhD, University of Arkansas.

Braude, B., Mitchell, K. G. & Harrington, G., 1957. Carcass length measurement in bacon pigs. *The Journal of Agricultural Science.* 49, 357-360.

Broderick, G. A. & Kang, J. H., 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. *Journal of Dairy Science.* 63, 64-75.

Butts, C. A., 1991. Endogenous ileal amino acid excretion in monogastric animals, USA, University of Massay.

Caine, W. R., Sauer. W. C., Tanminga, S., Verstegen. M. W. & Schulze, H., 1997. Apparent ileal digestibilies of amino acids in newly weaned pigs fed diets with protease treated soybean meal. *Journal of Animal Science.* 75, 61-63.

Chessson, A., 1993. Feed enzymes. *Animal Feed Science and Technology.* 45, 65-79.

Choct, M., 2006. Enzymes for the feed industry: Past, present and future. *World's Poultry Science Journal.* 62, 5-16.

Close, W. H., 2000, Producing pigs without antibiotic growth promoters. *Advances in Pork Production.* 11, 47.

Codogan, D. & Finn, A., 2010. Influence of increasing protease supplementation on two different types of sorghum. Co-operative research centre for an internationally competitive pork industry. Queensland, Australia.

Cone, J. W., Jongbloed, A. W., van Gelder, A. H. & De Lange, L., 2005. Estimation of protein fermentation in the large intestine of pigs using gas production technique. *Animal Feed Science and Technology. 12.*

Cowieson, A. J., 2010. Strategic selection of exogenous enzymes for corn soy based poultry diets. *Journal of Poultry Science.* 47.

Cowieson, A. J. & Roos, F. F., 2013. Bioefficacy of a mono- component protease in the diets of pigs and poultry: A meta-analysis of effect on ileal amino acid digestibility. *Journal of Applied Poultry Nutrition.* 2, 47.

Cromwell, G. L., 2013. Soybean meal-an exceptional protein source. Animal and Food Science Department, University of Kentucky.

CVB, 2007, Tables of composition and nutritional value of feedstuffs, pg 71

Dawson, K. A., 2006. Nutrigenomics: Feeding the genes for improved fertility. *Journal of Animal Reproduction Science.* 96 (3), 312-322.

Dewey, C. E., 1993. Ration induced diarrhea in grower pigs. *Swine health and Production.* 2, 1-16.

Douglas, M. W., Parsons, C. M. & Bedford, M. R., 2000. Effect of various soya bean meal sources and avizyme on chick growth performance and ileal digestible energy. *Journal of Applied Research.* 9, 74-80.

Dozier, W. A., Hess, I. B., 2014. Soybean meal quality and analytical techniques. In: Soybean and nutrition, El-Shemy, H., Auburn, USA, 60-65.

Ferket, P. R. & Gernat, A., 2002. Nutritional factors that affect gut health. *Journal for Poultry Nutrition.* 73, 17-18.

Fortin, A., Jones, S. D. M. & Haworth, C. R., 1984. A note on the accuracy of the New Zealand Hennessey grading probe and the Danish Fat-o-meter in measuring fat and muscle thickness in pig carcasses. *Journal for Animal Production.* 38, 507-510.

Fru-Nji, F., Kleunter, A. & Pontoppidan, K., 2011. A feed serine protease improves broiler performance and increases protein and energy digestibility. *The Journal of Poultry Science.* 48, 239-246.

Ghazi, S., Rooke, J., Galbraith, H. & Bedford, M., 2002. The potential for the improvement of the nutritive value of soya-bean meal by different proteases in broiler chicks and broiler cockerels. *British Poultry Science Journal.* 43, 70-77.

Gonzales-Vega, J. C., Kim, B. G., Htoo, J. K., Lemme, A. & Stein, H. H., 2011. Amino acid digestibility in heated soybean meal fed to growing pigs. *Journal of Animal Science.* 89, 3617-3625.

Han, K., Lee, J. H., 2000. The role of synthetic amino acids in monogastric animal production. *Asian Australian Journal of Animal Science.* 13, 1806-9061.

Harper, A. F. & Kornegay, E. T. & Schell, T. C., 1997. Phytase supplementation of low phosphorous growing-finishing pig diets improve performance, phosphorous digestibility, bone mineralization and reduced phosphorous excretion. *Journal of Animal Science.* 75, 3174-3186.

Hasting, W. H., 1946. Enzyme supplementation to poultry feeds. *Journal of Poultry Science.* 25, 584-586.

