UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

EFFICACY OF EXOGENOUS PHYTASE AND PROTEASE ENZYMES ON PERFORMANCE AND GASTRO-INTESTINAL HEALTH IN BROILER CHICKENS

By

Phokela Jonathan Segobola

Submitted in partial fulfilment of the requirements for the degree

MASTER OF SCIENCE IN AGRICULTURE

MSc. (Agric) ANIMAL SCIENCE

In the

Faculty of Natural and Agricultural Sciences
Department of Animal and Wildlife Sciences
University of Pretoria, Pretoria 0002
South Africa

November 2016

Department of Animal and Wildlife Sciences
University of Pretoria

DECLARATION

I, Phokela Jonathan Segobola declare that the thesis/dissertation, which I hereby submit for the degree M.Sc. (Agric) Animal Science at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other institution.

_____ Date: 30 November 2016

Mr. Phokela Jonathan Segobola

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I am grateful to have been given the opportunity to carry my project under the guidance of Dr Christine Jansen van Rensburg. Thank you for your scientific inputs, patience, constructive criticism and determination to see the project to success.

Many thanks to DSM Nutritional Products for providing financial support, premixes and enzymes for the trial. My sincere gratitude goes to Mrs. Annekie O'Rourke for giving me the opportunity to be involved with DSM research project. To Mr. Francois Nell, thank you for assisting with the trial set up and constant guidance throughout.

Thank you to the Nutri Lab staff for all your hard work. Marie Smith and Roelf Coertze for your assistance with statistical analysis, much appreciated.

Pennville Animal Nutrient Solutions for accurate mixing of trial feed.

Thanks to Bonita and James, for their dedicated assistance with the day to day management of the poultry house. Many thanks to Njabulo and the M.Sc. students for helping with the weighing and catching.

My parents, sister and brother for the courageous support, special thanks to my brother Madimetja for physically helping out during late hours. Special appreciation goes to my son Maduele and my wife Jane for their understanding and courageous support throughout.

Almighty God, for always giving me undistracted Strength, Wisdom, Courage, Persistence and for His grace.

LIST OF ABBREVIATIONS

AA	Amino Acids
AGP	Antimicrobial Growth Promoter
AIA	Acid Insoluble Ash
ANF	Anti-Nutritional Factors
AME	Apparent Metabolisable Energy
BBI	Bowman-Birk inhibitor
BWG	Body Weight Gain
BW	Body weight
CFI	Cumulative Feed Intake
CP	Crude Protein
CRD	Completely Randomised Design
CFCR	Mortality corrected feed conversion ratio
ECP	Embryonic Chicken Pepsin
GT	Granulated Thermostable
FCR	Feed Conversion Ratio
GE	Gross Energy
GRP	Gastrin Releasing Peptide
IP	Inositol Phosphate
KSTI	Kunitz Inhibitors
ME	Metabolisable Energy
MoFC	Margin Over Feed Cost
nPP	none Phytate Phosphorus
PA	Phytic Acid
Pi	Inorganic Phosphorus
PP	Phytate Phosphorus
PROT	Protein
ROI	Return On Investment
SMB	Soya Bean Meal
SOS	Sudden Death Syndrome
XAP	Xylanase, Amylase, Protease
WPS	Water Soluble Phosphate

ABSTRACT

The efficacy of exogenous phytase and protease, alone and in combination, or in combination with AGP, zinc bacitracin on performance and intestinal morphology in broilers was tested. Broilers were fed a diet containing nutrients at recommended levels (positive control, PC) with no enzyme supplementation or a diet with lower nutrient density than PC, either without enzyme supplementation (negative control, NC) or with enzyme(s) (Treatments, Trt). Nutrient specifications of NC1, 2 and 3 diets were decreased by levels expected to be made available by Ronozyme® HiPhos, Ronozyme® ProAct and a combination of Ronozyme® HiPhos and ProAct, respectively. The NC1, 2 and 3 diets contained the same level of nutrients than the NC counterpart, but with the respective enzymes supplemented to the diets. At 34d of age, body weight (BW), but not feed intake (FI), of NC1 was lower ($P<0.05$), and feed conversion ratio (FCR) not significantly different than PC. With the addition of 100 mg/kg of a phytase product (Trt1), no difference in performance of broilers was noted compared to PC. Broilers in NC2 and Trt2 groups did not differ in performance from PC, suggesting that nutrients in NC2 were not lowered sufficiently to limit performance. BW and FI, of NC3 was lower and FCR was not significantly different than PC. With the addition of phytase and protease (Trt3), performance of broilers was similar to PC. Addition of zinc bacitracin to Trt3 did not improve any of the performance parameters compared to Trt3. There was no difference in metatarsal bone ash mass, ash concentrations, densities and percentage Ca and P of birds fed PC or NC or diets supplemented with enzymes. While no differences were found for the ileum, phytase supplementation to the diet increased the duodenal villi length: crypt depth from 8.20 (PC) to 10.75 and 10.79 (Trt1 and Trt3, respectively). No differences in this ratio were noted between PC and the NC diets. The addition of phytase, alone and in combination with a protease, increased the available nutrient levels, improved duodenal villi height to crypt depth ratio and subsequently improved broiler production. The study was therefore conducted to determine the efficacy of exogenous phytase and protease, alone and in combination, or in combination with AGP, zinc bacitracin on performance and intestinal morphology in broilers from day old to 35 days of age.

CHAPTER 1

INTRODUCTION

Monogastric animals cannot synthesise phytase in sufficient quantities for an efficient utilisation of sources of phosphorus from plant materials (Nelson, 1967). Thus, much of the phytate phosphorus present in the diet is voided in the excreta. This is a source of environmental pollution, especially when environmental microbes capable of degrading phytate to release bound phosphorus act on the excreta. Furthermore, phytic acid has a strong chelating potential in the gut and can make up complexes with minerals, starch and protein (Kies *et al*., 2001), thereby reducing bioavailability of these nutrients substantially. Singh and Krikorian (1982) also suggested that phytate may inhibit proteolysis by altering the protein configuration. Phytate is also known to inhibit a number of digestive enzymes such as pepsin, alpha-amylase (Deshpande and Cheryan, 1984) and trypsin (Caldwell, 1992). Addition of various types of exogenous phytase enzymes and other exogenous enzymes has been widely accepted in broiler feed to address challenges described above. The additive, Ronozyme® HiPhos (DSM Nutritional Products, Kaiseraugst, Switzerland), has been described as a phytase enzyme preparation containing 6-phytase, produced by a genetically modified strain of *Aspergillus oryzae* (Lichtenberg *et al*., 2011).

The enzymes that degrade protein are called proteases. Proteases are characterised by their ability to hydrolyse peptide bonds before or after specific amino acids. Exogenous protease enzymes have been used as a feed additive over recent years to increase the digestibility of protein in the diets of monogastric animals. The wide range of endogenous proteases synthesised and released in the gastrointestinal tract (GIT) are generally considered sufficient to optimise feed protein utilisation (Le Heurou-Luron *et al*., 1993; Nir *et al*., 1993). Several authors have done studies on crude protein (CP) and amino acid (AA) digestibility in poultry diets and have reported that there is a valuable amount of crude protein (CP) passing through the GIT without being completely digested (Parsons *et al*.,1997; Wang and Parsons, 1998; Lemme *et al*., 2004). This undigested protein represents an opportunity for the use of supplemental exogenous proteases in broiler feeds to improve protein digestibility.

Ronozyme® ProAct (DSM Nutritional Products Kaiseraugst, Switzerland) is described by Fru-Nji *et al*. (2011) to be a purified mono-component serine protease expressed in *Bacillus licheniformis*, which is heat stable at the activity of 75,000 PROT/g. One protease unit is equivalent to one PROT which is defined as the amount of enzyme that releases 1mmol of p-nitro-aniline from 1mM substrate (Suc-Ala-Ala-Pro-Phe-pNa) per minute at pH of 9 and 37 ºC. Ronozyme® ProAct GT is stable to processing temperature of up to 95 ºC (Fru-Nji *et al*., 2011).

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Zakaria *et al*. (2010) described the use of exogenous feed enzymes in monogastric diets as an effective tool to allow flexibility in diet formulation and to lower feed cost, improve digestibility of feed and reduce environmental pollution. For optimal efficiency, exogenous enzymes need to complement the function of the endogenous enzymes secreted by the animal itself (Doskovic *et al*., 2013). In addition, the quality of the dietary ingredients or primarily the substrate concentration is crucial in the responsiveness of feed to enzyme supplementation (Cowieson and Ravindran, 2008).

The success of exogenous feed enzymes in improving utilisation of raw ingredients and in animal performance has resulted in the introduction of a number of commercial enzyme products to the feed industry as illustrated in Table 2.1.

Table 2.1: Types of exogenous enzymes, target substrates and production organism (Munir and Maqsood, 2013)

Enzyme name	Classification	Production organism	Targeted function
α – Amylase	Carbohydrase	*Aspergillus ssp, Bacillus spp, Rhizopus*	Starch hydrolysis
Maltogenic amylase	Carbohydrase	*Bacillus subtilis, d- Balillus stearothemophilus*	Starch hydrolysis and production of maltose
β – Amylase	Carbohydrase	*Barley malt*	Starch hydrolysis and production of maltose
Cellulase	Carbohydrase	*Aspergillus niger*	Cellulose breakdown
α –Galactosidase	Carbohydrase	*Aspergillus niger, Morteirella vinaceae var, Saccharomyces spp.*	Oligosaccharides hydrolysis
β – Glucanase	Carbohydrase	*Aspergillus spp, Bacillus spp,*	β-glucans hydrolysis
β – Glucosidase	Carbohydrase	*Aspergillus niger*	Hydrolyses cellulose degradation products to glucose
Glucoamylase (amylo-glucosidase)	Carbohydrase	*Aspergillus niger, Apergillus oryzae, Rhizopus niveus, Rhizopus oryzae*	Starch hydrolysis with production of glucose
Hemicellulase	Carbohydrase	*Aspergillus spp, Bacillus spp, Humicola spp, Trichoerma spp*	Break down hemicellulose
Invertase	Carbohydrase	*Aspergillus niger, Sacchatomyces spp*	Hydrolyse sucrose to glucose and fructose
Lactase	Carbohydrase	*Aspergillus niger, Aspergillus oryzae,*	Hydrolyse lactose to glucose and galactose
β – Mannanase	Carbohydrase	*Aspergillus niger, Bacillus lentus, Trichoderma spp. Trichoderma reeseic*	Beta-mannans hydrolysis
Pectinase	Carbohydrase	*Aspergillus niger, Rhizopus oryzae, Aspergillus aculeatus*	Pectin hydrolysis
Pullulanase	Carbohydrase	*Bacillus acidopullulyticus*	Starch hydrolysis
Xylanase	Carbohydrase	*Aspergillus spp, Bacillus spp, Humicola spp., Penicilin spp., Trichoderma spp.*	Xylan hydrolysis
Lipase	Lipase	*Aspergillus niger, Candida spp, Rhizomucor spp, Rhizopus spp.,*	Hydrolyses triglycerides, diglycerides and glycerol monoesters
Bromelian	Protease	*Pineapple (Ananas comosus) stem and fruit*	Protease hydrolysis
Ficain	Protease	*Ficus glabrata*	Protease hydrolysis
Keratinase	Protease	*Bacillus licheniformis*	Protease hydrolysis
Papain	Protease	*Papaya (Carica papaya)*	Protease hydrolysis
Pepsin	Protease	*Animal stomach*	Protease hydrolysis
Protease	Protease	*Aspergillus niger, Aspergillus spp, Bacillus spp.*	Protease hydrolysis
Trypsin	Protease	*Animal pancreas*	Protease hydrolysis
Catalase	Oxidoreductase	*Aspergillus niger variants, mycrococcus lysodeikticus*	Produces water and oxygen from hydrogen peroxide
Glucose oxidase	Oxidoreductase	*Aspergillus niger*	Degrade glucose to hydrogen peroxide and gluconic acid
Phytase	Phytase	*Aspergillus niger*	Phytate hydrolysis

2.2 Enzymes in maize-soya based diets

The early research work done on efficacy of exogenous enzymes focused on wheat and barley based diets and alleviation of anti-nutritional properties of soluble high molecular weight pentosans. However, much less work was done on non-viscous grain pentosans such as maize and sorghum (Choct, 2006). Bach Knudsen (1997) reported that enzyme supplementation to maize based diets has been ignored due to low concentrations of soluble NSPs of less than 1 g/kg compared to that of wheat of 25 g/kg. Cowieson *et al.* (2006), D'Alfonso (2005) and Ravindran *et al*. (2006) described the low maize solubility being related to variation in solubility and digestibility of starch and protein as well as the content and relative reactivity of phytate.

The effectiveness of exogenous enzymes provides the potential to strategically formulate maize-soya diets by taking into account the relative concentration of indigestible starch, protein and phytate in feed ingredients. The supplementation of a combination of exogenous carbohydrases, proteases and phytases to the maize-soya diets allow for the expected significant improvement in digestibility of Ca, P, energy and amino acids (Cowieson *et al*., 2006 a and b).

2.3 Mode of action of enzymes

The mechanism of action of supplemental exogenous enzymes to improve profitability of poultry production was described by Cowieson *et al*. (2010) as enhancing the apparent digestibility of dietary nutrients and reducing nutrient requirements of the animal itself. Successful application of exogenous enzymes to dry diets presupposes that the enzyme will be active in the digestive tract of the animal, and it must therefore fulfil a number of criteria (Thorpe and Beal, 2001). The enzyme must be active under the physiological conditions prevailing in the animal's digestive tract, meaning it must be able to resist proteolysis by the animal's endogenous proteases rather than antagonise the animal's digestive enzymes. Species differences in the anatomy and physiology of the digestive tract are likely to affect exogenous enzyme activity in this respect. Partridge (1993) and Dierick and Decuypere (1994) illustrated some of the species differences in the utilisation of enzymes between poultry and pigs as follows:

1. Anatomical: In poultry, feed passes into the crop, where any added enzymes can act for several hours at a pH of approximately 6.0 before passing into the acid environment of the gizzard, whereas in the pig, feed passes directly into the acid environment of the stomach immediately after ingestion.

2. Digestive capacity: Poultry have a relatively shorter small intestine and thus reduced possibilities for enzyme inactivation by the microflora, a shorter mean retention time in the small intestine (1 - 2 hours in poultry versus 4 - 5 hours in the pig) and lower water content in the upper part of the gastrointestinal tract.
3. Bacterial activity: The importance of the microflora in the gut of poultry is much less than in the pig.
4. Fibre fermentation: Less fermentation of fibre in poultry than in pigs, due to the much smaller hind gut in poultry.

Effectiveness of the enzymes in the digestive tract of animals varies widely, for example, Thacker and Baas (1996) and Baas and Thacker (1996) demonstrated that 84% of pentosanase and 26% of alpha-glucanase activity was recovered in the duodenal digesta of pigs 4 hours after feeding diets supplemented with these enzymes. Approximately 75% of exogenous protease has been detected in the ileal digesta of young pigs fed protease-supplemented diets.

2.4 Limitation to enzyme digestion

There is extensive research work reporting that maize-soya diets are highly digestible with at least 85% to 90% of starch, protein and lipids digested. In some diets, digestibility can be as low as 75% allowing for significant response to enzyme supplementation. Ravindran (2013) described that the digestibility of maize-soya diets could not be improved close to 100% due to restriction by the amount of substrate, enzyme characteristics and physiological limitation of the bird. This author reported that in maize-soya poultry diets, undigested nutrient fractions targeted by enzyme contribution is 25% to 35%. Ravindran (2013) further described prerequisites for effective exogenous enzyme action as: enzyme source, specific catalytic activity, resistance to pepsin proteolytic action; substrate concentration and accessibility; and the physiological state of the digestive tract (pH, moisture content, temperature and the resident time of the digesta in the gut, especially in the gastric phase as the main point of enzymatic action).

2.5 The role of enzymes in feed ingredient and nutrient digestion

2.5.1 Phosphorus and calcium

In animal feedstuffs, much so in poultry, phosphorus (P) is the third most expensive ingredient after energy and amino acids. Therefore, sustainable animal production requires optimal utilisation of P to reduce the cost of feeding. The role of P in animal nutrition has been reviewed by Applegate and Angel (2008). In their review the authors explained some of the essential roles such as the contribution in cellular metabolism and energy currency of the cell, intracellular regulatory mechanisms as well as structural processes such as bone formation. Bone is the main storage organ for P containing 85% of the body's total P. Phosphorus is essential in biological systems due to its involvement in both structural and metabolic processes. It is therefore required for animal production to attain their optimal genetic potential in growth, feed efficiency and skeletal development.

The macro-minerals, Ca and P, are the two most abundant minerals in bone constituting approximately 37% and 17% bone ash, respectively (Doyle, 1979). Supply of Ca and P to the animal requires a careful balance to avoid imbalances that could potentially result in a deficiency of either one or both. The main consequence of suboptimal levels of these minerals is rickets, caused by either a Ca deficiency or P deficiency, which may occur when the dietary content of either nutrient is too low, or the dietary content of one is too high that induces a deficiency of the other.

Kling (1985) has associated elevated incidences of tibial dyscondroplacia (TD) as another consequence of a sub-optimal supply of Ca and P and subsequent imbalance of Ca: P ratios. There are several other factors that can lead to P metabolic conditions such as TD even under an optimal P feeding regime, but rickets should be preventable by correct diet formulation. Calcium has been found to be one of the minerals that are not only abundant but highly available from most sources. On contrary, P availability varies widely depending on the source (Whitehead *et al*., 2004). The differences in the availability of these nutrients and the importance of maintaining a balanced ratio, together with avoiding excessive use of P to minimise pollution, dietary levels often fails to meet the requirements (Whitehead *et al*., 2004). The availability of dietary P in cereal grains is further complicated by binding of the nutrient in phytate molecules.

2.5.2 Protein

Hughes and Choct (1999) discussed protein digestion and the complexity of high protein raw ingredients in poultry diets. The authors reported that protein raw ingredients differ in their chemical composition and also form linkages with other types of proteins, lipids and carbohydrates in a way that complexity may affect protein digestibility. Angkanaporn et al. (1994) quantified reduction in protein digestibility as a result of a depression in digestion of both endogenous and exogenous protein and a subsequent increase in endogenous secretion of amino acids. Anti-nutritive factors were reported by Hughes and Choct (1999) as one of the major factors affecting apparent protein digestion and utilisation by the animal. Bryden (1996) referred to protease inhibitors, lectins, poly-phenolic compounds, saponins, non-starch polysaccharides and phytate as some of the main anti-nutritive factors found in grain legumes and oil seed meals.

Proteins in their nature are heat labile and so are most of the oil seed meals that are extensively used in poultry diets as sources of protein. The research approaches to improve protein digestibility and utilisation of ingredients include feed processing and the application of exogenous enzymes. Optimal processing conditions were reported to be an important factor for improvement of protein digestion. Friedman (1996) reported that heat treatment may reduce protein quality in the presence of carbohydrates by the Maillard reaction, protein crosslinking and amino acid racemisation. The ingredients that are not plant based such as meat meal may vary in the amino acid digestibility due to slaughtering age, time between slaughter and rendering and the duration of the rendering process (Skurray, 1974).

2.6 Anti-nutritional factors and functions of exogenous enzymes

Enzymes have been used for poultry diets to neutralise the effects of different types of anti-nutritive factors in grains and cereals. These anti-nutritive factors are undesirable as they reduce digestion and absorption of nutrients in the diet. There has been an adequate amount of research data showing phytase enzymes as a feed additive, as it does not only increase the availability of phosphate in plants but also reduces environmental pollution by reducing the amount of undigested nutrients egested by the animal. Several other enzyme products are available in the market and are still being evaluated in the feed industry. Those include protease to enhance protein digestion, lipases to enhance lipid digestion, ß-galactosidases to neutralise certain anti-nutritive factors in non-cereal feedstuffs, and amylase to assist in the digestion of starch in young monogastric animals (Khattak et al., 2006).

2.6.1 Non-starch polysaccharides

Non-starch polysaccharides (NSP) in animal feedstuffs are a complex group of components differing widely in chemical composition, physical properties and physiological activity. NSP includes celluloses, hemicelluloses and pectins. Masey O'Neill *et al*. (2014) described cellulose as the most abundant organic macromolecule consisting of hydrogen-bonded micro-fibrils, comprised of β-1,4-glucose chains while hemicelluloses include pentosans (in particular arabinoxylans) and β-glucans, as well as mannans, arabinans, galactans and xyloglucans. Cereal plants mostly consist of pectins, described by Theander *et al*. (1989) and Somerville *et al*. (2004) as pectic polysaccharides containing uronic acid.

Non-starch polysaccharides directly affect nutrient utilisation by either encapsulating nutrients and/or depressing digestion through gastrointestinal modifications. The viscous nature of the NSP is the primary cause for their anti-nutritive effect in poultry, because of the increased bulk and viscosity of the intestinal contents that decrease the rate of diffusion of substrates and digestive enzymes and hinder their effective interaction at the mucosal surface (Choct *et al*., 1996). The concentrations of soluble NSP in wheat are inversely correlated with their ME_n-values in broiler chickens (Annison and Choct, 1991). In addition to the direct effect of viscous NSP on gut physiology and morphology, there appear to be some indirect effects that may have important implications on the efficient utilisation of nutrients by the chicken (Danicke *et al*., 1999). One such indirect effect may be related to the gut microflora activity measured by intestinal concentration of volatile fatty acid, the result being colonisation of bacteria in epithelium of the small intestine as well as the caeca. Fermentation of NSP-rich diets in the gut of broilers will result in production and accumulation of volatile fatty acids (VFA) in the lumen of the small intestine resulting in proliferation of facultative microflora (Choct *et al*., 1996).

2.6.2 Non-starch polysaccharides enzymes (carbohydrases)

The 'favourable' mechanisms of dietary NSP-enzymes can be described: reducing the viscosity of the digesta in the small intestine, increasing the digesta passage and nutrient digestion rate, thereby giving less substrate and less time for the fermentative organisms to proliferate. This in turn may restore the normal and efficient 'endogenous' enzymatic digestion of nutrients in the small intestine. Enzymes are 'partially' counterbalancing the adverse effects of soluble NSP on zoo-technical performance (Bedford and Classen, 1992; Huyghebaert and De Groote, 1995). Moreover, the bio-efficacy of the NSP-enzymes is correlated with the fat level and degree of saturation of fatty acids in wheat based-diets (Danicke *et al*., 1999). It is, however, not possible to estimate the relative contribution of the improved nutrient utilisation as well as the 'selective' reduction in microbial population.

However, there is evidence that the consequence of a NSP-mediated reduced rate of digestion is much more radical in the presence of intestinal microflora than in their absence, being caused by the degradation of both digestive enzymes and bile salts and microbial colonisation of the absorptive surface area (Smits and Annison, 1996).

2.6.3 Anti-nutrition of phytate

Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) is a phosphorylated cyclic sugar alcohol as shown in Figure 2.1. The anion form of phytic acid, phytate, is the form present in all plants. Phytate in plants is usually chelated with cations, proteins and/or starches and this chelated form is called phytin. Ravindran *et al*. (2000) and Angel *et al*. (2002) demonstrated that in addition to reducing the availability of P, phytate also act as an anti-nutrient in the diet, reducing the metabolisable energy (ME) and overall digestibility of dietary cations and amino acids. Furthermore, it was reported that adverse effects of phytate on amino acid utilisation are associated with increased losses of endogenous amino acids that were proportional to the concentration of phytate added to the diet (Ravindran *et al*., 1999; Cowieson *et al*., 2004a; Cowieson and Ravindran, 2007).

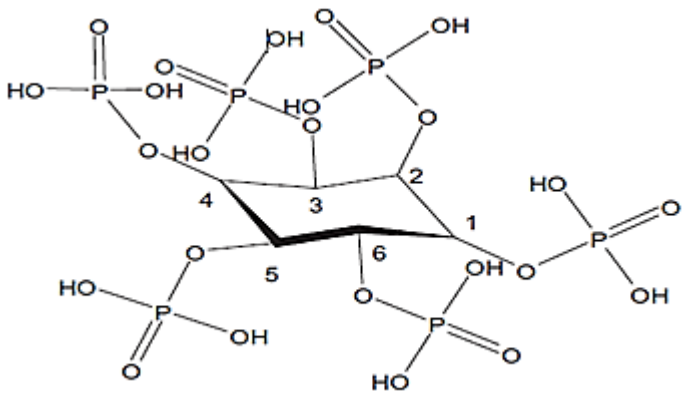

Figure 2.1: Myo-inositol 1, 2, 3, 4, 5, 6 – hexakis dihydrogen phosphate (IP6) (IUPAC, 1968)

Over two thirds of P in plant based feedstuffs is not readily available in poultry as it is bound to phytic acid (PA), which has been commonly thought to be due to the low levels of endogenous phytase (Bedford, 2000; Woyengo & Nyachoti, 2011). Recent studies have suggested that this is not the case, and in fact, chickens possess adequate phytase activity in the intestinal mucosa. The primary issue with phytate digestion is poor substrate solubility in the small intestine due to cation interactions, mainly with Ca (Maenz and Classen, 1998; Cowieson, *et al*. 2011). Calcium ions form insoluble complexes with phytate phosphorus (PP), which hinder phytase activity (Angel *et al*., 2002). Therefore, lowering dietary Ca levels can further improve the effect of exogenous phytase on PP degradation. The use of exogenous phytase to assist the bird in degrading PP has become common practice. Recent studies have shown that when adding phytase (500 FTU/kg), while at the same time lowering Ca in starter diets from 1.0% to 0.67% in combination with reduced non-PP (nPP) levels, performance of young birds was not affected (Létourneau-Montminy *et al*., 2010; Powell *et al*., 2011).

2.6.4 Phytase enzymes

Anti-nutritional effects of phytate can be alleviated partly by the use of exogenous phytase. A number of peer reviewed studies have shown increased P digestibility and utilisation and hence reduced P excretion into the environment due to phytase addition to poultry diets (Applegate *et al*., 2003; Penn *et al*., 2004; Angel *et al*., 2006). The liberation of P from phytic acid by phytase has been far from complete and studies presented in Table 2.2 have shown the level of phytate phosphorus released by addition of phytase in poultry diets. Although it is generally accepted that with phytase supplementation, the available phosphorus content of broiler chicken diets can be reduced by approximately 0.1 percentage point from 0.45% to 0.35%, the phytate P digestibility values reported in Table 2.2 did not show such a reduction.

Table 2.2: Ileal phytate P digestibility in broiler chicken fed maize/soybean meal based diets without and with phytase supplementation (Slominski, 2011)

				Phytate released (%)			
Study no.	**Trial length (d)**	**Phytate P (% of diet)**	**Phytase (U/kg)**	**Control**	**Phytase**	**Difference (%)**	**Reference**
1	1-21	0.31	250	0.068	0.139	0.071	Camden *et al*. (2001)
			500		0.149	0.081	
2	1-21	0.28	500	0.070	0.126	0.056	Tamim *et al*. (2004)
3	21-28	0.30	500	0.030	0.062	0.032	Rutherfund *et al*. (2004)
			750		0.062	0.032	
4	1-21	0.26	1000	0.076	0.130	0.054	Olukosi *et al*. (2007)
5	12-21	0.26	1000	0.008	0.057	0.049	Leytem *et al*. (2008)
6	1-21	0.31	600	0.077	0.109	0.032	Woyengo *et al*. (2010)
Mean		**0.29**		**0.055**	**0.106**	**0.051**	

2.6.5 Soybean and anti-nutritional factors

The level of anti-nutritional factors (ANF) in animal feed ingredients negatively affect nutrient utilisation of ingredients and limit the level at which it can be included in the diets of poultry. The type of anti-nutritional factors in ingredients vary between species and variety of plants (Warenham *et al*. 1994). Li *et al*. (1991) described the high molecular weight soya proteins, glycenin and β-conglycenin, acting as potential antigenic factors leading to the formation of serum antibodies particularly in young animals. The different types of ANF in animal feed ingredients include protease inhibitors, lectins, tannins, phytic acid, alkaloids, cyanogens and indigestible carbohydrates. The protease inhibitors, lectins and phytic acid, are well described as the main ANF in soybean meal which contribute 30 to 40% of the maize-soya poultry diets. The most abandoned serine protease inhibitors in SBM are the trypsin inhibitors namely Kunitz inhibitors (KSTI) and Bowman-Birk inhibitor (BBI). Trypsin inhibitors inactivate the digestive enzymes, trypsin and chymotrypsin, through binding to the active sites of these proteases, depressing the activity in the gut. The inhibition of these proteases can result in overstimulated secretion of digestive enzymes from exocrine pancreas and pancreatic hypertrophy (Rackis and Gumbman, 1981).

The mode of action of lectins as anti-nutritional factors in soybean meal was described by Oliveira *et al*. (1989). These glycoproteins were shown to have the ability to bind to cellular surfaces through specific oligosaccharides or glyco-peptides. Further research indicated that glycoproteins have the ability to bind to the epithelium of small intestine and resulting in the impairment of brush border. Excessive accumulation of these glycoprotein result in the ulceration of villi (Oliveira *et al*., 1989) which was believed to be the major cause for increased endogenous nitrogen losses and depressed growth rate in young animals (Pustzai *et al*., 1990).

In addition to lectins and trypsin inhibitors, complex high molecular weight oligosaccharides found in soybean meal are verbascose, stachyose and raffinose contributing approximately 6% of soybean meal dry matter. The limited intestinal capacity of monogastric animals to digest these oligosaccharides due to lack of endogenous β-galactosidase was reported by Gitzelmann and Auricchio (1965) in an experiment conducted with children. Wiggins (1984) described the ability of these complex oligosaccharides to accumulate in the gut causing fluid retention and subsequent increased flow rate of digesta, which reduces digestibility and absorption of nutrients.

2.6.6 Protease enzymes

Supplementation of poultry feed with protease enzyme has been applied in recent years as a strategy to improve protein digestibility. The effect of exogenous proteases added to poultry diets on live performance are frequently inconsistent (Cowieson and Ravindran, 2008).

The variability and inconsistencies of the performance results can be partially attributed to the following aspects:

a) the type of proteases tested,

b) experimental design, specifically the negative control diet nutrients and

c) the use of enzyme complexes rather than mono-component enzyme preparations.

A study was presented by Angel *et al*. (2011) where birds were fed a low protein diet that facilitated a negative effect on performance. The authors reported improvement in performance when protease was added at 200 mg/kg. Improvements in apparent CP digestibility of the protease-supplemented diets were observed when compared with the (PC) positive control (formulated at 22.50% crude protein without protease) or (LP0) low protein diets (formulated at 20.52% crude protein and amino acids lowered proportional to PC, no protease supplementation). The effect on amino acid digestibility and graded levels of supplemental exogenous protease enzymes are shown in Table 2.3.

Table 2.3: Apparent CP and ileal amino acid digestibilities (%) of diets supplemented with graded levels of protease and fed to 22 days old broiler chickens (Angel *et al*., 2011)

Nutrients	PC	LP0	LP100	LP200	LP400	LP800	SEM	P-value
CP	75.6^{b}	77.9^{b}	82.6^{a}	82.6^{a}	82.7^{a}	83.0^{a}	1.3	0.0009
Arginine	86.3^{b}	86.9^{b}	90.1^{a}	90.4^{a}	91.2^{a}	90.9^{a}	1.1	0.0287
Isoleucine	78.1^{b}	79.5^{ab}	82.6^{a}	82.3^{a}	82.9^{a}	83.1^{a}	1.8	0.0361
Leucine	85.4	85.9	86.8	87.1	86.5	86.9	2.6	0.2781
Lysine	78.4^{b}	80.6^{b}	85.9^{a}	85.1^{a}	86.4^{a}	86.6^{a}	1.9	0.0241
Methionine	76.1^{b}	78.4^{b}	82.7^{ab}	83^{ab}	83.9^{a}	83.2^{a}	2.2	0.0421
Phenylalanine	81	82.7	84.2	84.7	84.4	85.1	2.8	0.0828
Threonine	74.2^{b}	75.9^{b}	82.2^{a}	83.3^{a}	83.9^{a}	83.7^{a}	2.4	0.0261
Valine	82.2^{b}	83.6^{b}	86.4^{ab}	88.4^{a}	86.3^{ab}	87.1^{a}	1.4	0.0171
Alanine	77.7	78.5	80.2	80.6	80.1	81.1	2.1	0.0976
Histidine	80.2^{b}	82.1^{ab}	84.2^{a}	85.7^{a}	85.8^{a}	86.1^{a}	1.7	0.0198
Aspartic acid	74.5^{b}	76.1^{b}	82.3^{a}	81.6^{a}	82.5^{a}	82.8^{a}	1.6	0.0103
Cysteine	76.8^{b}	77.7^{b}	81.8^{a}	81.9^{a}	82.1^{a}	82.7^{a}	1.5	0.0219
Glutamic acid	84.3	85.1	86.9	87.1	86.8	87.3	2.2	0.4793
Serine	72.1b	73.6^{b}	78.2^{a}	79.1^{a}	78.7^{a}	78.8^{a}	1.7	0.0204
Tyrosine	77.9	78.9	78.4	79.9	78.4	79.2	2.5	0.1948
Proline	72.7	73.4	74.1	74.5	74	74.8	2.1	0.5159
Glycine	80	79.8	82.1	81.8	82.7	82.1	2.5	0.7434

a,b Means within rows with different superscripts differ (P < 0.05) based on Tukey's honestly significant difference test. Positive control (PC) and low protein (LP) diets had 22.50 and 20.52% formulated CP, respectively. Reductions in essential amino acids in LP diets were proportional to those in CP. LP0 = 0 mg of protease/kg; LP100 = 100 mg of protease/kg; LP200 = 200 mg of protease/kg; LP400 = 400 mg of protease/kg; LP800 = 800 mg of protease/kg. Formulated (analysed) protease (PROT units/kg) was as follows: PC, 0 (<LOD); LP0, 0 (<LOD); LP100, 7,500 (6,929); LP200, 15,000 (16,374); LP400, 30,000 (33,996); LP800, 60,000 (76,842). LOD = limit of detection

Three mono-component proteases were also tested by Ghazi *et al*. (2003). Proteases from *Bacillus subtilis* are active at neutral to alkaline pH and the others from *Aspergillus niger* are active at neutral to acid pH. Activities of these proteases were 92,000, 36,000, and 33,000 mg respectively, of α-amino groups formed (expressed as leucine equivalents) per minute per gram of product. These researchers reported improvements in TME and true nitrogen digestibility when using both proteases from *Aspergillus niger* but no effect for protease from *Bacillus subtilis*.

Walk *et al*. (2011) reported no effect on broiler performance with the addition of a protease extracted from *Bacillus subtilis*. However, in this study the enzyme was added to a summit diet, which may account for the lack of performance effect. Regression analysis of graded protease supplementation on performance and AA digestibility showed no significant linear or quadratic effects. More recent studies have shown that direct addition of a pure protease from *Nocardiopsis prasina* can lead to significant increases in CP and AA digestibility in broilers fed soybean meal or full fat soybean meal (Bertichini *et al*., 2009; Sorbara, 2009). It was concluded that AA utilisation was on average improved by about 5 % in soybean meal and 6 % in full fat soybean meal. Furthermore, the same protease has been demonstrated in several studies to have a positive impact on growth performance and N digestibility of broilers fed complete maize-soya based diets (Angel *et al*., 2011; Freitas *et al*., 2011; Fru-Nji *et al*., 2011).

2.7 Strategic selection of exogenous enzymes

The strategic selection of any additive or enzyme is dependent on the nature of the positive control (or normal diet) that is used as starting point for comparison. Cowieson and Bedford (2009) suggested that for every 10% improvement in the digestibility of the diets there is 50% drop in enzyme efficacy.

As indicated in section 2.6.3, the level of phytate phosphorus or undigested fraction of phosphorus in conventional poultry diets contributes 30% to 70% of the total phosphorus. The digestibility coefficient of phosphorus is 0.30. Phytase enzyme is the easiest selection of enzymes used for poultry and pig diets. The challenge with enzyme selection requires consideration of other non-phytate or multi-enzyme preparations and their contribution to starch, fat and protein digestion (Cowieson, 2010). When using different enzymes it might not be the best approach to assume that the nutrient contributions of all the enzymes will be fully additive. It was further suggested that using one or two enzymes might be an ideal option to target different substrates in feed ingredients. When the undigested fraction of the diets is considered, the use of phytase, xylanase, amylase and glucanase can be recommended to offer maximum response (Cowieson, 2010).

2.8 Benefits of feeding enzyme cocktails

The use of exogenous multi-enzyme cocktails has been receiving more focus as the number of formulated ingredients, enzyme types and products increase. Ohh (2011) reviewed multi-enzyme application and reported that multi-enzyme application enhance animal performance and an effective blend of enzymes exert relatively better response compared to single enzyme or no enzyme supplementation regardless of the cost effectiveness. Other authors suggested that the nutritive value of maize and soybean meal based broiler diets can be enhanced by the combination of amylase, xylanases and protease. Simbaya *et al.* (1996) reported a significant improvement in performance when feeding chick diets containing a combination of amylase, xylanase and protease (5.6% improvement in BWG and 4% in FCR). Cowieson and Adeola (2005) observed 1.9% improvement in BWG and 2.2% in FCR while Cowieson and Ravindran (2008) observed 5.8% improvement in BWG and 4.2% in FCR. Findings from these studies were not attributed to individual action of one enzyme.

In another experiment by Cowieson and Odeola (2005), a cocktail of xylanase, amylase, and protease (XAP, Danisco Animal Nutrition, UK Limited, Marlborough, Wiltshire, UK Danisco), was tested in combination with phytase (200 mg of XAP/kg provided a guaranteed minimum of 300 units of xylanase, 400 units of amylase, and 4,000 units of protease/kg; 200 mg of phytase/kg provided a guaranteed minimum of 1,000 units of phytase/kg). The authors reported an improvement in FCR when the cocktail was fed alone or in combination with phytase. Combination of XAP cocktail and phytase improved BWG significantly by 14% and ileal digestible energy by less than 100 kcal/kg. Cowieson and Odeola (2005) further concluded that supplementation of phytase and XAP individually in a maize-soya meal based diet is effective in improving nutrient digestibility and performance of broilers fed nutritionally marginal diets. Furthermore, there may be an additive effect of phytase and XAP on broiler performance, giving a cost-effective nutritional strategy for the profitable production of poultry products.

2.9 Enzymes and other additives

The mounting pressure to reduce the use of antimicrobial growth promoters (AGP) as feed additives and the popularity of exogenous enzymes have resulted in extensive work to evaluate the interaction of the two. Vukic-Vranjes and Wenk (1993) evaluated the effect of barley based diets supplemented with the antibiotic, Avoparcin, plus an enzyme complex containing β–glucanase and xylanase or Avoparcin without the enzyme complex on body weight gain of broiler birds and they did not observe significant difference between these two groups. Sarica et al (2005) evaluated wheat based diets supplemented with the antibiotic, flavomycin, plus xylanase based exogenous enzymes or thyme or garlic and the effect on body weight gain, feed intake and feed conversion ratio of broiler chickens. The author did not observe significant difference in body weight gain between treatments from days 14 to 35 and from days 1 to 42.

In another broiler study, avilamycin plus xylanase was supplemented to wheat based diets and feed intake was not significantly affected by this combination (Langhout & Schutte, 1995). In contrary to other authors, Esteve-Garcia *et al*. (1997) compared the effect of wheat or barley based diets without flavomycin with diets supplemented with flavomycin in combination with enzyme preparations (xylanase or β–glucanase) on broiler performance. Wheat based diets supplemented with flavomycin and xylanase improved feed efficiency with resultant reduction in intestinal viscosity, while xylanase preparation alone reduced percentage viscerae mostly due to reduction in intestines. Preparation of flavomycin and β–glucanase also improved feed efficiency and reduce viscosity and incidences of vent pasting.

2.10 Gut health and morphology

The gastro-intestinal tract of the broiler chicken is regarded as an important organ for nutrient digestion and absorption. It is essential for young broiler chicken to start developing this organ immediately post-hatching. Nitsan (1991) described the rapid growth of the GIT in young broilers as necessary to allow increased surface area for nutrient digestion and absorption. Gomide *et al*. (2004) correlated the development of the intestine to nutrient feed intake and consequently resulting in increased diameter and weight relative to body weight. This author further described the response of the intestinal mucosa to external stressors such as the absence or presence of feed and pathogens to be the main precursors of changes in the villus height (VH), crypt depth (CD), villus density and rate of epithelial turnover.

The increased villi height and crypt depth ratio (VH:CD) was directly correlated with increased epithelial cell turnover, although it leads to increased nutrient absorption and performance (Gomide *et al*., 2004). In contrast, Xu *et al*. (2003) reported that the reduced VH to CD ratio resulted in a marked increase in the endogenous enzyme secretion, reduced nutrient absorption, disease resistance and performance.

2.11 Motivation for the study

Most of the research work on supplementation of exogenous enzymes in broiler chickens published was done on grains grown under different production conditions compared to South Africa. These grain will differ in morphology and thus substrate composition. A study by Cromwell *et al*. (1999) compared crude protein content of maize from 15 different states in the United States of America and established variation of between 73.1 g/kg to 90.6 g/kg CP and 2.5 g/kg to 3.0 g/kg lysine. D'Alfonso (2002) found that total starch content of maize varied from 645.4 g/kg to 696.2 g/kg and amylopectin content of the starch varied from 732.5 g/kg to 828.6 g/kg across samples tested from 15 countries. Masey O'Neill *et al*. (2012) stated that grains such as cereals including maize vary considerably in composition as a result of environment, growth region, agronomic inputs and variety. Therefore, variability in the morphology of raw ingredients such as maize which comprises up to 55% of compound broiler diets may have a substantial effect on performance. The use of local ingredients offers an opportunity for low cost poultry feeds. However, there is wide variation amongst these ingredients within and between countries of origin and many times they contain high levels of anti-nutrients or components that variably affect nutrient utilisation by the broiler chicken.

Extrapolating the data from research done in other countries to local nutrition management in broiler production is not always ideal (Vieira *et al*., 2014). In addition, there is a need to understand the effect of combining exogenous enzymes in the broiler diets and also to quantify investment on return (ROI) through allocating sound nutrient matrix values to these products. The main question elaborating this problem is to understand whether a combination of different enzymes, targeting similar nutrients, work in a synergistic way which may be sub-additive, additive or antagonistic. Cowieson and Adeola (2005) and Juanpere *et al*. (2005) categorised the interaction between phytase and carbohydrase enzymes to be additive or sub-additive. Cell wall material encapsulates high NSP ingredients in the absence of carbohydrase enzymes and thus protects the phytate to be hydrolysed by exogenous phytase, thus limiting the efficiency of phytase enzyme. Furthermore, insufficiency of phytase enzyme will prevent carbohydrases from liberating other nutrients that may be bound with the phytate molecule.

Limiting nutrients may also compromise performance improvement and response of other enzymes even though sufficient phytase is present to liberate phosphorus from phytate (Cowieson and Adeola, 2005; Juanpere *et al*., 2005).

Very little is understood on the compatibility and synergy between feed protease and phytase when added to the feed simultaneously (Cowieson and Adeola, 2005). This limits the optimal use of both these enzymes in broiler diets as accurate matrix (nutrient) values cannot be allocated to the enzymes. There are countless interactions between enzymes and host animals, the host microflora and also dietary ingredients (Bedford, 2002). Cowieson and Adeola (2005) concluded that the use of a combination of different enzymes resulted in significant improvements in feed intake to weight gain ratio (from 0.8% to 10.5%) and body weight gain (from 1.9% to 6.9%) in broilers. They further indicated that phytase and protease may have an additive effect on broiler performance, and is a cost effective nutritional strategy for the profitable production of poultry product. The enzyme combination increased the metabolisable energy value of the feed by 3% and nitrogen retention by 11.7% of both adequate and reduced energy and amino acid diets (Cowieson and Ravindran, 2008). Woyengo *et al*. (2010) also found that a combination of phytase and protease enzymes can act synergistically in improving nutrient utilisation.