Herkelman, K. L., Cromwell, G. L., Stahly, T. S., Pfeiffer, T. W. & Knabe, D. A., 1992. Apparent digestibility of amino acids in raw and heated conventional and low trypsin inhibitor soybeans for pigs. *Journal of Animal Science.* 70, 818-826.

Honeyman, M. S., 1993. Environment friendly swine feed formulation to reduce nitrogen and phosphorous excretion. *American Journal of Alternative Agriculture.* 8, 128-132.

Hostamisligil, G. S., Arner, P., Caro, J. F., Atkinson, R. L. & Spiegelman, B. M., 1995. Increased adipose tissue expression of tumor necrosis factor alpha in human obesity and insulin resistance. *Journal of Clinical Investigation.* 95, 2409.

Hsu, J. & Satter, L., 1995. Procedures for measuring the quality of heat treated soybeans. *Journal of Dairy Science.* 78, 1353-1361.

Huang, H., Kwok, K. C. & Liang, H., 2004. Effects of tea polyphenols on the activities of soybean trypsin inhibitors and trypsin. *Journal of the Science of Food and Agriculture.* 84, 121-126.

Jana, B., Kozlowska, A., Andronowska, A. & Jedlinska-Krakowska, M., 2008. The effect of tumor necrosis factor alpha, inteleukin-1 and interleukin-6on chorioamnion secretion of prostaglandins PGF2-alpha and E2 in pigs. *Journal of Reproductive Biology.* 8, 57.

Jansman, A. J. M., Schulze, H., Van Leeuwen, P. & Verstegen, M. W. A., 1994. Effects of protease inhibitors and lectins from soya on the true digestibility and endogenous excretion of crude protein in piglets. Forschunginstitut fur die Biologie landwirtschaftlicher Nutztiere. In: Souffrant, W. B., Hagemeister, H., Proceedings of the 6th International Symposium on digestive physiology in pigs. EAAP Publications No. 80. Bad Doberan, 4-6 October 1994, pp. 322-324.

Jeaurond, E. A., Rademacher, M., Pluske, J. R., Zhu, C. H. & De Lange, C. F. M., 2008. Impact of feeding fermentable proteins and carbohydrates on growth performance, gut health and gastrointestinal function of newly weaned pigs. *Canadian Journal of Animal Science.* 88, 271-281.

Jegannathan, K. R. & Nielsen, P. H., 2013. Environmental assessment of enzyme use in industrial production – a literature review. *Journal of Cleaner Production.* 42, 228-240.

Jensen, M. S., Jensen, S. K. & Jakobsen, K., 1997. Development of digestive enzymes in pigs with emphasis on lipolytic activity in the stomach and pancreas. *Journal of Applied Animal Science.* 75, 437-445.

Jezierny, D., Mosenthin, R. & Bauer, E., 2010. The use of grain legumes as a protein source in pig nutrition: A review. *Animal Feed Science and Technology.* 157, 111-128.

Jha, R. & Berrocoso, J. F. D., 2012. Dietary fibre and protein fermentation in the intestine of swine and their interactive effects on gut health and on the environment: A review. *Animal Feed Science and Technology. 67, 319-338.*

Ji, F., Casper, D., Brown, P., Spangler, D., Haydon, K. & Pettigrew, J., 2008. Effects of dietary supplementation of an enzyme blend on the ileal and faecal digestibility of nutrients in growing pigs. *Journal of Animal Science.* 86, 1533-1543.

Kaankuka, F. G., Balogun, T. F. & Tegbe, T. S. B., 1996. Effects of the duration of cooking of full fat soya beans on proximate analysis, levels of anti-nutritional factors and digestibility by weanling pigs. *Animal Feed Science and Technology.* 62, 229-237.

Kim, J. C., Mullan, B. P., Hampson, D. J. & Pluske, J. R., 2008. Addition of oat hulls to an extruded rice based diet for weaner pigs ameliorates the incidence of diarrhea and reduces the incidences of protein fermentation in the gastro intestinal tract. *British Journal of Nutrition.* 99, 1217-1225.

Kittawornrat, A. & Zimmerman, J. J., 2011. Towards a better understanding of pig behavior and pig welfare. Animal Health Research Reviews. 12, 25-32.

Kleyn, R. 2013. Chicken Nutrition: A guide for nutritionists and poultry professionals. England, Context products Ltd. 68-99.