Studies have been conducted to investigate the interaction of exogenous enzymes and other feed additives to alleviate anti-nutritional effects of ingredients and improve gut health with subsequent improvement in broiler performance. Esteve-Garcia *et al*. (1997) reported the use of anti-microbial growth promoters (AGPs) in improving the nutritive value of non-starch polysaccharides in wheat based diets. The increasing pressure by the consumer to reduce the use of AGPs in poultry diets led to a need for the feed industry to find alternative feed additives (Humphrey *et al*., 2002; Botsoglou *et al*., 2004). The use of AGPs are banned in most countries but it is still routinely used as additives in broiler diets in the South African market. Most research conducted on exogenous enzymes was done without accounting for the inclusion of AGPs in the feed, and therefore the interaction of AGPs with exogenous enzymes, which is relevant to South Africa specifically, is not clearly understood. The work reported by Hock *et al*. (1997) suggests that there might be an interaction between exogenous enzymes and AGPs used in broiler diets, and that their effects are additive.

2.12 Hypotheses

a. H_0: Supplementing broiler diets with Ronozyme® ProAct or HiPhos will not improve broiler performance and gut health

H_A: Supplementing broiler diets with Ronozyme® ProAct or HiPhos will improve broiler performance and gut health

b. H_0: Supplementing broiler feed with a combination of exogenous enzymes and antimicrobial growth promoter will not improve broiler performance and gut health

H_A: Supplementing broiler feed with a combination of exogenous enzymes and antimicrobial growth promoter will improve broiler performance and gut health

c. H_0: Simultaneous inclusion of Ronozyme® ProAct and HiPhos in broiler diets does not have an additive effect on broiler performance and gut health (i.e. protease inclusion will not add to the benefits already obtained by phytase inclusion)

H_A: Simultaneous inclusion of Ronozyme® ProAct and HiPhos in broiler diets does have an additive effect on broiler performance and gut health (i.e. protease inclusion will add to the benefits already obtained by phytase inclusion, whether the additivity is complete or less than complete)

This study was undertaken to evaluate the effects of feeding broiler chicken's exogenous protease, Ronozyme® ProAct, and phytase, Ronozyme® HiPhos, alone or in combination or in combination with AGP, zinc bacitracin, on broiler performance parameters and intestinal health as measured by crypt depth and villi length and the ratios thereof.

CHAPTER 3

MATERIALS AND METHODS

Two separate experiments were carried out with a total of 1600 and 2080 birds, respectively. For Trial 1, 1600 Ross 308 separate sex day old chicks were randomly allocated to 32 pens with 50 birds per pen. For Trial 2, 2080 Ross 308 day old chicks were randomly allocated to 32 pens with 65 birds per pen. For both trials, the birds were sexed and the different sexes were placed in separate pens. Due to a limited number of pens available, the trial was duplicated in order to obtain more replicates per treatment. Eight treatments were included in the study, with four replicate pens per treatment (two male pens and two female pens) for each of Trial 1 and Trial 2, thus eight replicates in total.

The trail was approved by the Animal Ethics Committee of the Faculty of Natural and Agricultural Science of University of Pretoria (approval number EC029-12).

3.1 Preparation of the experimental diets

Chicks were fed maize-soya based diets fortified with a vitamin-mineral premix which also included a coccidiostat (Narasin/Nicarbazin, Maxiban®, Elanco Animal Health). The premix also included an enzyme and anti-microbial growth promoter (AGP) (Zinc bacitracin 15%, Albac®) where appropriate. The protease used was Ronozyme® ProAct, a commercial enzyme produced by submerged fermentation of *Bacillus licheniformis* containing transcribed genes from *Nocardiopsis prasina*. Ronozyme® ProAct GT (DSM Nutritional Products, Kaiseraugst, Switzerland) is a mono-component protease with activity of 75,000 PROT/g. One PROT unit is defined as the amount of enzyme that releases 1 µmol of p-nitroaniline from 1 µM of substrate (Suc-Ala-Ala-Pro-Phe-nitroaniline) per minute at pH 9.0 and 37 °C. The phytase which was used in the study was Ronozyme® HiPhos GT (DSM Nutritional Products, Kaiseraugst, Switzerland). This is a novel commercial enzyme of microbial 6-phytase expressed through the use of synthetic genes in *Aspergillus oryzae* with phytase activity of 10 000 phytase units (FYTs). One phytase unit is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate under standard conditions (0.25 M acetate buffer pH 5.5, 37 °C and 5 mmol sodium phytate).

Experimental diets were formulated to meet minimum nutrient requirements based on commercial feed specifications (sufficient to meet minimum breed recommendation). Premixes were manufactured by DSM Nutritional Products (South Africa) and added to compound raw materials at 3 g/kg. Feed was manufactured in 500 kg or 1000 kg batches and pressed through a cold pelleting system to yield either crumbles or a 3 mm size pellet and was weighed into 50 kg bags.

3.1.1 Feeding programme and experimental diets descriptions

A two-phase feeding programme was followed in the study. Starter crumbles were fed from placement to 19 days and finisher pellets were fed from 20 days to slaughter. The different dietary treatments used in the study is described in Table 3.1.

Table 3.1 : Description of the treatment groups and experimental diets

Groups	Experimental diets description
PC = Positive Control	Diet with standard commercial nutrient levels (No enzymes added)
NC1 = Negative Control 1	Diet with nutrient levels reduced equally to the recommended nutrient contribution of Ronozyme HiPhos (No enzyme addition)
NC2 = Negative Control 2	Diet with nutrient levels reduced equally to the recommended nutrient contribution of Ronozyme ProAct (No enzyme added)
NC3 = Negative Control 3	Diet with nutrient levels reduced equally to the recommended nutrient contribution of Ronozyme HiPhos and ProAct (No enzyme added)
Trt1 = Treatment 1	Diet with nutrient levels reduced equally to the recommended nutrient contribution of Ronozyme HiPhos (Added 100 mg/kg of HiPhos)
Trt 2 = Treatment 2	Diet with nutrient levels reduced equally to the recommended nutrient contribution of Ronozyme ProAct (Added 200 mg/kg of ProAct)
Trt3 = Treatment 3	Diet with nutrient levels reduced equally to the recommended nutrient contribution of Ronozyme HiPhos and ProAct (Added 100 mg/kg of HiPhos + 200 mg/kg of ProAct)
Trt4 = Treatment 4	Diet with nutrient levels reduced equally to the recommended nutrient contribution of Ronozyme HiPhos and ProAct (Added 100 mg/kg of HiPhos + 200 mg/kg of ProAct and 50 mg/kg of Albac (Zinc Bacitracin 15%)

3.1.2 Calculated nutrient values of the experimental diets and enzyme nutrient contribution

Nutrient matrices were included for the enzymes used in the study to calculate the nutrient specifications of the starter and finisher diets. Enzyme matrices were allocated as 100% of the nutrient value of Ronozyme® HiPhos and ProAct and combination of both enzymes on bases of recommended dose of 1000 FYTs/kg for Ronozyme® HiPhos equivalent to 100 mg/kg and 15,000 PROT/kg units equivalent to 200 mg/kg of Ronozyme® ProAct. Ronozyme® HiPhos contributed available P, total Ca, crude protein, amino acids and energy matrix values while ProAct contributed crude protein and amino acid matrix values. The combined effect of the enzymes were assumed to be 100% additive, where 1 + 1 = 2. Treatment diets were formulated based on the adjustment of nutrient values of the PC diet less enzyme nutrient contribution as indicated in Table 3.2 less enzyme nutrient contributions.

Table 3.2: Manufacturer's recommended enzyme nutrient contribution in g/kg of complete feed and enzymes matrix values of enzyme products in g/kg for meat type poultry

Nutrients for Meat Poultry	Units	Phytase nutrient contribution in feed	Phytase matrix (100g/kg)	Protease nutrient contribution in feed	Protease matrix (200g/kg)	Combination nutrient contribution in feed	Combined matrix values(300g/kg)
Crude Protein	g/kg	2.60	25,524	5.5	27,376	8.03	26,758
Digestible Lysine	g/kg	0.108	1,085	0.303	1,517	0.412	1,373
Digestible Methionine	g/kg	0.041	411	0.178	889	0.219	729
Digestible TSAA	g/kg	0.065	649	0.336	1,682	0.401	1,338
Digestible Threonine	g/kg	0.065	647	0.497	2,484	0.562	1,872
Digestible Tryptophan	g/kg	0.018	183	0.033	165	0.051	171
Digestible Iso-leucine	g/kg	0.105	1,053	0.220	1,102	0.326	1,086
Total Ca	g/kg	1.776	17,755			1.776	5,918
Available Phosphorus	g/kg	1.5	14,600			1.46	4,867
Digestible Phosphorus	g/kg	1.2	11,598			1.16	3,866
ME Broiler	MJ, ME/kg	0.3141	3,141			0.314	1,047
ME Broiler	Kcal, ME /kg	75	750,410			75.0	250,137

ME: Metabolisable energy

Table 3.3: Calculated enzymes matrix values for 3kg premix (g/kg) and percentage nutrient contribution for broiler starter and finisher diets

	Broiler starter							Broiler finisher						
Calculated nutrients values in % or g/kg	**Commercial Broiler Starter**	**Ronozyme® HiPhos**		**Ronozyme® ProAct**		**Ronozyme® HiPhos +ProAct**		**Commercial Broiler Finisher**	**Ronozyme® HiPhos**		**Ronozyme® ProAct**		**Ronozyme® HiPhos +ProAct**	
Units	**Min (%)**	**g/kg**	**%**	**g/kg**	**%**	**g/kg**	**%**	**Min (%)**	**g/kg**	**%**	**g/kg**	**%**	**g/kg**	**%**
Crude Protein	22.00	25.53	1.16	54.75	2.49	80.28	3.65	19.00	25.53	1.34	54.75	2.88	80.28	4.23
Digestible Lysine	1.100	1.08	0.98	3.03	2.75	4.11	3.74	1.00	1.08	1.08	3.03	3.03	4.11	4.11
Digestible Methionine	0.418	0.42	1.00	1.77	4.23	2.19	5.24	0.38	0.42	1.11	1.77	4.66	2.19	5.76
Digestible Total Sulphur AA	0.825	0.66	0.80	3.36	4.07	4.02	4.87	0.75	0.66	0.88	3.36	4.48	4.02	5.36
Digestible Threonine	0.693	0.66	0.95	4.98	7.19	5.61	8.1	0.63	0.66	1.05	4.98	7.90	5.61	8.90
Digestible Tryptophan	0.198	0.18	0.91	0.33	1.67	0.51	2.58	0.18	0.18	1.00	0.33	1.83	0.51	2.83
Digestible Isoleucine	0.693	1.05	1.52	2.19	3.16	3.27	4.72	0.63	1.05	1.67	2.19	3.48	3.27	5.19
ME Broiler (MJ/kg)	11.60	3.15	0.27			3.15	0.27	11.80	3.15	0.27			3.15	0.27
Calcium	1.050	17.76	16.91			17.76	16.91	0.95	17.76	18.68			17.76	18.68
Available Phosphorus	0.480	11.61	24.19			11.61	24.19	0.43	11.61	27.0			11.61	27
Ca:P ratio	2.180							2.209						

ME: Metabolisable energy

Table 3.4: Raw material composition of experimental starter diets expressed on a fed basis (g/kg)

Raw ingredients	PC	NC1	NC2	NC3	Trt1	Trt2	Trt3	Trt4
Yellow maize	525.96	545.87	545.91	568.17	545.87	545.91	568.17	567.84
Fullfat soya oilcake meal	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00
Soya Hi Pro oilcake meal (46%+)	263.22	256.08	246.77	237.61	256.08	246.77	237.61	237.61
Soya oil	10.92	4.43	7.36	0.42	4.43	7.36	0.42	0.42
Limestone	18.46	22.24	18.49	22.28	22.24	18.49	22.28	22.28
Monocalcium phosphate	18.59	8.53	18.76	8.72	8.53	18.76	8.72	8.72
Salt	4.38	4.38	4.39	4.39	4.38	4.39	4.39	4.39
Methionine (MHA)	3.32	3.27	3.05	3.03	3.27	3.05	3.03	3.03
Lysine HCL	0.78	0.84	0.90	1.01	0.84	0.90	1.01	1.01
Choline chloride 60%	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86
Maxiban (Narasin/Nicarbazin)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vit/Min broiler starter premix, no enzyme	3.00	3.00	3.00	3.00				
Vit/Min broiler starter premix+ HiPhos					3.00			
Vit/Min broiler starter premix + Pro Act						3.00		
Vit/Min broiler starter premix HiPhos+ProA							3.00	3.00
Zinc bacitracin (Albac)								0.335

Vitamin and mineral broiler starter premix in a 3kg unit and contribution per kg of complete feed: vitamin A: 10,000 IU; vitamin D3: 10,000 IU; vitamin E: 50 IU; vitamin K3: 4 mg; vitamin B1: 5 mg; vitamin B2: 8 mg; vitamin B6: 4 mg; pantothenic acid: 20 mg; biotin: 0.25 mg; folic acid: 2 mg; niacin: 50 mg; vitamin B12: 20 µg; I (calcium iodate): 2 mg; Se (sodium selenite): 0.3 mg; Cu: 20 mg; Mn : 120 mg; Zn: 110 mg; Fe: 40 mg ; Antioxidant: 125mg; Ronozyme® HiPhos: 100 mg/kg ; Ronozyme® ProAct: 200 mg/kg and Combination of ProAct (200 mg/kg) and HiPhos (100 mg/kg). Copper, Manganese and zinc sources are supplied in a form of Sulphates.

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

Table 3.5: Formulated nutrients values for broiler starter

Nutrients	Units	PC	NC1	NC2	NC3
Crude Protein	%	22.101	21.945	21.450	21.200
Digestible Lysine	%	1.100	1.089	1.070	1.059
Digestible Methionine	%	0.541	0.535	0.514	0.510
Digestible Total Sulphur	%	0.825	0.818	0.791	0.785
Digestible Threonine	%	0.693	0.686	0.670	0.657
Digestible Tryptophan	%	0.226	0.222	0.217	0.211
Digestible Isoleucine	%	0.826	0.816	0.798	0.781
Digestible Leucine	%	1.635	1.634	1.597	1.578
Digestible Valine	%	0.888	0.881	0.860	0.848
Digestible Histidine	%	0.524	0.520	0.509	0.500
Digestible Arginine	%	1.336	1.319	1.289	1.270
Digestible Glycine + Serine	%	1.695	1.679	1.641	1.616
ME BROILER	MJ/kg	11.60	11.28	11.60	11.28
Fat (ether extract)	%	6.771	4.988	6.446	4.902
Fibre	%	3.753	3.795	3.723	4.024
Ash	%	7.071	6.422	7.013	6.390
Calcium	%	1.050	1.020	1.050	1.020
Total P	%	0.781	0.572	0.779	0.576
Dig. P	%	0.480	0.300	0.480	0.300
Inositol P	%	0.253	0.255	0.249	0.258
Sodium	%	0.200	0.200	0.200	0.200
Potassium	%	1.026	1.015	0.992	0.961
Chloride	%	0.333	0.335	0.336	0.344

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

Table 3.6: Nutrient analysis of the experimental starter diets expressed on as is basis (g/kg)

Nutrients	PC	NC1	NC2	NC3	Trt1	Trt2	Trt3	Trt4
Gross Energy (MJ/kg)	16.55	15.89	16.39	16.47	16.37	16.27	16.45	16.33
Dry matter	914.43	879.11	902.57	901.59	899.42	905.47	906.25	902.14
Crude Protein	223.84	223.31	219.60	216.58	209.87	201.01	214.86	213.62
Crude Fat (ether extract)	59.50	51.97	58.69	52.44	59.74	56.54	51.84	58.04
Ash	56.43	55.57	54.34	49.26	58.89	59.58	57.62	58.24
Total Phosphorus	7.08	7.41	7.32	5.72	7.48	7.52	6.19	7.00
Total Calcium	10.58	10.42	10.23	11.06	11.13	11.85	10.30	11.07
Sodium	2.31	2.06	2.18	2.39	2.20	2.60	2.33	2.34
Potassium	7.70	6.90	6.80	6.30	7.60	6.90	7.30	7.19
Chloride	3.05	3.02	3.16	3.08	3.58	3.74	3.24	3.30

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

Table 3.7: Raw material composition of experimental finisher diets expressed on a fed basis (g/kg)

Raw ingredients composition	PC	NC1	NC2	NC3	Trt1	Trt2	Trt3	Trt4
Yellow maize	619.65	654.11	621.12	655.23	654.11	621.12	655.23	654.89
Fullfat soya oilcake meal	150.00	82.94	150.00	83.57	82.94	150.00	83.57	83.57
Soya Hi Pro oilcake meal (46%+)	173.71	221.07	173.49	220.41	221.07	173.49	220.41	220.41
Soya oil	8.27	0.00	8.33	20.75	0.00	8.33	20.75	20.75
Limestone	17.03	20.78	17.03	6.80	20.78	17.03	6.80	6.80
Monocalcium phosphate	16.76	6.73	16.76	4.42	6.73	16.76	4.42	4.42
Salt	4.45	4.42	4.45	2.51	4.42	4.45	2.51	2.51
Methionine (MHA)	3.08	2.96	2.66	1.78	2.96	2.66	1.78	1.78
Lysine HCL	2.18	2.17	1.80	0.00	2.17	1.80	0.00	0.00
L-Threonine	0.50	0.45	0.00	0.18	0.45	0.00	0.18	0.18
Choline chloride 60%	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86
Maxiban (Narasin/Nicarbazin)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vit/Min broiler finisher premix No Enzyme	3.00	3.00	3.00	3.00				
Vit/Min broiler finisher premix +HiPhos					3.00			
Vit/Min broiler finisher premix +Pro Act						3.00		
Vit/Min broiler finisher premix +HiPhos+ProA							3.00	3.00
Zinc bacitracin (Albac)								0.335

Vitamin and mineral broiler finisher premix in a 3kg unit and contribution per kg of complete feed: vitamin A: 10,000 IU; vitamin D3: 10,000 IU; vitamin E: 50 IU; vitamin K3: 4 mg; vitamin B1: 5 mg; vitamin B2: 8 mg; vitamin B6: 4 mg; pantothenic acid: 20 mg; biotin: 0.25 mg; folic acid: 2 mg; niacin: 50 mg; vitamin B12: 20 µg; I (calcium iodate): 2 mg; Se (sodium selenite): 0.3 mg; Cu: 20 mg; Mn : 120 mg; Zn: 110 mg; Fe: 40 mg ; Antioxidant : 125mg; Ronozyme® HiPhos: 100 mg/kg ; Ronozyme® ProAct: 200 mg/kg and Combination ProAct (200 mg/kg) and HiPhos (100 mg/kg). Copper, Manganese and zinc sources are supplied in a form of Sulphates.

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

Table 3.8: Formulated nutrient values for broiler finisher

Nutrients	Units	PC	NC1	NC2	NC3
Crude Protein	%	19.018	18.911	18.924	18.832
Digestible Lysine	%	1.000	0.989	0.970	0.958
Digestible Methionine	%	0.503	0.495	0.468	0.459
Digestible Total Sulphur	%	0.750	0.743	0.716	0.707
Digestible Threonine	%	0.630	0.623	0.581	0.596
Digestible Tryptophan	%	0.184	0.182	0.184	0.182
Digestible Isoleucine	%	0.685	0.683	0.685	0.683
Digestible Leucine	%	1.446	1.457	1.447	1.457
Digestible Valine	%	0.750	0.750	0.750	0.750
Digestible Histidine	%	0.447	0.447	0.447	0.447
Digestible Arginine	%	1.103	1.092	1.103	1.092
Digestible Glycine + Serine	%	1.425	1.425	1.425	1.425
ME BROILER	MJ/kg	11.800	11.480	11.800	11.480
Fat (ether extract)	%	5.727	3.824	5.736	3.837
Fibre	%	3.594	3.354	3.597	3.358
Ash	%	6.353	5.668	6.353	5.672
Calcium	%	0.950	0.920	0.950	0.920
Total P	%	0.710	0.498	0.710	0.500
Dig. P	%	0.430	0.250	0.430	0.251
Inositol P	%	0.233	0.232	0.233	0.232
Sodium	%	0.200	0.200	0.200	0.200
Potassium	%	0.857	0.855	0.857	0.854
Chloride	%	0.359	0.360	0.352	0.353

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

Table 3.9: Nutrients analysis of the experimental finisher diets expressed on as is basis (g/kg)

Nutrients	PC	NC1	NC2	NC3	Trt1	Trt2	Trt3	Trt4
Gross Energy (MJ/kg)	15.44	16.13	16.03	16.02	16.19	15.54	15.46	15.48
Dry Matter	902.78	889.38	902.31	905.14	900.73	880.88	873.89	883.10
Crude Protein	193.45	189.16	184.33	192.02	199.70	186.50	189.77	184.02
Crude Fat (ether extract)	52.97	47.35	39.49	46.34	44.09	35.00	32.12	35.74
Ash	49.43	47.62	51.69	50.02	49.89	48.62	45.97	50.31
Total Phosphorus	6.46	6.44	6.58	5.50	6.57	6.51	5.46	6.54
Total Calcium	9.70	9.09	9.67	9.15	8.53	10.35	9.46	9.70
Sodium	2.15	2.00	2.23	1.93	1.88	2.21	2.16	2.27
Potassium	5.95	5.90	5.55	5.65	6.15	5.65	5.70	5.55
Chloride	3.61	3.50	3.69	3.43	3.27	3.50	3.44	3.53

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

3.2 Performance trial

3.2.1 Housing and management

Birds were reared in an environmentally controlled broiler house, on the experimental farm of the University of Pretoria, Hatfield, Pretoria. Birds were randomly allocated to 32 pens demarcated by 3 x 1.15 m mesh (3.45 m^2). Each pen was fitted with a bell drinker connected to municipality water supply lines and two tube feeders per pen to allow *ad libitum* access to drinking water and feed. Each pen was fitted with two infrared heaters. The brooding temperature was initially controlled at 32 ^oC for the first 4 days prior to placement and 4 days after placement and then gradually lowered to reach approximately 26 ^oC by slaughter age. Temperature was monitored on a daily basis and the lighting program was gradually adjusted weekly according to the broiler management guide. Minimum ventilation was regulated to allow removal of toxic gases and flow of fresh air in the house.

Each pen was allocated a 50 L labelled bin of known empty weight, filled with its corresponding treatment feed. Feed was weighed into marked bins corresponding to each treatment pen. Feed was then scooped and transferred from the bin to the pen when required. Chicks were fed on chick trays for the first 3 days and changed to tube feeders for the remainder of the trial. Feed and water were offered *ad libitum* during the period of the trial. Tube feeders were re-filled when necessary and shaken twice a day to ensure consistent feed availability throughout the trial. Bell drinkers were cleaned at least once a day to ensure the availability of clean water.

3.2.2 Feed sample collection

A probe was used to sample every second bag of feed. Samples were pooled per production batch, labelled and stored in a zip lock bag for analysis. The feed samples were ground through a 1 mm sieve and analysed.

3.2.3 Vaccination program

Birds were vaccinated against most commonly prevalent poultry diseases in South Africa following a basic broiler vaccination program recommended by Onderstepoort Veterinary Faculty, University of Pretoria (Table 3.10). The water system was switched off to deprive the chickens' water for an hour prior to vaccine administration. Vaccines were prepared by breaking vaccine vials in cool water in a 5 litre plastic bucket and mixing to volume in a big plastic drum. Required dose was calculated for birds' age per number of birds in a pen and added to each pen in fountain drinkers.

Table 3.10: Vaccination program used during the trial period

Age	Disease	Application route
Day Old	Infectious Bronchitis (IB)	Course Spray (Hatchery)
Day Old	New Castle Diseases (NCD)	Course Spray (Hatchery)
Day 14	Infectious Bursal Disease (IBD) (Gumboro)	Drinking water (On farm)
Day 18	New Castle Disease (NCD)	Drinking water (On farm)
Day 18	Infectious Bursal Disease (IBD) (Gumboro)	Drinking water (On farm)

3.3 Gut health measurements

3.3.1 Intestinal sample collection

Birds were sacrificed by cervical dislocation, subjected to dissection and tissue sampling at an abattoir situated at the Meat Science laboratory on the same experimental farm (University of Pretoria, Hatfield). Approximately 1 cm cross sectional cuts of the duodenum and the jejunum were collected and stored. The duodenum was defined as the section of the small intestines extending from the pylorus to the entrance of the main biliary and pancreatic duct. The jejunum was defined as the portion of the small intestine between the duodenum and Meckel's diverticulum (Jaroni *et al*., 1999).

Samples were thoroughly rinsed with physiological saline solution and fixed in a 10% Millonig's buffered formalin solution to prevent shrinking of the tissues. The sections of the intestinal samples were processed and stained at the Department of Histopathology, University of Pretoria, Onderstepoort. The tissue samples were embedded in paraffin wax after which sections of 4 to 5 µm were cut by a microtome blade and smeared onto a slide. The slides were dehydrated with Millonig's buffer solution and washed with distilled water. The dehydration process involved washing for 3 minutes in ethanol solutions of graded concentration of 100, 96 and 70 %, one minute for each concentration. The dehydrating fluid and the paraffin wax were removed with a clearing agent, xylene, for 5 minutes. The slides were stained in haematoxylin for 10 minutes then rinsed with tap water. To be differentiated, the slides were dipped in acid alcohol solution only once. The specimen turned blue after rinsing with tap water for 10 minutes. The slides were then rinsed in 70% ethanol for 3 minutes followed by counter staining in Eosin for 2.5 minutes. The slides were differentiated and dehydrated in 96% ethanol for 3 minutes and dehydrated again in 100% ethanol for 3 minutes. The slides were then cleared in xylol and mounted in Entallan (Bancroft, 2003).

3.3.1.1 Preparation of 20 L physiological saline solution

180g of NaCl was weighed on an analytical balance and dissolved in 1000 mL of distilled water. The solution was transferred to a 20 L bucket and filled to make up 20 L volume.

3.3.1.2 Preparation of 25 L of 10% Millonig's buffer solution

An analytical balance was used to weigh each dry chemical (428.6g KH_2PO_4, 87.0 g NaOH, and 121.4 g $C_6H1_2O_6$). Three 2 L volumetric flasks were filled with 1800 mL tap water, labelled for each chemical and placed on a magnetic stirrer. Each of the three weighed dry chemicals was carefully added to the corresponding volumetric flasks to be dissolved. 2.5 L H_2CO 40% formalin (Methanal) solution was poured into a 25 L bucket and topped with 3 solutions of dissolved dry chemicals. Tap water was added to the bucket to make up 25 L volume

3.3.1.3 Preparation of Haematoxylin and Eosin staining

a) Preparation of Haematoxylin (Lillie-Mayer)

1. 25 g haematoxylin
2. 3,500 mL Distilled water
3. 250 g Aluminium ammonium sulphate dodecahydrate
4. 100 mL glacial acetic acid
5. 2.5 g sodium iodate

Distilled water was heated to 40^{o}C. Haematoxylin and aluminium ammonium sulphate dodecahydrate were completely dissolved in distilled water to avoid the strains from breakage. The metal sheen appearing on top of the stain was then discarded. Glycerol was added to a solution followed by glacial acetic acid and then the stain was ripened by adding the Sodium Iodate. The mix was then left to stand for at least 18 hours before it was used. The solution was used within 18 to 24 hours of preparation.

b) Acid alcohol preparation

Concentrated hydrochloric acid (HCL) was added to 1980 mL of 70% ethanol.

c) Eosin preparation

25 g of Eosin (yellow) was dissolved in 1400 mL distilled water followed by addition of 3600 mL of 96% ethanol.

d) Expected results

The nuclei of the cells were expected to turn blue and the cytoplasm were expected to turn pink. (Bancroft, 2003)

3.3.2 Measurement of the villi length and crypt depth

The slides were micro graphed at 1: 2, 5 x 40 magnification using a Zeiss light microscope HB0100 equipped with AXIOCAM MRC5 imaging software. Villus length was measured as the length between the villus-crypt axis and the tip of the villus. Crypt depth was measured from the villus-crypt axis to the base of the specific crypt. A scale of x 100 µm was used for the measurements.

3.4 Bone measurements

The right metatarsus of each slaughtered bird was removed and stored in a zip lock bag and frozen at -20 °C for further processing. Seldin (1965) reported that when wet bones are stored at -20 °C there is no effect or alteration to the bone matrix.

3.5 Chemical analysis of feed

Feed samples were analysed in duplicate to determine the accuracy of formulated diets. Samples were milled to pass through 1 mm screen before being analysed for dry matter (DM), crude ash, crude protein (CP), ether extracted crude fat (EE), gross energy (GE), calcium (Ca), phosphorus (P), sodium (Na), chloride (Cl) and potassium (K).

3.5.1 Dry matter analysis

Dry matter was determined using the AOAC (2000), Official Method of Analysis 934.01. A 5 grams of feed sample was weighed into oven dried porcelain crucibles. Each sample was weighed in duplicate. The crucibles were placed into an oven overnight at 105ºC and cooled in a desiccator before weighing.

3.5.2 Ash analysis

Ash of the feed was determined using the AOAC (2000), Official Method of Analysis 942.05. After drying the samples for DM determination, the samples were transferred to a furnace and incinerated at 200 ºC for 1 hour and then at 600 ºC for four hours. The samples were transferred to the desiccator to cool overnight. The ceramic crucibles were weighed to determine the ash content.

$$\% \text{ Ash} = \frac{\text{Ash Mass}}{\text{Wet Mass Sample}} \times 100$$

3.5.3 Ether extracted crude fat (ether extract) analysis

Crude fat was determined using the Büchi Soxhlet (AOAC 2000), Official Method of Analysis 920.39. A No. 1, 125mm Ø Whatman filter paper was tared on the mass balance. A 2 g sample was then weighed into a filter paper, after which oven dried Büchi beakers were marked and weighed. The sample was then folded into the filter paper to prevent spillage and placed into marked extraction thimbles. The extraction thimbles were then placed in the soxhlet extraction tube. A volume of 80 mL petroleum-ether (60 – 80 °C boiling points) was poured into the flat bottomed Büchi beaker. The beakers and extraction tube (thimbles) were connected to the apparatus. Beakers were tightly fixed to the Büchi digestion block using a lever. The water taps were then opened and the tap of the steam generator was turned to the horizontal. The process involved: 1-hour digestion or fat extraction with petroleum ether, 2 hours of rinsing, 20 min drying or ether recovery. After the process, the ether was evaporated and collected in the soxhlet tube and filtered into a waste bottle. The Büchi beaker was then placed in an oven at 70 °C for 1 hour and transferred to a desiccator to cool for 30 minutes. The mass of the Büchi beaker was then determined. The difference between the mass of the flask before and after the extraction was the mass of crude fat of the sample.

$$\% \text{ Crude Fat} = \frac{(\text{Mass of beaker + residue} - \text{Mass of empty beaker})}{\text{Sample Mass}} \times 100$$

3.5.4 Crude protein (CP) analysis (Dumas/Leco)

The determination of crude protein was done following the A.O.A.C (2000), using the Dumas' method, with the Leco FP-428 version 2.4 of the Leco Corporation St. Joseph MI USA.

Sample preparation

1. Approximately 0.2 g of EDTA was weighed into cups. The cups were carefully folded to conceal the sample.
2. Approximately 0.2 g of feed sample was weighed in duplicate into Leco cups and the cups were folded.

The results were displayed as percent nitrogen. The conversions of nitrogen to crude protein of samples were calculated using a conversion factor of 6.25 (AOAC, 2000).

3.5.5 Gross energy (GE) analysis

Gross energy of feed was determined by a complete oxidation using an adiabatic calorimeter with oxygen ignition (MC 1000 Modular Calorimeter). Approximately 0.4 g sample was weighed into calorimeter crucibles lined up with a thread (as a fuse). Benzoic acid tablet weighing approximately 0.5 g was used as standard (calorific value of at least 26.453 ME/kg) and analysed before start of every batch. The sample and crucible were inserted into the calorimeter chamber for processing.

3.5.6 Mineral analysis

Feed samples were analysed for Ca, P, Cl, Na and K and faecal samples were analysed for P using the Official Method of Analysis 935.13 (AOAC, 2000).

The heating block was switched on and set to a temperature of 240 °C. Duplicate samples of approximately 0, 5 g were weighed out and transferred into the digestion tube. A volume of 25 mL nitric acid (HNO3) was then added to each sample. The samples were then placed on the pre-heated block and boiled for 15 minutes and then removed from the block to cool for 5 minutes.10 mL perchloric acid (HClO4) was added to each sample before the tubes were returned to the digestion block. The samples were allowed to boil for 40 minutes until they showed orange yellow colouration. Samples were then removed from the block and allowed to cool in a fume cupboard. Distilled water was added to the samples to make up to 50 mL mark.

Phosphorous concentrations were analysed using a Technicon Auto Analyser and concentration was obtained from a calibration curve determined according to Official Method of Analysis 965.17 (AOAC, 2000).

3.5.7 Bone ash and mineral analysis

Metatarsus samples were defatted according to Official Method of Analysis 932.16 (AOAC, 2005) and the method described by Zhang and Coon (1997).

3.5.7.1 Metatarsal bone defatting

a) The metatarsus bones samples were placed in separate voile bags, each with a numbered metal disk. The bags were closed with a ribbon to secure the bone and the disk

b) The bags were placed in Soxhlet fat extraction apparatus.

i. The Soxhlet fat extraction apparatus was filled to the marked line in bulb container with 60 - 80 °C petroleum ether.

ii. The tap water cooling system was turned on and the Soxhlet heater was switched on to 55 °C.

iii. The procedure was monitored after 30 minutes and 1 hour after turning on the heater to ensure all orifices are closed and boiling and condensation is occurring.

iv. The Soxhlet was allowed to run for 16 hours.

c) The bags were removed from Soxhlet and the ether was allowed to evaporate in the fume hood for 1 to 2 hours.

3.5.7.2 Metatarsal bone drying

a) The bones were removed from bags and weighed in water to record density after defatting.

b) The bones were cut to fit into crucibles and weighed into oven dried crucibles

c) The bones were dried in an oven at 70 °C for 12 hour

d) The crucibles were placed in a desiccator to cool then weighed.

Weight of dry defatted bone = (Dry crucible + Dry bone weight) – Dry crucible weight

3.5.7.3 Metatarsal bone ashing

a) Oven dried bones were ashed in a muffle furnace at 600 °C overnight for 12 hours

b) The furnace was turned off and allowed to cool to approximately 100 °C. The crucible with bones were removed from the furnace and place directly into a desiccator and allowed to completely cool down.

c) The ash weight was recorded.

d) The ash samples were transferred into labelled containers for further mineral analysis.

3.6 Measurements of broiler performance

Performance parameters were measured weekly at day 0, 7, 14, 21, 28 and 34. Birds were counted every week during weighing days. Birds were weighed weekly in groups per pen. Body weight was calculated as total weight of group divided by number of birds. Feed intake was calculated weekly or with each change over to a next phase feed. Average daily gain (ADG) was calculated as group weight divided by number of birds and number of days. Feed intake (FI) was measured as weight of feed offered less weight of left overs and cumulative feed intake (CFI) calculated as feed consumed times number of days divided by number of birds. Feed conversion ratio (FCR) was calculated as feed intake over the week divided by body weight gain for the week. All FCR values were corrected for mortality including weight of dead birds added to the weekly body weight gain (weekly feed intake divided by weekly body weight gain + weight of dead birds including culls. Mortalities were collected twice daily, evaluated for probable cause of death and weighed. Dead birds were stored in a freezer to be disposed of accordingly.

3.7 Measurements of bone density

Metatarsal bone samples were left to thaw at room temperature and soft tissues were mechanically removed through dissection and clean bones were weighed in air. Bones were not boiled to avoid the risk of Ca/P salt formation in water that could result in alteration of the mineral matrix of the bone. The volume of wet bone was determined by the method described by Zhang and Coon (1997) where bones were weighed in air and in water.

Graduated glass cylinder was filled to a set level with water at room temperature. The weight change equals the weight of water replaced by the bone. The metatarsal bones were dipped into the water and the level of water displaced by the weight of the bone was recorded. Khan *et al.* (1997) reported the density of water at 0 °C to be 0.9998395 g/mL, while at 20 °C and 25 °C was 0.9982041 and 0.9970449 g/mL, respectively. The authors reported that at the temperature of 4°C the density of water in closest to 1.0 g/mL at 0.9999720 g/mL. In this case the density was rounded to 1.0 g/mL. The density was calculated by dividing the mass by volume expressed in g/cm^3 (p = m/v) where p is density, m is mass and v is volume by displacement.

3.8 Statistical analysis

GenStat® statistical program (Payne, 2014) was used to analyse data.

3.8.1 Statistical analysis for performance parameters (BW, ADG, FI and FCR)

Linear mixed models (LMM) meta-analysis over the two trials was applied to test for differences between 8 treatment effects of repeated measurements for BW, ADG, FI, CFI, FCR per week over 34 days. The fixed effects were specified as day, treatment and the day x treatment interaction. The random effect was specified as pen number and the residual effects were not normally distributed and treatment effects were homogenous. Means were separated using Tukey LSD at the 5% confidence level.

3.8.2 Statistical analysis for mortality

Mortality data was analysed using Generalised linear models (GLM). GLM extend the usual regression framework to cater for non-Normal distributions. For example: binomial data recording r "successes" out of n trials; in this study the numbers of mortalities per week out of the number of chicks per pen. The logit link function, that defines the transformation required to make the model linear, was applied. The standard errors were considered appropriate for interpretation of the predictions as summaries of the data rather than as forecasts of new observations.

3.8.3 Statistical analysis of bone minerals and villi data

Linear mixed model analysis was applied testing for differences between treatment effects. Means were separated with the alternative Fisher's protected LSD test where the Studentized Range statistic is used instead of Student's t statistic at the 5% level of significance.

CHAPTER 4

RESULTS

4.1 Performance trail

4.1.1 Body weights (BW) from 0 to 34 days of age

Meta-analysis of body weights of broilers measured during Trial 1 and 2 are summarised in Table 4.1 and illustrated by Figure 4.1.

The body weights of broiler chicks at placements were not significantly different between any of the treatments. At the end of day 7, body weights of broilers fed NC1 and NC3 were significantly lower compared to the positive control (PC) while NC2 did not differ significantly from PC.

The addition of Ronozyme® HiPhos to NC1 (Trt1) or addition of Ronozyme® ProAct to NC 2 (Trt2) or combination of Ronozyme® HiPhos and ProAct to NC3 (Trt3) diets significantly improved 7-day body weight by 19%, 8.0% and 11.0%, respectively, when compared to deficient NC diets.

The supplementation of zinc bacitracin to the enzyme combination diets NC3 (Trt4), significantly improved growth rate by 11% compared to NC3, but there was no significant difference when compared to the broilers that received the combination of enzymes without zinc bacitracin (Trt3).

Addition of Ronozyme® HiPhos or combination of Ronozyme® HiPhos and ProAct to the restrictive NC1 and NC3 diets improved body weight significantly from day 14 to 34 days. Addition of HiPhos alone improved body weight at 14, 21, 28 and 34 day of age by 20%, 22%, 18% and 22%, respectively, while addition of combination of Ronozyme® HiPhos and ProAct to the restrictive NC3 diets improved weight at 14, 21, 28 and 34 days of age by 18%, 17%, 15.6% and 17%, respectively. Addition of Ronozyme® ProAct alone (Trt2) did not show significant effect on body weight from day 14 to slaughter, compared to both the PC and NC2 groups.

However, for Trt 1 and Trt 3, the addition of the exogenous enzymes to the respective negative control diets improved growth rate of broilers to such an extent that it did not significantly differ from the positive control diets.

Table 4.1: Least square means of weekly average body weights (BW) of broilers on day 0 to 34 days of age (g/bird)

Treatments	Day 0	Day 7	Day 14	Day 21	Day 28	Day 34
PC	42.72	169.7^{ab}	475.6^{a}	954.0^{a}	1576^{a}	2154^{a}
NC1	43.22	149.2^{d}	378.2^{b}	777.8^{b}	1273^{b}	1737^{b}
NC2	42.45	161.3^{bc}	463.4^{a}	938.9^{a}	1533^{a}	2093^{a}
NC3	43.22	157.0^{cd}	396.6^{b}	796.6^{b}	1280^{b}	1729^{b}
Trt1	43.16	177.5^{a}	471.3^{a}	950.6^{a}	1544^{a}	2127^{a}
Trt2	43.39	174.7^{a}	478.2^{a}	954.3^{a}	1539^{a}	2157^{a}
Trt3	42.32	174.4^{a}	467.7^{a}	933.5^{a}	1480^{a}	2025^{a}
Trt4	42.88	174.0^{a}	469.9^{a}	938.9^{a}	1482^{a}	2023^{a}
Standard error of means	0.26	2.63	5.69	15.96	35.02	54.10
P value	0.045	<0.001	<0.001	<0.001	<0.001	<0.001

^{abcd} Means with common superscripts within a column do not differ significantly (P > 0.05)

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

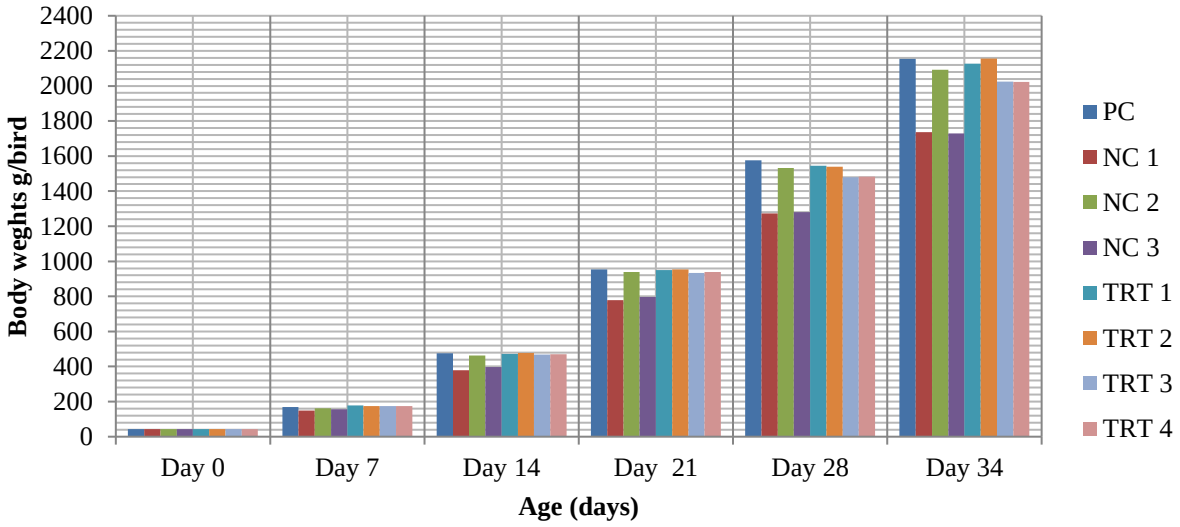

Figure 4.1: Body weight of broilers from day 0 to 34 of age

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

4.1.2 Daily weight gains of broilers (ADG) from 0 to 34 days of age

Meta-analysis of average daily weight gains of broilers measured during Trial 1 and 2 are summarised in Table 4.2 and illustrated by Figure 4.2, respectively.

As indicated in Table 4.2, the average daily weight gain at 7 days was significantly lower for broilers fed restrictive negative control diets compared to supplemented diets. The Ronozyme® HiPhos treatment significantly improved weekly ADG at days 7 and 14 by 28% and 29%, respectively. Ronozyme® ProAct treatment significantly improved weekly ADG of the deficient NC2 diet at day 7 by 11%.

The addition of exogenous enzymes Ronozyme® HiPhos to nutrient deficient NC1 diet significantly improved weekly ADG at days 21 and 34 by 19% and 27 %, respectively, while there was no significant improvement at day 28. Addition of combination of Ronozyme® HiPhos and ProAct to the nutrient deficient NC3 diets significantly improved weekly ADG at days 21 by 17%. There were no significant improvements when Ronozyme® ProAct or a combination of Ronozyme® HiPhos and ProAct were added to nutrient deficient NC diets at ages 28 and 34 days.