Kunitz, M. & Northrop, J. H., 1963. Isolation from beef pancreas of crystalline trypsinogen, trypsin, a trypsin inhibitor and an inhibitor trypsin compound. *The Journal of General Physiology.* 19, 991-1007.

Laerke, H. & Hedemann, M., 2015. Nutritional physiology of pigs. In: The digestive enzyme of the pig. South Africa. 1-26.

Larson, G., Albarella, U., Dobney, K., Rowly-Conwy, P., Schibler, J., Tresset, A., Vigne, J. D., Edwards, C. J., Schlumbaum, A., Dinu, A., Balacsescu, A., Dolman, G., Tagliacozzo, A., Manaseryan, N., Miracle, P., Van Wijngaarden-Bakker, L., Masseti, M., Bradley, D. G. & Cooper, A., 2007. Ancient DNA, pig domestication and the spread of the Neolithic into Europe. Proceedings of the National Academy of Science, 104, 15276-15281.

Le Bellego, L., Van Milgen, J., Dubois, S. & Noblet, J., 2001. Energy utilization of low protein diets in growing pigs. *Journal of Animal Science.* 79, 1259-1271.

Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M. & Moller, K., 2002. Culture independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Applied and Environmental Microbiology.* 68, 673-690.

Li, P., Piao, X., Ru, Y., Han, X., Xue, L. & Zhang, H., 2012. Effects of adding essential oil to the diet of weaned pigs on performance, nutrient utilization, immune response, and intestinal health. *Asian-Australasian Journal of Animal Science.* 25, 1617-1618.

Liener, I., 1981. Factors affecting the nutritional quality of soya products. *Journal of the American Oil Chemists Society.* 58, 406-415.

Lindemann, M., Cornelius, S., El Kandelgy, S., Moser, R. & Pettigrew, J., 1986. Effects of age, weaning and diet on digestive enzyme levels in the piglet. *Journal of Animal Science.* 62, 1298-1307.

Maré, L., 2009. The use of prebiotics and probiotics in pigs: A review. *South African Pork Producers Organization Scientific Reviews.*

Mc Alpine, P., O'Shea, C., Varley, P. & O'Doherty, J., 2012. The effect of protease and xylanase enzymes on growth performance and nutrient digestibility in finisher pigs. *Journal of Animal Science.* 90, 375-377.

Meyer, F., Davids, T. & Van der Burgh, G., 2013. Evaluating the South African pork value chain. *BFAP.*

National Research Council, 2012. Nutrient requirements of swine. *Animal Nutrition Series.*

Nyachoti, C., Omogbenigun, F., Rademacher, M. & Blank, G., 2006. Performances responses and indicators of gastrointestinal health in early weaned pigs fed low protein amino acid supplemented diets. *Journal of Animal Science.* 84, 125-134.

O'Doherty, J. V. & Forde, S., 1999. The effect of protease and α-Galactosidase supplementation on the nutritive values of peas for growing and finishing pigs. *Irish Joural of Agriculture and Food Research.* 38(2), 217-226.

O'Shea, C. J., Mc Alpine, P. O., Solan, P., Curran, T., Varley, P. F., Walsh, A. M. & Doherty, J. V. O., 2014. The effect of protease and xylanase enzymes on growth performance, nutrient digestibility and manure odour in grower finisher pigs. *Animal Feed Science and Technology.* 189, 88-97.

Odetallah, N., Wang, J., Garlich, J. & Shih, J., 2003. Keratinase in starter diets improves growth of broiler chicks. *Journal of Poultry Science.* 82, 664-670.

Olukosi, O. A., Beeson, L. A., Englyst, K. & Romero, L. F., 2005. Effects of exogenous proteases without or with carbohydrases on nutrient digestibility and disappearance of non-starch polysaccharides in broiler chickens. *Journal of Poultry Science.* 94, 2662-2669.

Otto, E., Yokoyama, M., Hengemuehle, S., Von Bermuth, R., Van Kempen, T. & Trottier, N., 2003. Ammonia, volatile fatty acids, phenolics and odour offensiveness in manure from growing pigs fed diets reduced in protein concentrations. *Journal of Animal Science.* 81, 1754-1763.

Palliyeguru, M. C. D., Rose, S., Mackenzie, A., 2011. Effect of trypsin inhibitor activity in soya bean on growth performance, protein digestibility and incidence of sub-clinical necrotic enteritis in broiler chicken flocks. *British Journal of Poultry Science.* 52, 359-367.