There was no significant difference between the various treatment diets (Trt1, Trt2, Trt3 and Trt4). Supplementation of exogenous enzymes Ronozyme® HiPhos alone or in combination with Ronozyme® ProAct to the nutrient deficient negative control diets significantly improved weekly ADG from 0 to 34 days. The improvement was significantly positive to the extent that the performance did not significantly differ from the PC.

Table 4.2: Least square means of average daily weight gain (ADG) for broilers from day 0 to 34 days of age (g/bird)

Treatment	Day 0-7	Day 7-14	Day 14-21	Day 21-28	Day 28-34	Day 0-34
PC	15.85^{ab}	43.49^{a}	69.72^{a}	89.35^{a}	96.04^{a}	62.11^{a}
NC 1	13.40^{d}	32.29^{b}	57.74^{b}	70.90^{bc}	76.38^{b}	49.83^{b}
NC 2	15.03^{bc}	42.44^{a}	67.96^{a}	83.90^{ab}	91.84^{ab}	60.33^{a}
NC 3	14.10^{cd}	34.09^{b}	57.56^{b}	67.83^{c}	74.98^{b}	49.59^{b}
TRT 1	17.14^{a}	41.68^{a}	68.95^{a}	84.06^{ab}	97.03^{a}	61.30^{a}
TRT 2	16.72^{a}	43.13^{a}	69.22^{a}	82.72^{ab}	96.39^{a}	62.17^{a}
TRT 3	16.61^{a}	41.79^{a}	67.11^{a}	77.71^{abc}	90.78^{ab}	58.34^{a}
TRT 4	16.27^{ab}	42.10^{a}	67.30^{a}	77.02^{abc}	90.72^{ab}	58.25^{a}
Standard error of mean	0.3417	0.6646	1.857	3.338	4.043	1.587
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

^{abcd} Means with common superscripts within a column do not differ significantly (P > 0.05)

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

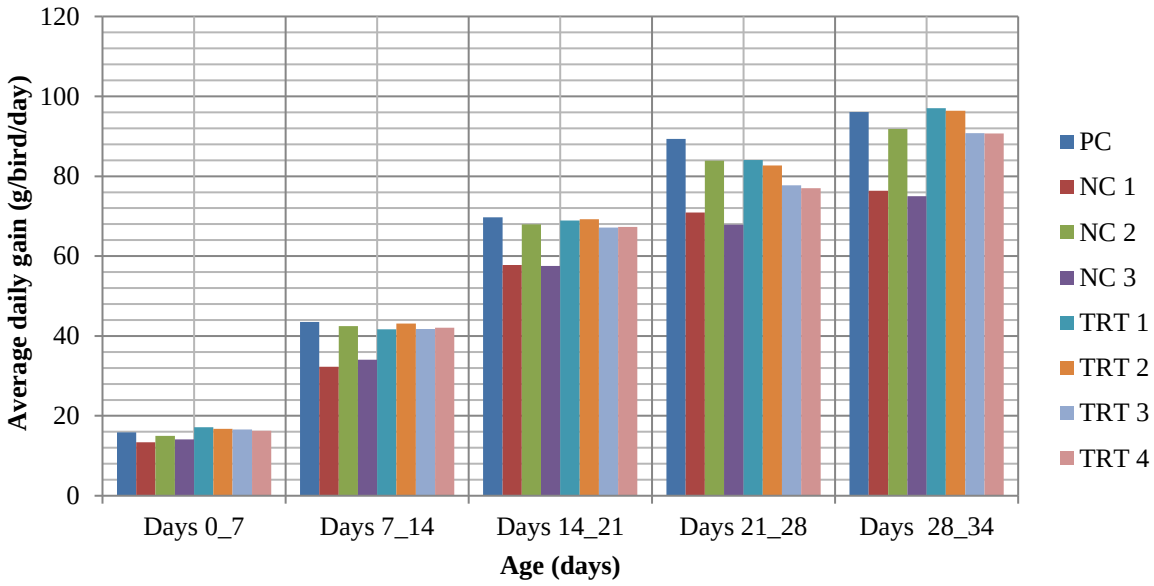

Figure 4.2: Weekly average daily weight gain from day 0 to 34 days of age

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

4.1.3 Feed intake (FI) for broilers from 0 to 34 days of age

The average weekly feed intake of broilers measured during Trial 1 and 2 are summarised in Table 4.3 and illustrated by Figure 4.3, respectively.

The average weekly feed intake of broilers fed nutrient deficient diets (NC1 and NC2) was lower in days 7, 21 and 34, respectively, as compared to PC. When nutrient deficient diets (NC) were fortified with exogenous enzymes Ronozyme® HiPhos or ProAct, average feed intakes did not increase significantly. The average feed intake of broilers fed PC diets improved during the first week (0-7 days) compared to enzyme supplemented diets (Trt1, 2, 3, 4). In subsequent weeks, average feed intake of PC diets was not higher than that of treatment diets.

Table 4.3: Least square means of average daily feed intakes for broilers from day 0 to 34 days of age (g/bird)

Treatments	Day 0-7	Day 7-14	Day 14-21	Day 21-28	Day 28-34
PC	32.46^{a}	53.68^{ab}	88.48	127.0	180.5^{a}
NC 1	28.03^{cd}	48.63^{b}	84.56	106.9	145.1^{b}
NC 2	30.42^{ab}	51.08^{ab}	90.40	118.9	161.6^{ab}
NC 3	26.35^{d}	48.54^{ab}	77.31	115.1	137.7^{b}
TRT 1	28.39^{bcd}	54.23^{ab}	93.67	130.9	166.2^{ab}
TRT 2	29.34^{bc}	55.72^{ab}	87.16	134.3	171.1^{ab}
TRT 3	28.55^{bcd}	56.29^{a}	89.86	130.2	171.6^{ab}
TRT 4	28.62^{bcd}	55.16^{ab}	92.40	128.1	160.2^{ab}
Standard error of means	0.5205	1.762	4.181	6.697	7.724
P value	<0.001	0.011	0.142	0.085	0.006

^{abcd} Means with common superscripts within a column do not differ significantly (P > 0.05)

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

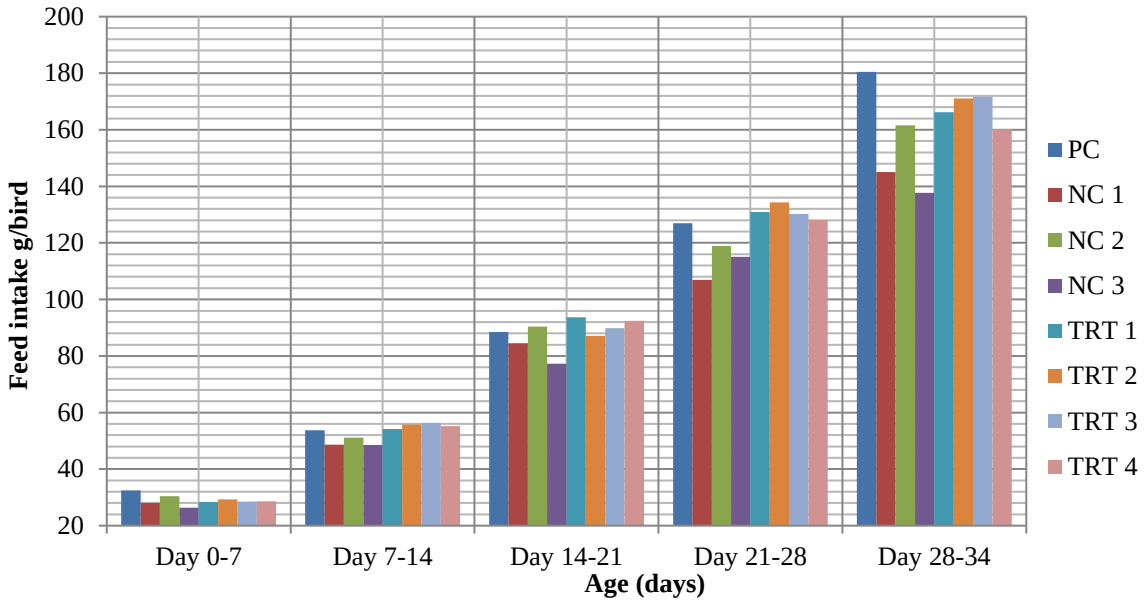

Figure 4.3: Weekly average feed intakes from day 0 to 34 days of age

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

4.1.4 Cumulative feed intakes (CFI) for broilers from 0 to 34 days of age

Meta-analysis of cumulative feed intakes of broilers measured during Trial 1 and 2 are summarised in Table 4.4 and illustrated by Figure 4.4, respectively.

The cumulative feed intake of birds fed nutrient deficient diets differed significantly to the birds supplemented with exogenous enzymes from 0 to 21 days of age. The addition of Ronozyme® HiPhos to the nutrient deficient NC1, significantly increased cumulative feed intake at 28 days by 15%, while addition of enzyme combination to nutrient deficient NC3 (Trt3) significantly increased cumulative feed intake at day 34 by 15.8%.

Table 4.4: Least square means of cumulative feed intakes (CFI) from day 0 to 34 days of age (g/bird)

Treatment	Day 0 - 7	Day 0 - 14	Day 0 - 21	Day 0 - 28	Day 0 – 34
PC	226^{a}	599^{b}	1174^{ab}	2138^{abc}	3165^{a}
NC1	196^{cd}	525^{a}	1095^{ab}	1910^{c}	2764^{ab}
NC2	214^{ab}	573^{ab}	1206^{ab}	2095^{abc}	3080^{a}
NC3	185^{d}	524^{a}	1076^{a}	1920^{bc}	2742^{b}
TRT 1	198^{bcd}	571^{ab}	1237^{b}	2192^{a}	3191^{a}
TRT 2	205^{bc}	589^{b}	1176^{ab}	2166^{a}	3165^{a}
TRT 3	201^{bcd}	584^{ab}	1160^{ab}	2150^{ab}	3174^{a}
TRT 4	200^{bcd}	590^{b}	1222^{ab}	2173^{a}	3130^{a}
Standard error of means	3.81	13.98	35.45	55.56	83.4
P value	<0.001	<0.001	0.025	<0.001	<0.001

^{abcd} Means with common superscripts within a column do not differ significantly (P > 0.05)

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

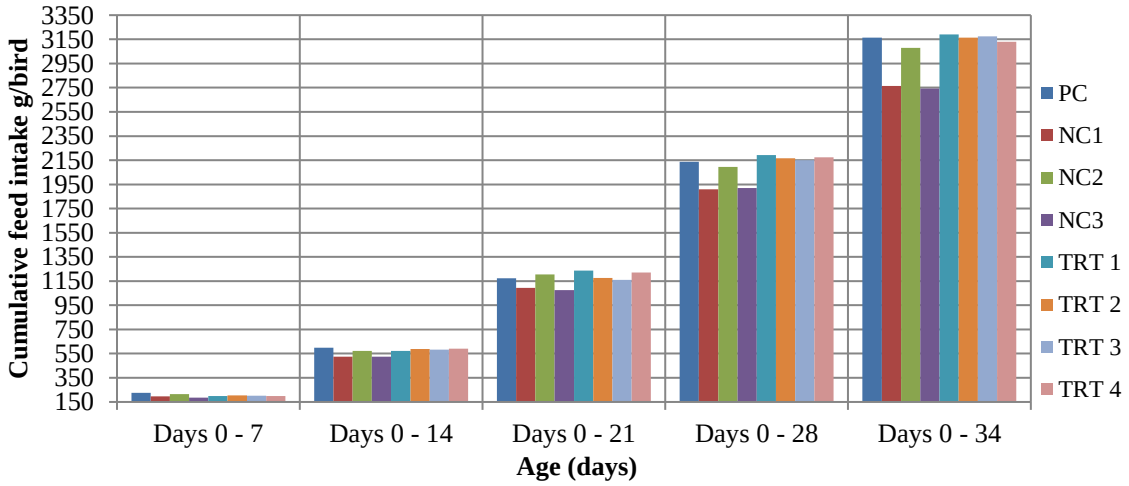

Figure 4.4: Cumulative feed intakes from day 0 to 34 days of age

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

4.1.5 Weekly feed conversion ratio (FCR)

Meta-analysis of weekly conversion ratio of broilers measured during Trial 1 and 2 are summarised in Table 4.5 and illustrated by Figure 4.5; respectively. Feed conversion ratio (FCR) was corrected for birds that were weighed as mortalities and culls. The addition of Ronozyme® HiPhos alone to nutrient deficient diet NC1 significantly improved FCR in first week of production by 17%, but there was no significant improvement in days 7 to 14, 14 to 21, 21 to 28 and 28 to 34. The broilers supplemented with Ronozyme® ProAct added to the nutrient deficient NC2 has shown a significant improvement in FCR of 13% in the first week of production but no significant difference in subsequent weeks.

There was no significant improvement in weekly FCR when NC3 diets were supplemented with combination of Ronozyme® HiPhos and ProAct from first week to last week of production. The addition of zinc bacitracin to Trt3 did not show any additional benefits to the FCR.

Table 4.5: Least square means of weekly feed conversion from day 0 to 34 days of age (g/bird)

Treatment	Day 0-7	Day 7-14	Day 14-21	Day 21-28	Day 28-34
PC	1.322^{c}	1.227^{a}	1.177^{a}	1.594	1.568
NC 1	1.295^{bc}	1.430^{b}	1.414^{b}	1.598	1.640
NC 2	1.316^{c}	1.220^{a}	1.182^{a}	1.665	1.519
NC 3	1.179^{ab}	1.382^{ab}	1.236^{ab}	2.011	1.618
Trt 1	1.105^{a}	1.254^{ab}	1.256^{ab}	1.675	1.555
Trt 2	1.159^{a}	1.317^{ab}	1.229^{ab}	1.736	1.470
Trt 3	1.144^{a}	1.317^{ab}	1.29^{ab}	1.732	1.670
Trt 4	1.156^{a}	1.332^{ab}	1.329^{ab}	1.757	1.501
Standard error of means	0.03082	0.04197	0.04962	0.0856	0.08008
P value	<0.001	0.013	0.025	0.05	0.613

^{abcd} Means with common superscripts within a column do not differ significantly (P > 0.05)

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

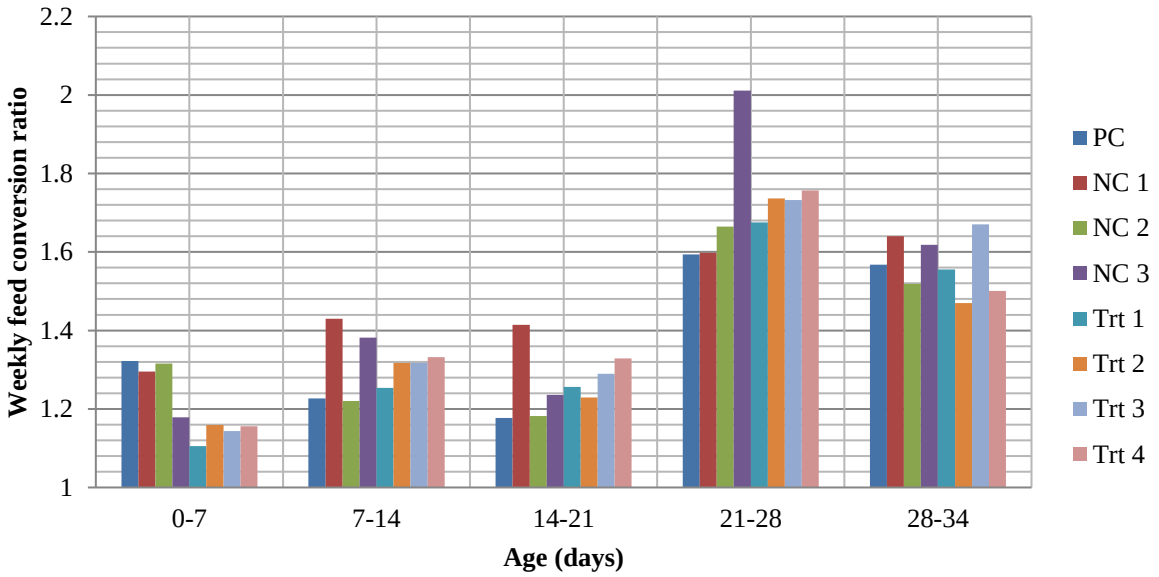

Figure 4.5: Graphical representation of weekly feed conversion ratios from 0 to 34 days of age

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

4.1.6 Feed conversion ratio from 0 to 34 days of age

Meta-analysis of feed conversion ratio corrected for mortality of broilers measured during Trial 1 and 2 are summarised in Table 4.6 and illustrated by Figure 4.6, respectively.

The feed conversion ratio (FCR) was corrected for birds that were weighed as mortalities and culls. The addition of Ronozyme® HiPhos alone to NC1 diet significantly improved FCR significantly in first week of production, 0 to 14 days and 0 to 21 days by 17%, 13% and 15%, respectively. There was no significant improvement in FCR in periods 0 to 28 and 0 to 34 days respectively. The broilers supplemented with Ronozyme® ProAct added to the nutrient deficient NC2 has shown a significant improvement of 13% in weekly feed conversion ratio only in the first week of production.

There was no significant improvement in feed conversion ratio when NC diets were supplemented with combination of Ronozyme® HiPhos and ProAct from 0 to 34 days. The broilers fed treatments diets (Trt1, 2, 3 and 4) significantly converted feed efficiently compared to PC during the first week of production by 20%, 14%, 16% and 14% respectively. The addition of zinc bacitracin to Trt3 did not show any additional benefits to the FCR.

Table 4.6: Least square means for feed conversion ratio for different treatments from day 0 to day 34

Treatment	Day 0 – 7	Day 0 - 14	Day 0 – 21	Day 0 - 28	Day 0 – 34
PC	1.322^{c}	1.261^{ab}	1.209^{a}	1.429	1.606
NC1	1.295^{bc}	1.368^{b}	1.395^{b}	1.577	1.687
NC2	1.316^{c}	1.273^{ab}	1.201^{a}	1.444	1.603
NC3	1.179^{ab}	1.255^{ab}	1.233^{a}	1.571	1.741
TRT 1	1.105^{a}	1.208^{a}	1.215^{a}	1.452	1.644
TRT 2	1.159^{a}	1.288^{ab}	1.245^{a}	1.494	1.598
TRT 3	1.144^{a}	1.252^{ab}	1.273^{ab}	1.517	1.713
TRT 4	1.156^{a}	1.253^{ab}	1.285^{ab}	1.528	1.680
Standard error of means	0.03082	0.02687	0.03107	0.0367	0.04246
P value	<0.001	0.022	0.004	0.05	0.116

^{abcd} Means with common superscripts within a column do not differ significantly (P > 0.05)

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

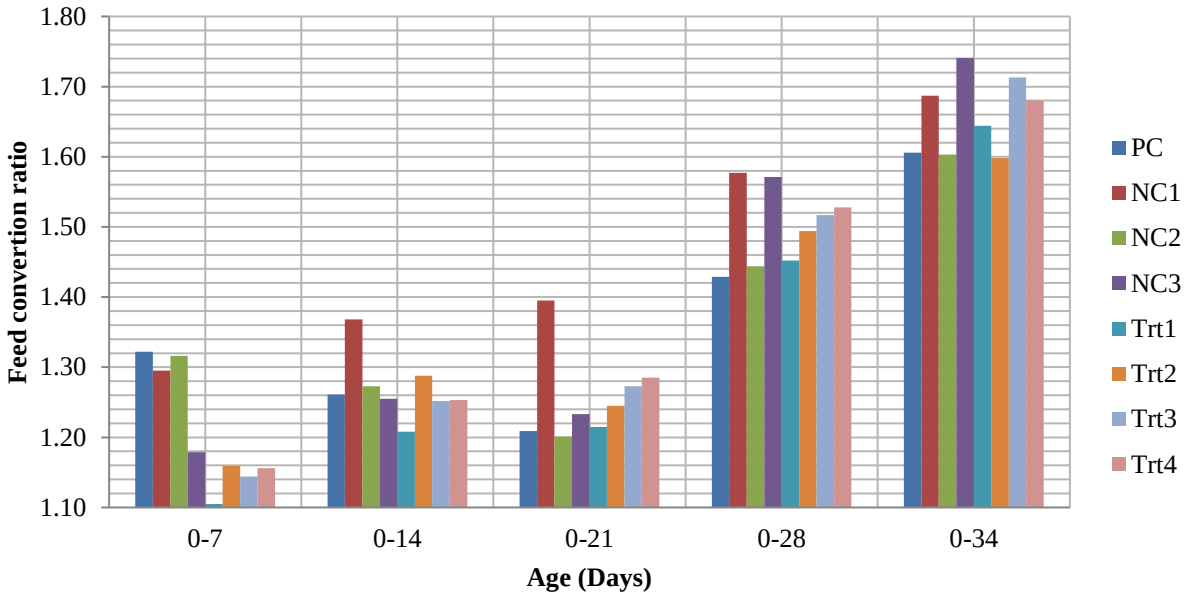

Figure 4.6: Graphical representation of feed conversion ratio from 0 to 34 days of age

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

4.1.7 Mortality during the course of the experiment

Mortalities were variable for each week of the growing phase. There was no observation of specific deaths associated with dietary treatments. High incidences of mortality were observed in treatment groups where nutrient contribution of Ronozyme® HiPhos matrix alone and combination of Ronozyme® HiPhos and ProAct matrix values were reduced with no enzyme supplementation (NC1 and NC3), compared to the rest of other treatments. These number of deaths were observed during weeks 3 to 4 with high percentage of non-specific causes of deaths from lethargic birds.

Specific bone related incidences were very low in all groups. Birds that died of sudden death syndrome (SDS) were observed at different ages in all the treatment groups. Although broiler house conditions were managed to the best of ability, some of the deaths could have been related to ventilation and heat stress as a result of sub-optimal housing conditions.

Table 4.7: Least square means for average weekly mortality (%) for different treatments from day 0 to day 34 of age

Treatments	Day 0-7	Day 7-14	Day 14-21	Day 21-28	Day 28-34
PC	1.10	0.67	0.74	1.89	0
NC 1	1.52	1.57	2.11	5.88	5.34
NC 2	1.68	0	0.71	1.97	0.52
NC 3	2.13	1.81	1.27	9.06	3.21
Trt 1	1.93	0.90	0.24	1.77	0.75
Trt 2	1.56	0.44	0.46	1.23	0.50
Trt 3	1.93	1.80	0.68	0.25	1.32
Trt 4	1.28	0.89	1.49	1.71	2.12
Standard error of means	0.223	0.201	0.178	0.244	0.174
P value	0.973	0.495	0.547	0.11	0.114

[abcd] Means with common superscripts within a column do not differ significantly (P > 0.05)

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and Zinc bacitracin 15% at 50 mg/kg.

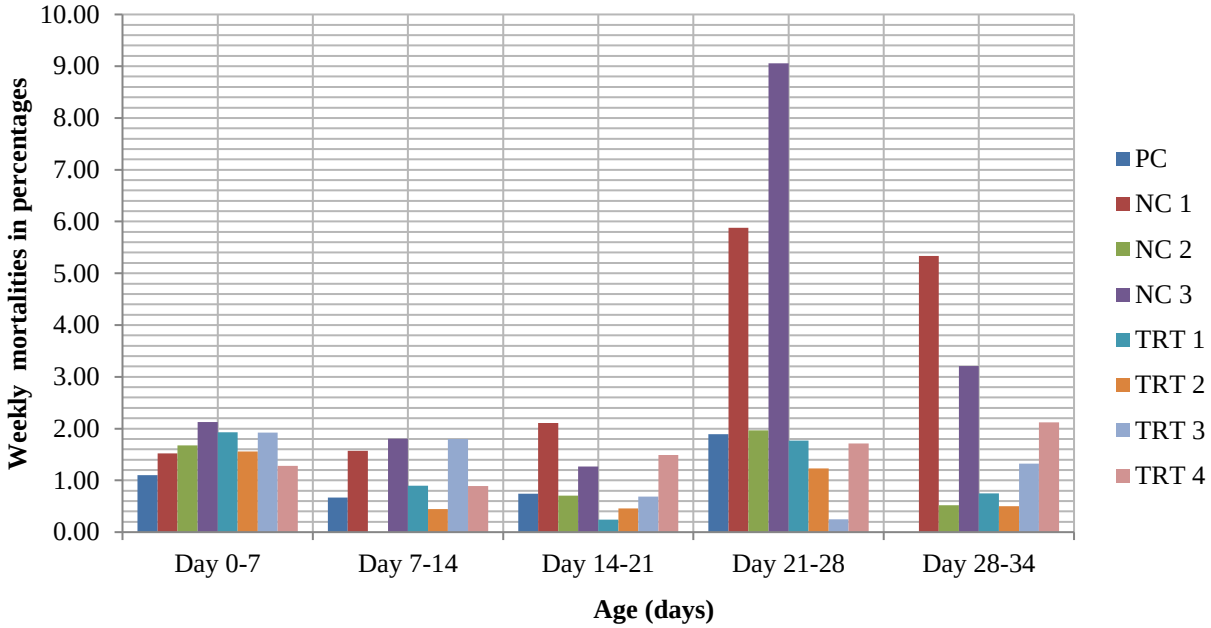

Figure 4.7: Weekly mortalities from 0 to 34 days of age

[abcd] Means with common superscripts within a column do not differ significantly (P > 0.05)

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

4.2 Bone and gut parameters

4.2.1 Duodenum and jejunum villi length and crypt depth

Duodenal villi were longer and crypt deeper in broilers that were fed a diet with reduced protein level (NC2) compared to broilers fed PC, NC and all other treatment diets. The duodenal villi length to crypt depth ratios were significantly better in the broilers fed Ronozyme® HiPhos (Trt1) or combination of HiPhos and ProAct (Trt3) when compared to birds fed PC. There were no significant differences in jejunum villi lengths and crypt depths and their ratio between broilers from all experimental groups. The addition of zinc bacitracin (Trt4) did not affect any of the measurements compared to Trt3.

Table 4.8: Least square means of the duodenal and jejunum villi length (VL) and crypt depth (CD) and VL:CD

Treatment	Duodenum			Jejunum		
	Villi length	Crypt Depth	VL:CD Ratio	Villi length	Crypt Depth	VL:CD Ratio
PC	111.6^{b}	13.62^{b}	8.20^{b}	99.59	10.12	9.68
NC 1	110.3^{b}	11.83^{b}	9.39^{ab}	72.78	8.77	8.33
NC 2	220.2^{a}	24.07^{a}	9.06^{ab}	83.66	8.82	9.63
NC 3	77.9^{b}	8.59^{b}	9.07^{ab}	83.78	8.17	10.24
Trt 1	102.5^{b}	9.68^{b}	10.75^{a}	75.82	8.77	8.63
Trt 2	117.9^{b}	11.62^{b}	10.46^{ab}	74.99	8.29	9.07
Trt 3	116.1^{b}	10.66^{b}	10.79^{a}	66.79	8.08	8.12
Trt 4	108.1^{b}	10.47^{b}	10.31^{ab}	79.44	8.95	8.99
Standard error of means	13.43	1.29	0.55	9.047	0.715	0.66
P value	<0.001	<0.001	0.021	0.315	0.505	0.325

^{abcd} Means with common superscripts within a column do not differ significantly (P > 0.05)

VH:CD = Villi height and crypt depth ratio

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

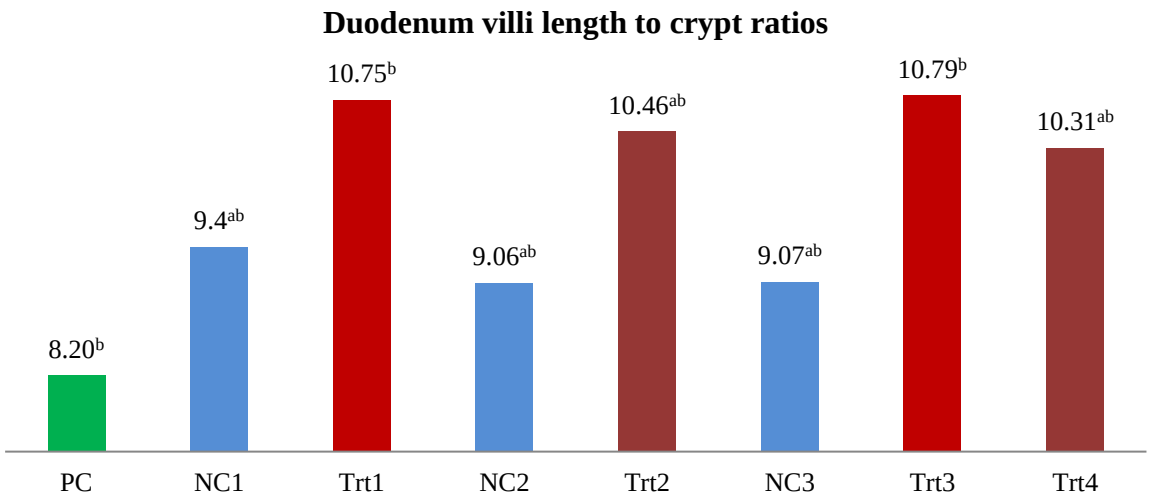

Figure 4.8: Graphical representation of villi length to crypt depth ratios of duodenum of a broiler at 28 days of age

VL:CD = the ratio between villi height and crypt depth ratio

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

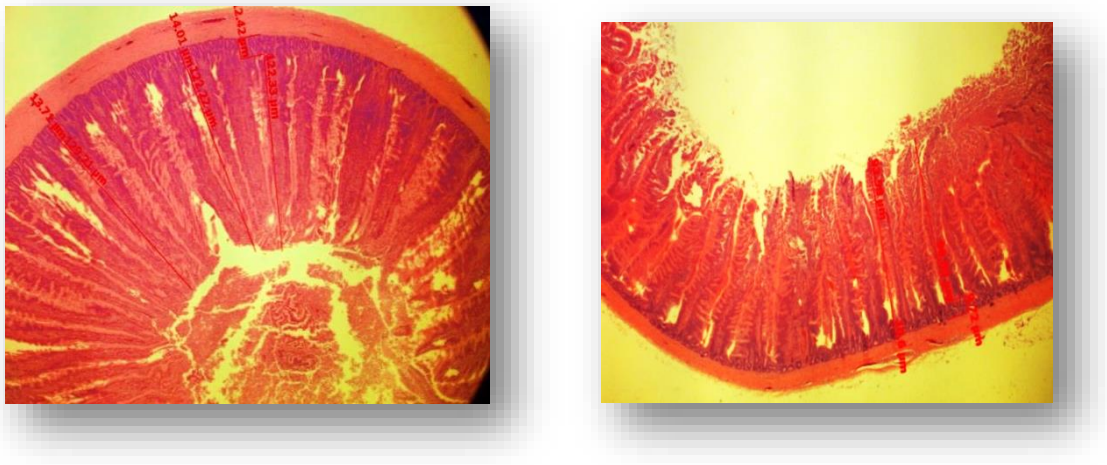

Figure 4.9: Haematoxylin and eosin staining. Cross section of the duodenum tissue specimen of a broiler chicken at 28 days of age.

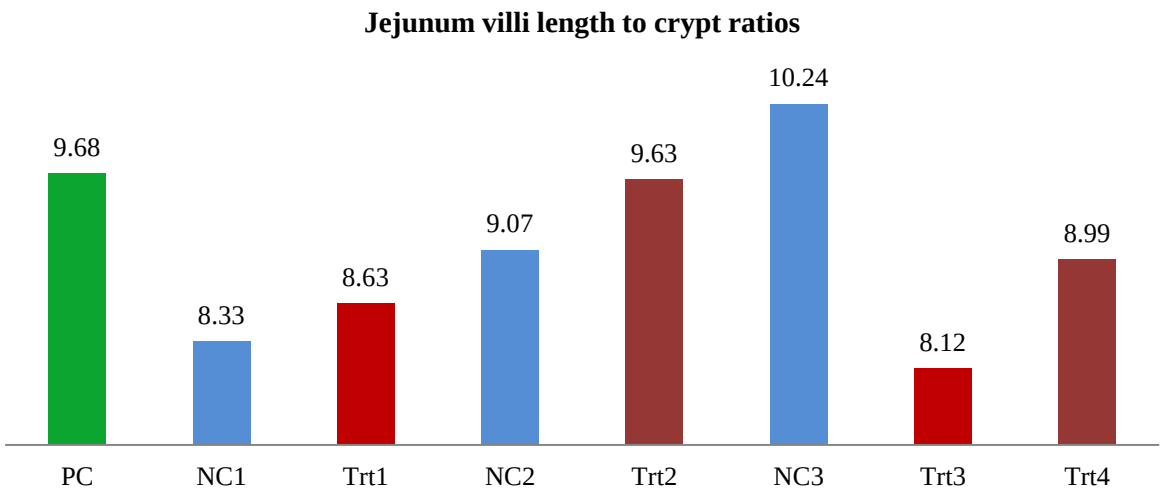

Figure 4.10: Graphical representation of villi length to crypt depth ratios of the jejunum of a broiler at 28 days of age

VL:CD = the ratio between villi height and crypt depth ratio.

PC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

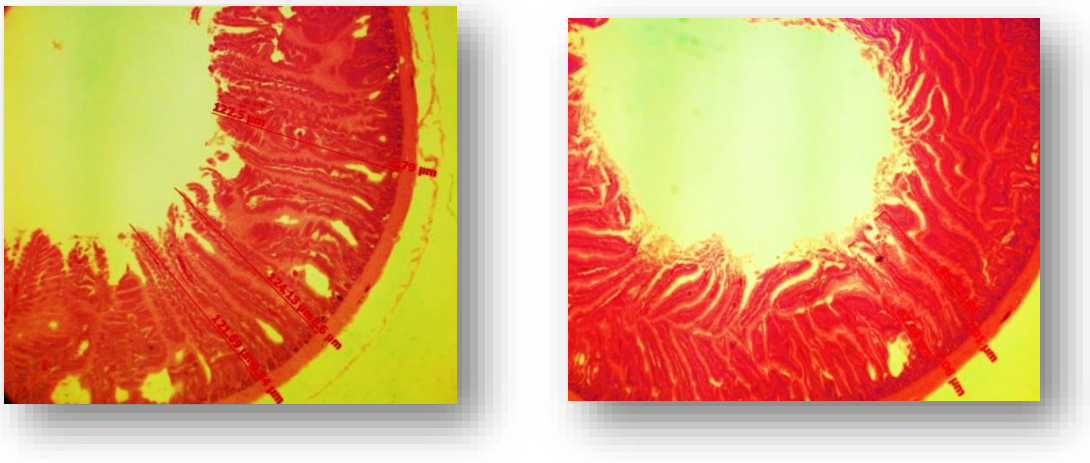

Figure 4.11: Haematoxylin and eosin staining slides. Cross section of the jejunum tissue specimen of a broiler at 28 days of age.

4.2.2 Metatarsus bone parameters

The metatarsus bone mineral volume, ash, Ca and P were measured in group of birds per treatment pen. Fresh and dry bone density and fat free dry matter calculated. The addition of enzymes to negative control diets did not have any significant effect on bone density, bone mass (fresh and dry), ash concentration, percentage ash, Ca and P. Bone ash mass of broilers fed diets deficient in nutrient NC3 (reduced nutrients matrix contribution of Ronozyme® HiPhos and ProAct) was significantly lower compared to the NC2 and Trt1.

Table 4.9: Least square means of metatarsal bone mineral measurements

Treatments	FBD (g/cm^3)	DBD (g/cm^3)	FBM (g)	FFDBM (g)	Ash Mass (g)	Ash/FFDBM (g/g)	Bone ash conc. (g/cm^3)	Ash %	% Ca (as % of ash)	%P (as % of ash)	Ca: P Ratio
PC	0.499	0.897	1.079	0.745	0.419^{ab}	0.402	0.191	40.21	41.84	18.11	2.31
NC 1	0.524	0.950	1.111	0.709	0.392^{ab}	0.357	0.184	35.68	42.43	17.79	2.39
NC 2	0.516	0.976	1.226	0.881	0.513^{a}	0.428	0.212	42.74	42.10	18.03	2.34
NC 3	0.411	0.783	0.995	0.632	0.329^{b}	0.332	0.136	33.22	41.83	17.74	2.36
Trt 1	0.642	0.846	1.368	0.846	0.509^{a}	0.441	0.235	38.12	42.00	17.90	2.35
Trt 2	0.578	0.849	1.156	0.772	0.432^{ab}	0.382	0.216	38.19	42.61	18.09	2.36
Trt 3	0.704	0.865	1.408	0.865	0.502^{ab}	0.362	0.251	36.18	41.51	17.77	2.34
Trt 4	0.544	0.818	1.243	0.818	0.472^{ab}	0.379	0.205	37.91	41.52	17.81	2.33
Standard error of means	0.092	0.062	0.145	0.064	0.039	0.028	0.026	2.513	0.621	0.113	0.028
P value	0.488	0.398	0.643	0.136	0.030	0.398	0.125	0.281	0.749	0.049	0.564

^{abcd} Means with common superscripts within a column do not differ significantly (P > 0.05)

FBD = fresh bone density (fresh bone mass/fresh bone volume; DBD = dry bone density (dried bone mass/dried bone volume); FBM = fresh bone mass; FFDBM = fat free dried bone mass; Bone ash conc. = ash concentration; Ca: P ratio = ratio of percentage Ca to percentage PPC = Positive control (commercial diet); NC1 = Negative control 1 (reduced nutrients for phytase contribution); NC2 = Negative control 2 (reduced nutrients for protease contribution); NC3 = Negative control 3 (reduced nutrients for a combination of phytase and protease contribution); Trt1 = NC1 + addition of 100 mg/kg Ronozyme® HiPhos; Trt2 = NC2 + addition of 200 mg/kg Ronozyme® ProAct; Trt3 = NC3 + addition of Ronozyme® HiPhos at 100 mg/kg and ProAct at 200 mg/kg ; Trt4 = NC3 + addition of Ronozyme® HiPhos at 100mg/kg and ProAct at 200 mg/kg and zinc bacitracin 15% at 50 mg/kg.

CHAPTER 5

DISCUSSION

Exogenous enzymes have always been under continuous scrutiny and remain recognised as innovative animal feed additives that are effective in improving digestibility of traditional feed raw ingredients. The economic benefits of supplementing exogenous enzymes in poultry nutrition was described by Costa *et al*. (2008) as being related to feed cost reduction, opportunity for flexibility in feed formulation, better performance, improved litter quality and health status with subsequent saving on total cost of poultry production. The use of combination of enzymes in this study offered an opportunity to lower feed cost and total cost of production.

The supplementation of exogenous enzymes to the poultry feeds is only rational if the targeted substrate is well understood and sufficient for the enzymes it is specified for. Therefore, it is also important to acknowledge that the complementary hydrolysis produced by the exogenous enzymes on substrates depends on the undigested fraction of the substrates passing through the gastrointestinal tract (Vieira *et al*., 2014). In addition, the choice of which enzyme or enzyme combination to use in feed require that nutritionists follow a strategic approach focusing on the substrate concentration and indigestible fraction of ingredients.

Nutritionist are tasked to ensure that their strategic approaches to enzyme supplementation take into account sensible nutrient contributions to give advantage to cost saving and efficacy of these enzymes. The performance response of broilers to supplementation of exogenous enzymes and the magnitude of which these enzymes can lower feed cost are dependent on these strategic approaches. Pack and Bedford (1997) described two approaches in which exogenous enzymes can be applied to the diets. The first approach was described as manipulating the formulation of the standard diet by reducing the nutritional level and adding enzymes to restore nutritional value of the standard diet. The second method was through addition of enzymes to standard rations without altering the nutrient level. This method was referred to as “over the top”. In this second method, cost saving may not be realised and additional cost have to be justified. The most commonly used approach is to change the diet formulation by reducing nutrient concentration and supplementing exogenous enzymes to provide identical nutritional values to the diet without adversely affecting bird performance. Matrix values are thus assigned to the enzyme product.

The latter strategy was followed in this study with standard commercial broiler starter and finisher diets (PC) as the base and the nutrient levels were reduced to elicit a negative effect in the chicken that received the negative control diets. The enzymes were added to these diets with reduced nutrients and it was hypothesised that the performance of the broilers should improve to the same level or better than the broilers from the PC group.

The results of this study indicated that supplementation of exogenous enzymes Ronozyme® HiPhos and ProAct to reduced nutrients speciation of maize-soya based diets of broiler chickens has shown not to negatively affect nutrient utilisation and performance parameters. Supplementation of Ronozyme® HiPhos or combination of HiPhos and ProAct to NC diets significantly improved BW and ADG throughout the production cycle. The benefit of adding Ronozyme® ProAct was only observed during the first 7 days of production for BW, ADG and FCR. This can be explained by the inability of young broilers chicks to secrete sufficient endogenous enzymes to digest complex plant substrates. Douglas *et al*. (2000) reported that chicks cannot produce sufficient endogenous enzymes and the use of exogenous enzymes will enhance catalytic and hydrolytic activities and increase the rate of digestion, absorption and metabolism of complex organic feed ingredients. Olukosi *et al*. (2007) reported that supplementation of exogenous enzymes to young chicks improved nutrient digestibility. Angel *et al*. (2011) also found that the addition of protease at concentrations of 200 mg/kg to the low protein starter diets fed to birds for 3 weeks of age completely compensated for performance losses, resulting in improvement of amino acid digestibility and subsequently ADG and FCR similar to the birds fed the PC diets. These differences could be a result of under development of the birds' capacity to secrete endogenous enzymes.

Bao *et al.* (2013) conducted an extensive review of the pattern of different exogenous enzymes on feed ingredients. In their review these authors indicated that although phytase is able to show positive response in many enzyme trials, carbohydrases and proteases results were seldom consistent in their responses. These authors stated a number of possible reasons causing such inconsistencies. These reasons were said to vary from application of inappropriate matrix values, nutrient imbalances, variance in underlying ingredient quality and changes in ingredients proportions with reformulation. In this study, the addition of protease (Ronozyme® ProAct) significantly improved BW, ADG and FCR only in the first week of the broilers' life. This improvement in performance can be associated with complementary effect of exogenous protease to the limited levels of endogenous proteases and other secretion in the GIT of young broilers. It is possible then that part of the beneficial effect of exogenous protease is mediated via a reduction in the loss of muco-protein from the intestine (Cowieson *et al*., 2014).

This may be associated with a reduction in mucin secretion or an increase in the autolytic recovery of mucin, or both. The mechanism by which exogenous protease may result in autolytic recovery of the mucin was suggested to be a result of reduction of HCl and pepsin in the gastric phase of digestion (Cowieson *et al*., 2009). It is possible that exogenous protease reduced the secretion of HCl and pepsin in the gastric phase of digestion, reducing the need for mucin as a protective agent in the intestine. Although part of the benefits protease enzymes offer can be related to the work by Cowieson *et al*. (2014), one of the main reason to inconsistencies of protease enzymes was pointed out by Bao *et al.* (2013) and it was ascribed to 'responsiveness' of the diet modification by enzymes. The authors further emphasised that the 'responsiveness' of a diet or an ingredient to exogenous enzymes was difficult to accurately determine. There are factors such as substrate concentration and accessibility, inherent digestibility, nutrient interactions, anti-nutrients, and solubility in water that are at the core of responsiveness of exogenous protease or carbohydrases. In relation to this study, it can be concluded that although Ronozyme® ProAct did not affect performance negatively, the results were positively consistent from starter to finisher although the birds performed significantly better in the early stages of the bird's life. Therefore, more extended knowledge of the responsiveness of Ronozyme® ProAct in different ages of the birds, feeding phases and dietary protein concentration may assist in optimising the economic benefits of proteases.

It is important to note that in the current experiment there were no significant differences found in any of the parameters measured between PC and NC2 groups. This could mean that the broilers receiving the NC2 diet were not deficient in any nutrient and that the addition of Ronozyme® ProAct to the NC2 diets could not improve the nutritional status of the birds and therefore performance. This experiment was thus not successful in evaluating the efficacy of Ronozyme® ProAct.