Parsons, C., Hashimoto, K., Wedekind, K. & Baker, D., 1991. Soybean protein solubility in potassium hydroxide: an in vitro test of in vivo protein quality. *Journal of Animal Science.* 69, 2918-2924.

Pie, S., Lalles, J., Blazy, F., Laffitte, J., Seve, B. & Oswald, I., 2004. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. *The Journal of Nutrition.* 134, 641-647.

Pierzynowski, S., Szwiec, K., Piedra, J. V., Gruijc, D., Szymanczyk, S., Swieboda, P., Prykhodko, O., Fedkiv, O., Kruszewska, D. & Filip, R., 2012. Exogenous pancreatic like enzymes are recovered in the gut and improve growth of exocrine pancreatic insufficient pigs. *Journal of Animal Science.* 90, 324-326.

Qaisrani, S. N., Van Krimpen, M. M., Kwakkel, R. P., Verstegen, M. W. A. & Hendriks, W. H., 2015. Dietary factors affecting hindgut protein fermentation in broilers: A review. *World's Poultry Science Journal.* 71, 139-160.

Qin, G., Elst, E. R., Bosch, M. W. & van der Poel, A. F. B., 1996. Thermal processing of whole soybeans: Studies on the inactivation of antinutritional factors and effects on ileal digestibility in piglets. *Animal Feed Science and Technology.* 57, 313-324.

Rantzer, S. R., Kiela, P., Thael, M. J., Svedsen, J., Ahren, B., Karlsson, S. & Pierzynowski, S. G., 1997. Pancreatic exocrine secretion during the first days after weaning. *Journal of Animal Science.* 1324.

Redlich, J., Souffrant, W. B., Laplace, J. P., Hennig, U., Berg, R. & Mouwen, J., 1997. Morphometry of the small intestine in pigs with ileo rectal anastomosis. *Canadian Journal of Veterinary Research.* 61, 21.

Rist, V. T.S., Weiss, E., Eklund, M. & Mosenthin, R., 2013. Impact of dietary protein on microbiota composition and activity in the gastrointestinal tract of piglets in relation to gut health: A review. *Joural of Animal Science.* 7, 1067-1078.

Rooke, J. A., Slessor, M., Fraser, H. & Thomson, J. R., 1997. Growth performance and gut function of piglets weaned at four weeks of age and fed protease treated soybean meal. *Animal Feed Science and Technology.* 70, 175-190.

Saleh, F., Ohtsuka, A., Tanaka, T. & Hayashi, K., 2004. Carbohydrases are digested by proteases present in enzyme preparations during in vitro digestion. *The Journal of Poultry Science.* 41, 229-235.

Song, J., Xiao, K., Ke, Y., Jiao, L., Hu, C., Shi, B. & Zhou, X., 2014. Effect of a probiotic mixture on intestinal microflora, morphology and barrier integrity of broilers subjected to heat stress. *Journal of Poultry Science.* 93, 581-588.

Spoelstra, S. F., 1997. Simple phenols and indoles in anaerobically stored piggery wastes. *Journal of the Science of Food and Agriculture.* 28, 415-423.

Sultan, A., Gan, C., Li, X., Zhang, D. & Bryden, W., 2011. Dietary enzyme combinations improve sorghum ileal protein and starch digestibility during the broiler starter phase. 22nd Annual Australian Poultry Science Symposium. 82.

Suryawan, A., Jeyapalan, A. S., Orellana, R. A., Wilson, F. A., Nguyen, F. A. & Davis, T. A., 2008. Leucine stimulates protein synthesis in skeletal muscle of neonatal pigs by enhancing mTORC1 activation. *American Journal of Physiology-Endocrinology and Metabolism.* 295, E868-E875.

Thacker, P. A., 2005. Effect of Xylanase and Protease on performance of growing-finishing pigs fed corn based diets. *Journal of Animal Research.* 28, 17-23.

Thorpe, J., Beal, J., Bedford, M. & Partridge, G., 2001. Vegetable protein meals and the effects of enzymes. *Enzymes in Animal Nutrition.* 125-143.

Torrallardona, D., Roura, E., 2009. Voluntary feed intake in pigs, Wageningen Academic Publications.

Twinning, S. S., 1994. Regulation of proteolytic activity in tissues. *Critical reviews in biochemistry and molecular biology*. 29, 315-383.

Upadhaya, S. D., Park, J. W., Lee, J. H. & Kim, I. H., 2016. Ileal digestibility of nutrients and amino acids in low quality soybean meal sources treated with ß-mannase for growing pigs. *Animal Science Journal*, 10:7, 1148-1154.