The intestinal morphology was measured as an indication of gut health and nutrient utilisation. It was hypothesised that addition of enzymes will improve gut health as measured by improvement in the villi length and crypt depth in the duodenum and jejunum sections of the small intestine. Duarte *et al*. (2014) reported that an increase in crypt depth was related to mucosal cell turnover. Sayrafi *et al*. (2011) studied the responses of histo-morphometrical parameters to AGP and alternative additives and reported that an increase in crypt depth indicates accelerated villi renewal which occurs as a result of damaged mucosal cells. The process of cell renewal is a nutrient expenditure process. Wyatt *et al*. (2008) reported that if digestion is improved in the upper intestine it may lower endogenous secretions by the gut villi as a result reducing the maintenance requirement spent on digestion with subsequent improvement in nutrient retention.

In this study, the duodenal VH to CD ratio of Trt1 and Trt3 was significantly higher than PC and other treatment groups. This is an indication that the addition of phytase alone or phytase with protease was positively related to the increased duodenal VH to CD ratio. In the jejunum, VH to CD ratio did not differ between any of the treatments. Improvement in VH, CD and VH to CD ratios of the duodenum could have contributed to improvement in GIT health and production performance.

Wu *et al*. (2004) fed broilers microbial phytase or xylanase or combination of the two enzymes to wheat based diets. Microbial phytase increased villi height in the duodenum as compared to the jejunum or ileum. The authors concluded that performance improvement in the phytase supplemented group could be due to the increase in absorptive surface area of the duodenum that improved nutrient absorption. They also observed that the addition of microbial phytase reduced the number of goblet cells in villi of the jejunum compared to un-supplemented group. These authors attributed the reduction in number of goblet cells to a reduction in mucin production and endogenous protein losses. Findings of Wu *et al*. (2004) were consistent with Cowieson *et al*. (2003) where they reported that addition of phytic acids increased production of silicic acid which is an indicator of mucin production. The results of our study was in agreement with both studies where the addition of phytase alone or in combination with protease improved the villi length and crypt depth and its ratio in the duodenum as compared to the un-supplemented PC control group.

Soybean meal contains relatively high levels of non-starch polysaccharides, which are potentially antagonistic to nutrient utilisation and can negatively affect the intestinal morphology (Zanella *et al*., 1999). The authors further explained that supplementing maize-soya based diets with NSP degrading enzymes can potentially yield improved nutrient digestibility and reduce endogenous amino acid losses, resulting in the conservation of endogenous utilisable energy that may be otherwise used for protein accretion (Zanella *et al*., 1999). Most of the literature consider a combination of phytase with NSP degrading carbohydrases as an effective approach to improve gut morphology in maize-soya diets. In this study, significant improvement in the duodenum villi was observed even without carbohydrase enzymes which indicates the efficacy of phytase or phytase and protease combination.

The incorporation of AGP, zinc bacitracin to Trt3, (Trt4) was evaluated to consider the effect when protease and phytase were supplemented. There was no difference in performance in both enzymes supplemented diets and NC diets. Studies reported enzymes to be an effective tool improve efficiency when AGP free diets are fed to poultry. Attia *et al*. (2016) studied the effect of two different sources of phytase with and without AGP, zinc bacitracin.

These authors did not notice any improvement in performance parameters when zinc bacitracin was added at a rate of 500mg/kg to the diets with or without exogenous phytase. It was clear from the study that exogenous enzymes could act as a gut modulator reducing the substrate available for the pathogens in the gut.

The bone mineral concentration and density did not respond to reduced specification treatments groups (NC) or in enzyme supplemented treatment groups. Yan *et al*. (2005) conducted experiments to evaluate the ability of broiler chickens to adapt to Ca and P deficiency. They fed diets low in P (49.5 vs. 56.0%) and Ca (60.9 vs. 71.1%) and measured the absorptive ability on 18, 21 and 23 days. The birds fed moderately deficient P and Ca were able to adapt to the deficiency. In another study, Blahos and Sommerville (1987) established that chicks fed Ca deficient diets for 2 weeks exhibited an increase in duodenal and ileal absorption of P labelled as ^{32}P. When diets deficient in P were fed for two weeks, a slightly significant increase in the duodenal P absorption was observed. These authors suggested that this interchangeable adaptation process was due to a compensatory increase in 1, 25-dihydroxy-vitamin D_3. The level of vitamin D_3 in this study was 10 000 IU/kg which was relatively higher than most published recommended levels from literature. In part, these levels of vitamin D_3 could have contributed to supporting the compensatory increase in 1, 25-dihydroxy-vitamin D_3.

However, Ravindran *et al*. (2000) further challenged the Ca and P adaptation process by investigating the role of phytase enzymes in the adaptation process. The authors revealed that decreasing nPP from 0.45% to 0.23% did not improve ileal P absorption when phytase is absent in the diet. The lack of negative response to bone mineralisation observed in our study when low specification diets (NC) were fed can be due to the birds' ability to adapt to moderate Ca and P deficiency during the period when bones were harvested at 28 day of age. Several authors have reported different factors explaining some of the adaptation process. The increase in the plasma level of calbindin reported by Morrisey and Wasserman (1971), duodenal Ca pump mRNA observed in broiler chicks (Cai *et al*., 1993) and intestinal phosphate co-transporter observed in humans (Xu *et al*., 2002) were reported to be correlated to adaptation to Ca and P deficiency. Although it is widely reported in literature that 1, 25-dihydroxy-vitamin D_3 is involved in the initial signalling process, the actual initial process is still not well understood in poultry. Hence, the lack of response in bone mineralisation to enzyme supplementation was not clearly highlighted.

FINANCIAL EVALUATION

Table 5.1: Financial calculation of margin over feed cost for exogenous enzyme(s) supplementation in broiler starter feed compared to Positive control diets

Treatments	PC	NC1	NC2	NC3	Trt1	Trt2	Trt3	Trt4
Live weight gain (kg)	0.954	0.778	0.939	0.797	0.951	0.954	0.934	0.939
Income R/bird (weight gain)	R 9.44	R 7.70	R 9.30	R 7.89	R 9.41	R 9.45	R 9.24	R 9.30
Live weight gain cost saving cents/bird					*-R 0.03*	*R 0.00*	*-R 0.20*	*-R 0.15*
Feed intake (kg)	1.174	1.095	1.206	1.076	1.237	1.176	1.16	1.222
Cost cents/kg	379.16	347.61	369.78	339.93	348.09	373.56	343.80	344.98
Feed cost cents/bird	R 4.45	R 3.81	R 4.46	R 3.66	R 4.31	R 4.39	R 3.99	R 4.22
Enzyme inclusion cost saving c/bird					*-R 0.15*	*-R 0.06*	*-R 0.46*	*-R 0.24*
MoFC (cents)	499.32	389.39	483.56	422.87	510.51	505.45	525.35	507.95
***Increased MoFC (cents)**					R 11.19	R 6.13	R 26.03	R 8.63
****Total savings (feed + live weight gain)***					*-R 0.18*	*-R 0.06*	*-R 0.67*	*-R 0.39*

Raw material prices were based on market prices. The cost of broiler production of R9.90/bird was used for the financial calculation.

MoFC – Margin over feed cost

* Increase MoFC - negative value is an indication of decrease in MoFC.

** Total savings (cent per bird) – negative value indicate saving as a results of improvement in weight gain and enzyme(s) supplementation.

Table 5.2: Financial calculation of margin over feed cost for exogenous enzyme(s) supplementation in broiler starter feed compared to Positive control diets

Treatments	PC	NC1	NC2	NC3	Trt1	Trt2	Trt3	Trt4
Live weight gain (kg)	1.200	0.959	1.154	0.932	1.176	1.203	1.092	1.084
Income R/bird	R 11.88	R 9.50	R 11.43	R 9.23	R 11.65	R 11.91	R 10.81	R 10.73
Live weight gain cost saving cents/bird					*-R 0.23*	*R 0.03*	*-R 1.07*	*-R 1.15*
Feed intake (kg)	1.991	1.669	1.874	1.666	1.954	1.989	2.014	1.908
Cost cents/kg	342.33	319.84	339.96	335.46	320.29	343.59	339.54	340.71
Feed cost cents/bird	R 6.82	R 5.34	R 6.37	R 5.59	R 6.26	R 6.83	R 6.84	R 6.50
Enzyme incl. cost saving cents/bird					*-R 0.56*	*R 0.02*	*R 0.02*	*-R 0.31*
MoFC (cents)	506.43	415.80	505.48	364.20	538.80	507.28	396.75	423.18
***Increased MoFC (cents)**					R 32.37	R 0.85	-R 109.68	-R 83.25
****Total savings (feed and live weight gain)***					*-R 0.79*	*R 0.04*	*-R 1.05*	*-R 1.46*

Raw material prices was based on market prices. The cost of broiler production of R9.90/bird was used for the financial calculation.

MoFC – Margin over feed cost

* Increase MoFC - negative value is an indication of decrease in MoFC.

** Total savings (cent per bird) – negative value indicate saving as a results of improvement in weight gain and enzyme(s) supplementation.

CHAPTER 6

CONCLUSIONS

The interaction between the use of multi-enzymes, other feed additives such as AGPs, the animal, gut microflora and the type of diet remains topics of interest. The focal point with enzymes still revolves around understanding underlying factors such as how to allocate accurate nutrient matrix values to multi-enzymes and justify broiler performance response. In this study it was evident that diets supplemented with phytase, protease or combination of the two enzymes to nutrient deficient diets were effective in improving performance and gut health of broiler chickens compared to nutrient deficient diets without enzyme(s).

The effect of phytase alone showed a positive response on body weight, FCR, ADG and gut health as observed when Ronozyme® HiPhos was added to negative control diets, indicating that supplementation of Ronozyme® HiPhos improved nutrient utilisation.

It can be concluded that phytase Ronozyme® HiPhos offered an opportunity to reduce the inclusion of inorganic phosphates. Ronozyme® HiPhos proved to be an efficient phytase that offers a feasible and cost effective phytase to replace expensive and scarce raw ingredients such as mono di calcium phosphate and improve the nutritive values of low quality ingredients without negatively affecting performance. The replacement of mono di calcium phosphate by phytate P released by phytase without loss in performance was a clear indication that Ronozyme® HiPhos is an efficient phytase to release phytate phosphorus from the plant material. This subsequently shows that the total concentration of P excreted to the environment could be reduced substantially by supplementing Ronozyme® HiPhos without negatively affecting performance of broiler chickens.

In this trial, it was not clear whether performance improvement in Trt3 was attributed to supplementation of Ronozyme® HiPhos or ProAct or combination of the two. It is eminent that the effect of the two enzymes were not additive as their effect was not better than individual contribution of phytase alone or protease alone. Addition of AGP (zinc bacitracin) Trt4 did not show any response to improvement in gut morphology when compared to Trt 1, 2, 3. Since there were no significant differences when comparing gut morphology with broilers in Trt4, it can be concluded that addition of enzymes phytase and protease was able to maintain the gut health in a manner similar to what AGP could offer.

Although not measured directly, it can be assumed that exogenous enzymes improve digestibility of feed, minimising accumulation of undigested fractions and resulting in reduction in proliferation of facultative microbes and proteolytic fermentation of undigested feed in the hindgut and subsequent improved gut health. Exogenous enzymes can be considered as alternative nutritional additives to AGP.

In this study, there was no response in the ash concentration or any other bone mineralisation parameters measured. Samples were collected from four birds and pooled for analysis. In part, it can be concluded that the lack of response could be ascribed to variation in the mineral intakes, nutrient utilisation and bone development of the different birds within and between treatments. Secondly, it can be concluded that birds fed diets moderately deficient in Ca and P for the first 18 days may exhibit partial ability to adapt to the deficiency. This study was focused on measuring the bone development parameters to prove the efficacy of exogenous phytase on bone development. The underlying factors such as the ability of the birds to partially adapt when subjected to moderate deficiency as in our negative control require careful scrutiny and approach. It is therefore imperative to consider the degree of challenge when designing negative control diets or lower nutrient specification diets when conducting exogenous enzyme studies.

GENERAL CONCLUSIONS

The use of exogenous enzymes in low nutrients density diets improved performance and gut health of broilers. This offers an opportunity to lower dietary specification and subsequently lower feed cost, improve nutrient digestibility and reduce environmental contamination from excess loss in nutrients such as protein and phosphorus. Most of the multi-enzyme combination studies reported in literature were conducted with a combination of phytase, carbohydrase and protease to target different types of substrates. In this study, the focus was on phytase or protease or combination to better understand nutrient contribution or additivity effect of the two enzymes. More work is still needed to understand additivity of two or more enzymes. Most importantly, the extend at which the nutrient density can be lowered in negative control treatments and subsequent contribution of enzymes added to the diets remains the key component in better measuring the efficacy of enzymes to an inherently varying degree of feed ingredient.

CRITICAL REVIEW

The following observations were made during the study that might have affected the outcome:

1. There was challenge in manufacturing reasonable pellet size and quality, especially in starter diets. The use of large and harder pellets in the starter diets resulted in spillages and wastage which could have slightly skewed some of the measurement of the feed consumption due to unaccounted feed leftovers.

2. Enzyme analysis was not conducted to verify the mixing accuracy of the enzymes. It is always an important parameter to execute when carrying out enzymes trial. The drawback with analysis is the fact that routine analysis for most of the enzymes cannot be done on any standard laboratory method, but through suppliers' specific laboratories which mostly have a long turnaround time as these are done at international laboratories.

3. We have attempted to measure nutrient digestibility with few drawbacks to the process. Firstly, we have chosen acid insoluble ash as the marker of choice as it is reported in literature to be one of the better indigestible markers. The major drawback was that we could not find an analytical method locally to accurately extract and recover the marker from the feed and faecal samples. The other complication was with the metabolic cages. The poor state of the metabolic cages resulted in the mixing of feed and faeces. Therefore, we could not report any of the data. It is important to consider the type of inert marker to be used which can be easily recovered, such as the mineral based markers.

CHAPTER 7

REFERENCES

AOAC, 2000. Official methods of analysis, 17th ed., Association of Official Analytical Chemists, AOAC International, Maryland, USA.

AOAC, 2005. Official methods of analysis, 18th ed., Association of Official Analytical Chemists, Washington DC, USA.

Angel, R., Tamim, N.M., Applegate, T.J., Dhandu, A.S. & Ellestad, L.E., 2002. Phytic acid chemistry: influence on phytin-phosphorus availability and phytase efficacy. J. Appl. Poult. Res. 11, 471-480

Angel, C. R., Saylor, W. W., Mitchell, A. D., Powers, W. & Applegate, T. J., 2006. Effect of dietary phosphorus, phytase, and 25-hydroxycholecalciferol on broiler chicken bone mineralisation, litter phosphorus, and processing yields. Poult. Sci. 85, 1200-1211

Angel, C. R., Saylor, W., Vieira, S. L. & Ward, N., 2011. Effects of a mono-component protease on performance and protein utilisation in 7 to 22 day-old broiler chickens. Poult. Sci. 90, 2281-2286

Angkanaporn, K., Choct, M., Bryden, W. L., Annison, E. F. & Annison, G., 1994. Effects of wheat pentosans on endogenous amino acid losses in chickens. J. Sci. Food Agric. 66, 399-404

Annison, G. & Choct, M., 1991. Anti-nutritive activities of cereal non-starch polysaccharides in broiler diets and strategies minimising their effects. World Poult. Sci. J. 47, 232-242

Applegate, T. J., Joern, B. C., Nussbaum-Wagler, D .L. & Angel, R., 2003. Water-soluble phosphorus in fresh broiler litter is dependent upon phosphorus concentration fed but not on fungal phytase supplementation. Poult. Sci. 82, 1024-1029

Applegate, T.J., Powers, W.J., Angel, R. & Hoehler, D., 2008. Effect of amino acid formulation and acid supplementation on performance and nitrogen excretion in turkey toms. Poult. Sci. 87, 514-520

Applegate, T. J. & Angel., R., 2008. Phosphorus requirements for poultry. Purdue Extension, Anim. Sci. AS-583-W

Attia, Y. A., Bovera, F., Abd El-Hamid, A. E., Tag El-Din, A. E., Al-Harthi M. A. & El-Shafy, A. S., 2016. Effect of zinc bacitracin and phytase on growth performance, nutrient digestibility, carcass and meat traits of broilers. J. Anim. Physiol. and Anim. Nutri. 100, 485-491.

Baas, T.C. & Thacker, P.A., 1996. Impact of gastric pH on dietary enzyme activity and survivability in swine fed β-glucanase supplemented diets. Can. J. Anim. Sci. 36, 245-252

Bach Knudsen, K. E., 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Anim. Feed Sci. Technol. 67, 319-338.

Bao, Y.M., Romero, L.F. & Cowieson, A.J., 2013. Functional patterns of exogenous enzymes in different feed ingredients. World's Poult. Sci. J. 69, 759-774

Bancroft, J.D., 2003. Theory and practice of histological techniques. (5th ed). pp 130.

Bedford, M.R. & Classen, H.L., 1992. Reduction of intestinal viscosity through manipulation of dietary rye and pentosanase concentration is effected through changes in the carbohydrate composition of the intestinal aqueous phase and results in improved growth rate and food conversion efficiency of broiler chicks. J. Nutr. 122, 560-569

Bedford, M. R., 2000. Exogenous enzymes in monogastric nutrition. Their current value and future benefits. Livest. Prod. Sci. 86, 1-13

Bedford, M.R., 2002. The foundation of conducting feed enzyme research and the challenge of explaining the results. J. Appl. Poult. Res. 11, 464-470.

Bertichini, A.G., Carvalho, J.C.C., Mesquita, F.R., Castro, S.F., Meneghetti, C. & Sorbara, J.O.B., 2009. Use of a protease to enhance the utilisation of soybean meal amino acids by broilers. Poult. Sci. Abstr. 221, Raleigh NC

Blahos, J., Care, A. D., & Sommerville, B. S., 1987. Effect of low calcium and low phosphorus diets on duodenal and ileal absorption of phosphate in chick. Endocrinol. Exp. 21, 59-64.

Bohak, Z., 1969. Purification and characterisation of chicken pepsinogen and chicken pepsin. J. Biol Chem. 244, 4638-4648.

Bohak, Z., 1973. The kinetics of the conversion of chicken pepsinogen to chicken pepsin. European J. Biochem. 32, 547-554.

Botsoglou, N.A., Christaki, E., Florou-Paneri, P., Gieannenas, I., Papageorgiou, G. & Spais, A.B., 2004. The effect of a mixture of herbal essential oils or α–tocopheryl acetate on performance parameters and oxidation of body lipid in broilers. S.A. J. Anim. Sci. 34, 52-61.

Bryden, W. L., 1996. Antinutritive factors in protein metabolism. In Protein Metabolism and Nutrition, (editors Nunes, A.F., Portugal, A.V., Costa, J.P. & Ribeiro, J.R.) Estacao Zootecnica Nacional. Vale de Santarem, Portugal, pp. 517-518.

Cai, Q., Schandler, J.S., Wasserman, R.H., Kumar, R. & Penniston, J.T., 1993. Vitamin D3 and adaptation to dietary calcium and phosphate deficiencies increase intestinal plasma membrane calcium pump gene expression. Proc. of the Nat. Academy of Sci. USA. 90, 1345-1349.

Caldwell, R. A., 1992. Effect of calcium and phytic acid on the activation of trypsinogen and the stability of trypsin. J. Sci. Food Agric. 40, 43-46.

Camden, B. J., Morel, P.C.H., Thomas, D.V., Ravindran, V. & Bedford, M.R., 2001. Effectiveness of exogenous microbial phytase in improving the bio-availabilities of phosphorus and other nutrients in maize-soya-bean meal diets for broilers. Anim. Sci. 73, 289-297.

Choct, M., Hughes, R. J., Wang, J., Bedford, M. R., Morgan A. J. & Annison, G., 1996. Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. Br. Poult. Sci. 37, 609-621.

Choct, M., 2006. Enzymes for the feed industry: past, present and future. World's Poult. Sci. J. 62, 5-15.

Costa, F.G.P., Goulart, C.C., Figueiredo, D.F., Oliveira, C.F.S. & Silva, J.H.V., 2008. Economic and Environmental Impact of Using Exogenous Enzymes on Poultry Feeding. Int. J. Poult. Sci. 7, 311-314.

Cowieson, A.R., 2010. Strategic selection of exogenous enzymes for corn/soy-based poultry diets. J. Poult. Sci. 17, 1-7.

Cowieson, A. J., Acamovic, T. & Bedford, M. R., 2003. The effect of phytase and phytic acid on endogenous losses from broiler chickens. Br. Poult. Sci. 44, S23-S24.

Cowieson, A.J., Acamovic, T. & Bedford, M.R., 2004a.The effects of phytase and phytic acid on the loss of endogenous amino acids and minerals from broiler chickens. Br. Poult. Sci. 45, 101-108.

Cowieson, A.J., Acamovic, T. & Bedford, M.R., 2004b. The effects of supplementing maize-based diets with exogenous phytase on amino acid digestibility and nitrogen retention by young broiler chicks. Br. Poult. Sci. 45, (S6).

Cowieson, A. J., Acamovic, T. & Bedford, M. R., 2006a. Phytic acid and phytase: Implication for protein utilisation by poultry. Poult. Sci. 85, 878-885.

Cowieson, A.J., Acamovic, T. & Bedford, M.R., 2006b. Supplementation of corn–soy-based diets with an Eschericia coli-derived phytase: Effects on broiler chick performance and the digestibility of amino acids and metabolisability of minerals and energy. Poult. Sci. 85, 1389-1397

Cowieson, A. J. & Adeola, O., 2005. Carbohydrases, protease, and phytase have an additive beneficial effect in nutritionally marginal diets for broiler chicks. Poult. Sci. 84, 1860-1867.

Cowieson, A.J. & Bedford, M.R., 2009. The effect of phytase and carbohydrase on ileal amino acid digestibility in monogastric diets: complimentary mode of action? World Poult. Sci. J. 65, 609-623.

Cowieson, A.J., Bedford, M.R., Selle, P.H. & Ravindran, V., 2009. Phytate and microbial phytase: implications for endogenous nitrogen losses and nutrient availability. Worlds. Poult. Sci. J. 65, 401-418.