Van Kempen, T., 2004. Towards zero waste swine production, London Swine Conference. 73.

Varley, M. A., Wiseman, J., 2001. The weaner pig: Nutrition and management , New York, CABI Publishing.

Vasconcelos, I. M. & Oliveira, J. T. A., 2004. Antinutritional properties of plant lectins. *Toxican.* 44, 385-403.

Ven, J. T., Hymowitz, T. & Jensen, A. H., 1974. Effects of soybeans of different trypsin inhibitor activities on performance of growing swine. *Journal of Animal Science.* 38.

Walsh, G. A., Power, R. F. & Headon, D. R., 1993. Enzymes in the animal feed industry. *Trends in Biotechnology*. 11, 424-430.

Wang, T. & Fuller, M., 1989. The optimum dietary amino acids pattern for growing pigs. *British Journal of Nutrition.* 62, 77-89.

Wellock, I., Fortomaris, P., Houdijk, J. & Kyriazakis, I., 2008. Effects of dietary protein supply, weaning age and experimental enterotoxigenic Escherichia coli infection on newly weaned pigs health. *Journal of Animal Science.* 6, 834-842.

Williams, B. A., Bosch, M. W., Boer, H., Verstegen, M. W. A. & Tamminga, S., 2001. An in vitro batch culture method to assess potential fermentability of feed ingredients for monogastric diets. *Animal Feed Science and Technology.* 123, 445-462.

Willis, S., 2003. The use of soybean meal and full fat soybean meal by the animal feed industry, 12th Australian Soybean conference, Toowomba, Queensland: North Australian Soybean industry Association, pp 1-8.

Windley, K., De Preter, V. & Verbeke, K., 2012. Revelance of protein fermentation to gut health. *Molecular Nutrition and Food Research Journal.* 56.

Woyengo, A., 2014. Non Ruminant nutrition symposium: Controlling feed costs by including alternative ingredients into the pig diets: A review. *Journal of Animal Science.* 92.

Xu, J., 2014. Effect of glycinin and B Conglycinin on the absorbing capacity of mouse intestinal epithelial cells. *Asian Journal of Animal Science.* 8.

Yin, Y. L., Baidoo, S. K., Jin, L. Z., Liu, Y. G., Schulze, H. & Simmins, P. H., 2001. The effect of different carbohydrase and protease supplementation on apparent (ileal and overall) digestibility of nutrients of five hulless barley varieties in young pigs. *Livestock Production Science.* 71, 109-120.

Yokoyama, M. T., Tabori, C., Miller, E. R. & Hogberg, M. G., 1982. The effects of antibiotics in the weanling pig diet on growth and the excretion of volatile phenolic and aromatic bacterial metabolites. *American Journal of Clinical Nutrition.* 35, 1417-1424.

Zanella, I., sakomura, N., Silversides, F., Fiquerido, A. & Pack, M., 1999. Effect of enzyme supplementation of broiler diets based on corn and soybeans. *Journal of Poultry Science.* 78, 561-568.

Zhou, L., Fang, L., Sun, Y., & Zhu, W., 2015. Effects of the dietary protein level on the microbial composition and metabolomic profile in the hindgut of the pig. *Anaerobe.* 8, 61-69.

Zijlstra, R. T., Owusu-Asiedu, A. & Simmins, P. H., 2010. Future of NSP degrading enzymes to improve nutrient utilization of co-products and gut health in pigs. *Journal of Livestock Science.* 134, 255-257.

Appendix:

A Treatment 3 pen (Negative control + 250 mg/kg Cibenza DP100)

These pens were observed to be much cleaner with fewer incidences of diarrhea

Figure A1: Faecal scoring in pens (Protease supplemented pen)

Treatment 2 pens (Negative controls) were visually observed to be dirtier and have a higher incidence of diarrhea

Figure A2: Visual pen scores (Negative control pen)

A view of the pig house

Figure A3: Pig house

Pigs at 116 days of age

Figure A4: Pigs-day one of finisher phase

University of Pretoria: washing the pig house out with antiseptics prior to the arrival of the pigs

Figure A5: Disinfecting of house prior to pig arrival

Final feeds bagged and ready to be sampled and labeled

Figure A6: Feed batch

Manual mixing of the trial feeds using a fountain blender. Each treatment was mixed in 2 batches, and then blended again to ensure proper mixing and product distribution

Figure A7: Fountain mixer used to mix feed