Cowieson, A.J., Bedford, M.R. & Ravindran, V., 2010. Interaction between xylanase and glucanase in maize-soy-based diets for broilers. Br. Poult. Sci. 51, 246-257

Cowieson, A.J. & Ravindran, V., 2007. Effect of phytic acid and microbial phytase on the flow and amino acid composition of endogenous protein at the terminal ileum of growing broiler chickens. Br. J. Nutr. 98, 745-752.

Cowieson, A. J. & Ravindran, V., 2008. Effect of exogenous enzymes in maize-based diets varying in nutrient density for young broilers: Growth performance and digestibility of energy, minerals and amino acids. Br. Poult. Sci. 49, 37-44.

Cowieson, A. J., Ravindran, V. & Selle, P. H., 2008. Influence of dietary phytic acid and source of microbial phytase on ileal endogenous amino acid flows in broiler chickens. Poult. Sci. 87, 2287-2299

Cowieson, A. J., Wilcock, P. & Bedford, M. R., 2011. Super-dosing effects of phytase in poultry and other monogastrics. Worlds Poult. Sci. J. 67, 225-236.

Cowieson, A. J. & Roos, F. F., 2014. Bio-efficacy of a mono-component protease in the diets of pigs and poultry: a meta-analysis of effect on ileal amino acid digestibility. J. Appl. Anim. Nutr. 2, (e13), 1-8

Cromwell, G. L., Calvert, C. C., Cline, T. R., Crenshaw, J. D., Easter, R. A., Ewan, R. C., Hamilton, C. R., Hill, G. M., Lewis, A. J., Mahan, D. C., Miller, E. R., Nelsson, J. L. Pettigrew, J. E., Tribble, L. F., Veum, T. L. & Yen, J. T., 1999. Variability among sources and laboratories in nutrient analyses of corn and soybean meal. J. Anim. Sci. 77, 3262-3273.

D' Alfonso, T.H. & McCracken, K., 2002. Global corn quality variability. Proceedings of the Multi-state poultry meeting. Indianapolis, Indiana May, 14-16.

D' Alfonso, T.H., 2005 Sources of variances of energy digestibility in corn-soy poultry diets and effect on performance: starch, protein, oil and fibre. Krmiva. 2, 83-86.

Danicke, S., W. Vahjen, W., Simon, O. & Jorech, H., 1999. Effect of dietary fat type and xylanase supplementation to rye-based broiler on selected bacterial groups adhering to the intestinal epithelium on transit time of feed and on nutrient digestibility. Poult. Sci. 78, 1292-1299.

Deshpande, S. S. & Cheryan, M., 1984. Effects of Phytic Acid, Divalent Cations, and Their Interactions on α-Amylase Activity. J. Food Sci. 49, 516-519

Dierick, N.A. & Decuypere, J.A., 1994. Enzymes and growth in pigs. In: Cole, D.J.A., Wiseman, J. & Varley, M. Principles of Pig Science. Nottingham University Press, Nottingham. pp 169 -195.

Doyle, J.J., 1979. Toxic and essential elements in bone - a review. J. Anim. Sci. 49, 482-497.

Dosković, V., Bogosavljević-Bošković, S., Pavlovski, Z., Milošević, B., Škrbić, Z., Radonjac, S. & Petričević, V., 2013. Enzymes in broiler diets with special reference to protease. W. Poult. Assoc. 69, 343-360.

Douglas, M. W., Peter, C. M., Boling, S. D., Parsons, C. M. & Baker, D. H., 2000. Nutritional evaluation of low phytate and high protein corns. Poult. Sci. 79, 1586-1591.

Duarte, C.R.A., Eyng, C., Murakami, A.E. & Santos, T.C., 2014. Intestinal morphology and activity of digestive enzymes in broilers fed crude propolis. Can. J. Anim. Sci. 94, 105-114.

Engberg, R.M., Hedemann, M.S., Leser, T.D. & Jensen, B.B., 2000. Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. Poult. Sci. 79, 1311-1319.

Esteve-Garcia, E., Brufau, J., Perez-Vendrell, A., Miquel, A. & Duven, K., 1997. Bioefficacy of enzyme preparations containing β-glucanase and xylanase activities in broiler diets based on barley or wheat, in combination with flavomycin. Poult. Sci. 76, 1728-1737.

Friedman, M., 1996. Food Browning and Its Prevention: An Overview. J. Agricul. & Food Chem. 44, 631-653.

Freitas, D.M., Vieira, S.L., Angel, C.R., Favero, A. & Maiorka, A., 2011. Performance and nutrient utilisation of broilers fed diets supplemented with a novel mono-component protease. J. Appl. Poult. Res. 20, 322-334

Fru-Nji, F., Kluenter, A.M., Fischer, M. & Pontoppidan, K., 2011. A feed serine Protease improves broiler performance and increase protein and energy digestibility. J. Poult. Sci. 48, 239-246.

Ghazi, S., Rooke, J. A. & Galbraith, H., 2003. Improvement of the nutritive value of soybean meal by protease and α-galactosidase treatment in broiler cockerels and broiler chicks. Br. Poult. Sci. 44, 410-418.

Gitzelmann, R. & Auricchio, S., 1965. The handling of soy β-galactosidase by a normal and galactosemic child. Paediatrics 36, 231-232.

Gomide, M. H. J., Sterzo, E. V., Macari, M., & Boleli, I. C., 2004. Use of scanning electron microscopy for the evaluation of intestinal epithelium integrity. Revista Brasileira de Zootecnia. 33, 1500-1505.

Hock, E., Halle, I., Matthes, S. & Jeroch, H., 1997. Investigations on the composition of the ileal and caecal microflora of broiler chicks in consideration to dietary enzyme preparation and zinc bacitracin in wheat-based diets. Agribiological Research-Zeitschrift fur Agrarbiologie Agrikulturchemie Okologie. 50, 85–95

Hughes, R. J. & Choct, M., 1999. Chemical and physical characteristics of grains related to variability in energy and amino acid availability in poultry. Aust. J. Agric. Res. 50, 689-701.

Humphrey, B.D., Huang, N. & Klasing, K.C., 2002. Rice expressing lactoferrin and lysozyme has antibiotic-like properties when fed to chick. J. Nutr. 132, 1214-1218.

Huyghebaert, G. & De Groote, G. 1995. The effect of specific enzymes on the MEn value and nutrient utilisation of target feedstuffs in broiler and layer diets. ln: Proc. 10th Eur. Symp. on Poult. Nutr., 15-19 Oct. 1995, Antalya, Turkey. pp. 176-19

Juanpere, J., Perez-Vendrell, A.M., Agulo, E. & Brufau, J., 2005. Assessment of potential interaction between phytase and glycosidase enzyme supplementation on nutrient digestibility in broilers. Poult. Sci. 84, 571-580.

Jaroni, D., Scheideler, S.E., Beck, M.M. & Wyatt. C., 1999. The effect of dietary wheat middlings and enzyme supplementation II: Apparent nutrient digestibility, digestive tract size, gut viscosity and gut morphology in two strains of Leghorn hens. Poult. Sci. 78, 1664-1674.

Khan, A., Khan, R., Khan, M.F. & Khanam, F., 1997. A cluster model explaining quantitatively the anomalous variation of density of water with temperature. Chemical Physics Letters 266, 473-480

Khattak, F.M., Pasha, T. N., Hayat, Z. & Mahmud, A., 2006. Enzymes in poultry Nutrition. J. Anim. Poult. Sci. 16, 1-2.

Kies, A. K., Van Hemert K. H. F. & Sauer, W. C., 2001. Effect of phytase on protein and amino acid digestibility and energy utilisation. World's Poult. Sci. J. 57, 110-124.

Kling, L. J., 1985. The effect of calcium and phosphorus on the incidence of tibial dyschondroplasia in young chicks. Nutrition Reports International 32, 1473-1480.

Langhout, D. J. & Schutte, J.B., 1995. Effects of avilamycin and a xylanase enzyme preparation alone or in combination on broiler performance and ileal viscosity. Proc. 10th Eur. Symp. on Poult. Nutr., 15-19 Oct. 1995, Antalya, Turkey. pp. 379-380.

Le Heurou-Luron, I., Lhoste, E., Wicker-Planquarl, C., Dakka, N., Toullec, R., Corring, T., Guilloteau, P. & Puigserver, A., 1993. Molecular aspects of enzyme synthesis in the exocrine pancreas with emphasis on development and nutritional regulation. Proceeding in Nutrition Society 52, 301-313.

Lemme A., Ravindran V. & Bryden, W.L., 2004. Ileal digestibility of amino acids in feed ingredients for broilers. Worlds' Poult. Sci. J. 60, 423-438.

Létourneau-Montminy, M. P., Narcy, A., Lescoat, P., Bernier, J. F., Magnin, M., Pomar, C., Nys, Y., Sauvant, D. & Jondreville, C., 2010. Meta-analysis of phosphorus utilisation by chicks: Influence of dietary calcium and microbial phytase content. Anim. 4, 1844-1853.

Leytem, A.B., Willing, B.P. & Thacker, P.A., 2008. Phytate utilisation and phosphorus excretion by broiler chickens fed diets containing cereal grains varying in phytate and phytase content. Anim. Feed Sci. Technol. 146, 160-168.

Li, D. F., Nelssen, J. L., Reddy, P. G., Blecha, F., Klemm, R. & Goodband, R. D. 1991. Interrelationship between hypersensitivity to soybean proteins and growth performance in early-weaned pigs. J. Anim. Sci. 69, 4062.

Lichtenberg, J., Pedersen, P. B., Elvig-Joergensen, S. G., Skov, L. K., Olsen, C. L. & Glitsoe, L. V., 2011. Toxicological studies on a novel phytase expressed from synthetic genes in Aspergillus oryzae. Regul. Toxicol. Pharmacol. 60, 401-410.

Maenz, D. D. & Claasen, H. L., 1998. Phytase activity in the small intestinal brush border membrane of the chicken. Poult. Sci. 77, 557-563.

Maguire, R. O., Dou, Z., Sims, J. T., Brake, J. & Joern, B. C., 2005. Dietary strategies for reduced phosphorus excretion and improved water quality. J. Environ. Qual. 34, 2093-2103.

Masey O'Neill, H.V., Liu, N., Wang, J. P., Diallo, A. & Hill, S., 2012. Effect of Xylanase on performance and apparent metabolisable energy in starter broilers fed diets containing one maize variety harvested in different regions of China. Asian Austral. J. Anim. Sci. 25, 515 - 523.

Masey O'Neill, H. V., Smith, J.A. & Bedford, M.R., 2014. Multi-carbohydrase enzymes for non-ruminants. Asian Austral. J. Anim. Sci. 27, 290-301.

Moran, E.T., 1985. Digestion and absorption of carbohydrates in fowl and events through perinatal development. J. Nutr. 115, 665-674.

Morrisey, R. L. & Wasserman, R. H., 1971. Calcium absorption and calcium-binding protein in chicks on differing calcium and phosphorus intakes. Am. J. Physiol. 220, 1509-1515.

Munir, K. & Maqsood, S., 2013. A review on role of exogenous enzyme supplementation in poultry production. J. Food Agric. 25, 66-80.

Nelson, T.S., 1967. The utilisation of phytate phosphorus by poultry. Poult. Sci. 46, 862-871.

Nir, I., Nitsan, Z., & Mahagna, M., 1993. Comparative growth and development of the digestive organs and of some enzymes in broiler and egg type chicks after hatching. Br. Poult. Sci. 34, 523-532.

Nitsan, Z., Dunnington, E. & Siegel, P., 1991. Organ growth and digestive enzyme levels to 15 days of age in lines of chickens differing in body weight. Poult. Sci. 70, 204 & 2048.

Ohh, S. J., 2011. Meta-analysis to draw the appropriate regimen of enzyme and probiotic supplementation to pigs and chicken diets. Asian Austral. J. Anim. Sci. 24, 573-586.

Oliveira, A. C., Vidal, B. C. & Sgarbieri, V. C., 1989. Lesions of intestinal epithelium by ingestion of bean lectins in rats. J. Nutr. Sci. Vitaminol. 35, 315-322

Olukosi, O. A., Bedford, M. R. & Adeola, O., 2007. Xylanase in diets for growing pigs and broiler chicks. Can. J. Anim. Sci. 87, 227-235.

Pack, M. & Bedford, M., 1997. Feed enzymes for corn and soybean broiler diets. World Poult. 13, 87-93.

Partridge, G.G. 1993. In feed enzymes and antibodies. Pig Vet. J. 31, 34-50.

Parsons, C. M., Castanon, F. & Han, Y., 1997. Protein and amino acid quality of meat and bone meal. Poult. Sci. 76, 361-368.

Payne, R.W. (Ed.) 2014. Introduction to GenStat® for Windows™ (17th Edition), VSN International, Hemel Hempstead, Hertfordshire, United Kingdom ©, VSN International. Website: http://www.genstat.co.uk/

Penn, C. J., Mullins, G. L., Zelazny, L. W., Warren, J. G. & McGrath, J. M., 2004. Surface run-off losses of phosphorus from Virginia soils amended with turkey manure using phytase and high available phosphorus corn diets. J. Environ. Quality 33, 1431-1439

Piper, D.W. and Fenton, B.H. 1965. pH stability and activity curves of pepsin with special reference to their clinical importance. Gut 6, 506

Powell, S., Bidner, T. D. & Southern, L. L., 2011. Phytase supplementation improved growth performance and bone characteristics in broilers fed varying levels of dietary calcium. Poult. Sci. 90, 604-608.

Pustzai, A., Ewen, S.W.B., Grant, G., Peumans, W. J., van Damme, E.J.M., Rubio, L. & Bardocz, S., 1990. Relationship between survival and binding of plant lectins during small intestinal passage and their effectiveness as growth factors. Digest. 46, 308-316

Rackis, J. J. & Gumbman, M. R., 1981. Protease inhibitors: physio-logical properties and nutritional significance. In: ORY, R. L. (Ed.), Anti-nutrients and Natural Toxicants in Foods. Westport, CT. Food and Nutrition Press, pp. 203-237

Ravindran, V., Cabahug S., Ravindran G. & Bryden, W.L., 1999. Influence of microbial phytase on apparent amino acid digestibility of feedstuffs for broilers. Poult. Sci. 78, 699-706. 681

Ravindran, V., Cabahug, S., Ravindran, G., Selle, P.H. & Bryden, W.L., 2000. Response of broiler chickens to microbial phytase supplementation as influenced by dietary phytic acid and non-phytate phosphorus levels. II. Effects on apparent metabolisable energy, nutrient digestibility and nutrient retention. Br. Poult. Sci. 41, 193-200.

Ravindran, V., Morel, P., Partridge, G., Hruby, M. & Sands, J., 2006. Influence of an Escherichia coli derived phytase on nutrient utilisation in broiler starters fed diets containing varying concentrations of phytic acid. Poult. Sci. 85, 82-89.

Ravindran, V., 2013. Feed enzymes: The science, practice, and metabolic realities. J. Appl. Poult. Res. 22, 628-636

Rutherfurd, S. M., Chung, T.K, Morel, P.C. & Moughan, P. J., 2004. Effects of microbial phytase on the ileal digestibility of phytate phosphorus, total phosphorus, and amino acids in a low phosphorus diet for broilers. Poult. Sci. 83, 61-68

Sarica, S., Ciftci, A., Demir, E., Kilinc, K. & Yildirim, Y., 2005. Use of an antibiotic growth promoter and two herbal natural feed additives with and without exogenous enzymes in wheat based broiler diets. S. Afr. J. Anim. Sci. 35, 61-72.

Seldin, E. D., 1965. A rheologic model for cortical bone. Acta Orthopaedica Scandinavica 36, 1-77.

Simbaya, J., Slominski, B. A., Guenter, W., Morgan, A. & Campell, L. D., 1996. The effects of protease and carbohydrase supplementation on the nutritive value of canola meal for poultry: In vitro and in vivo studies. Anim. Feed Sci. Technol. 61, 219-234.

Singh, P. K., & Krikorian, A. D., 1982. Inhibition of trypsin activity in vitro by phytate. J. Agricul Food Chem. 30:799-800.

Slominski, B. A., 2011. Review: Recent advances in research on enzymes for poultry diets. 2011 Poult. Sci. 90, 2013-2023

Skurray, G.R., 1974. The nutritional evaluation of meat meals for poultry. World Poult. Sci. J. 30, 129-136.

Somerville, C., Bauer S., Brininstool G., Facette, M. Hamann, T., Milne, J., Osborne, E., Paredez, A., Persson, S., Raab, T., Vorwerk, S. & Youngs, H., 2004. Toward a systems approach to understanding plant cell walls. Sci. 306, 2206-2211.

Smits, H.M., & Annison, G., 1996. Non-starch plant polysaccharide in broiler nutrition towards a physiologically valid approach to their determination. Worlds' Poult. Sci. J. 52, 203-221.

Sorbara, J.O.B., 2009. Use of a protease to enhance the utilisation of soybean meal amino acids by broilers. Poultr. Sci. Assoc. 98th Annual Meeting, Raleigh, North Carolina. Poult. Sci. 88, Abstr. 221, 68.

Sayrafi, R., Soltanalinejad, F., Shahrooz, R. & Rahimi, S., 2011. Comparative study of the effect of alternative and antibiotic feed additives on the performance and intestinal histo-morphometrical parameters of broiler chickens. Afr. J. Agric. Res. 6, 2794-2799.

Thacker, P.A. & Baas, T.C., 1996. Effects of gastric pH on the activity of exogenous pentosanase and the effect of pentosanase supplementation of the diet on the performance of growing–finishing pigs. Anim. Feed Sci. Technol. 63, 187- 200.

Tamim, N. M., Angel, R. & Christman, M., 2004. Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. Poult. Sci. 83, 1358-1367

Theander, O., Westerlund, E., Aman, P. & Graham, H., 1989. Plant cell walls and monogastric diets. Anim. Feed Sci. Technol. 23, 205-225.

Thorpe, J. and Beal, J.D. 2001. Vegetable protein meals and the effects of enzymes, In: Bedford, M. R. and Patridge, G.G. (Eds) Enzymes in Farm Animal Nutrition, pp. 125 -143

Vieira, S.L., Stefanello, C. & Sorbara, J.O.B., 2014. Possible substrates for exogenous enzymes formulating poultry diets based on their indigestible components. Poult. Sci. 93, 1-6.

Vukic-Vranjes, M. & Wenk, C., 1993. Influence of dietary enzyme complex on broiler performance in diets with and without antibiotic supplementation. Enzymes in Animal Nutrition. Proc. 1st Symp. 13-16th October, Kartause, Ittingen, Switzerland. pp. 152-155

Walk, C. L., Cowieson, A. J., Remus, J. C., Novak, C. L. & McElroy, A. P., 2011. Effects of dietary enzymes on performance and intestinal goblet cell number of broilers exposed to a live coccidian oocyst vaccine. Poult. Sci. 90, 91-98.

Wang, X. & Parsons, C.M., 1998. Effect of raw material source, processing systems, and processing temperatures on amino acid digestibility of meat and bone meals. Poult. Sci. 77, 834 -841.

Warenham, C. N., Wiseman, J. & Cole, D. J. A., 1994. Processing and anti-nutritive factors in feedstuff. In: Cole, D.J.A, Wiseman, J, Varley M. A. Eds. Principles of Pig Science. Nottingham University Press, Nottingham, UK. pp. 141 - 167

Wiggins, H. S. 1984. Nutritional value of sugars and related compounds undigested in the small gut. Proc. Nutr. Soc. 43, 69-85.

Whitehead, C.C., McCormack, H.A., McTier, L. & Fleming, R.H., 2004. High Vitamin D3 requirements in broilers for bone quality and prevention of tibial dyschondroplasia and interactions with dietary calcium, available phosphorus and Vitamin A. Br. Poult. Sci. 45, 425-436.

Woyengo, T. A., Adeola, O., Udenigwe, C. C. & Nyachoti, C. M., 2010. Gastro-intestinal digesta pH, pepsin activity and soluble mineral concentration responses to dietary phytic acid and phytase in piglets. Livest. Sci. 134, 91-93

Woyengo, T. A., & Nyachoti, C. M., 2011. Review: Supplementation of phytase and carbohydrases to diets for poultry. Can. J. Anim. Sci. 91, 177-192

Wu, Y.B., Ravindran, V., Thomas, D.G., Birtles, M.J. & Hendriks, W.H., 2004. Influence of phytase and xylanase, individually or in combination, on performance, apparent metabolisable energy, digestive tract measurements and gut morphology in broilers fed wheat-based diets containing adequate level of phosphorus. Br. Poult. Sci. 45, 76-84

Wyatt, C. L., Parr, T, and Bedford, M.R. 2008. Mechanisms of action for supplemental NSP and phytase enzymes in poultry diets. Proc. 35th Annual Carolina Poult. Nutr. Conference. pp 12-22.

Xu, H., Bai, L., Collins, J. F. & Chishan, F.K., 2002. Age-dependent regulation of rat intestinal type IIb sodium-phosphate co-transporter by 1,25-(OH)2 vitamin D3. Am. J. Physiol. Cell. Physiol. 282 , 487-493.

Xu, Z.R., Hu, C.H., Xia, M.S., Zhan, X.A. & Wang, M.Q., 2003. Effects of dietary fructo-oligosaccharides on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poult. Sci. 82, 1030-1036.

Yan, F., Angel, R., Ashwell, C., Mitchell, A. & Christman, M., 2005. Evaluation of the broiler's ability to adapt to an early moderate deficiency of phosphorus and calcium. Poult. Sci. 84, 1232-1241.

Zakaria, H.A.H., Jalal, M.A.R. & Abu Ishmais, M.A., 2010. The Influence of Supplemental Multi-enzyme Feed Additive on the Performance, Carcass Characteristics and Meat Quality Traits of Broiler Chickens. Int. J. Poult. Sci. 9, 126 -133

Zanella, I., Sakamuro, N. K., Silversides, F. G., Fiqueirdo, A. & Pack, M., 1999. Effect of enzyme supplementation of broiler diets based on corn and soybeans. Poult. Sci. 78, 561-568.

Zhang, B. & Coon, C.N., 1997. The relationship of various tibia bone measurements in hens. Poult. Sci. 76, 1698-1701.