INFLUENCE OF DIETARY ELECTROLYTE BALANCE ON PHYTASE EFFICACY IN POULTRY

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Abstract

The aims of the studies reported in this thesis were to investigate the possible effects of changes in dietary electrolyte balance (dEB) levels on phytase efficacy on growth performance, bone mineralisation and nutrient utilisation; and the effects of phytase supplementation, alone or in combination with xylanase, on growth performance, bone ash, volatile fatty acids (VFA) and pH at different parts of the gastrointestinal tract (GIT) in diets marginally deficient in dEB. Two experiments were performed and each experiment used 336 day-old Ross 308 male broiler chicks which were allocated to 7 treatments in a randomised complete block design. The dietary treatments were corn-soybean meal based and were fed in a mash form. For both experiments, each treatment had 6 replicate pens with 8 birds each. A 3×2 factorial arrangement of treatments was used in each experiment along with a positive control (PC) with nutrient levels meeting breeder recommendation. Data in both experiments were analysed using the linear mixed model of GenStat. In experiment 1, three levels of dEB (214, 234 and 266 mEq/Kg) and 2 levels of phytase (0 FTU/Kg and 1500 FTU/Kg) were used. Birds and feed were weighed on day 0 and 21. Excreta were collected from pens on days 19 and 20. On day 21 one randomly selected bird from each pen was killed, blood was collected from the jugular vein and blood pH measured. The remaining birds in each pen were euthanised and ileal digesta were collected from each pen. The left tibia bones from two randomly selected birds in each raised pen were collected for bone mineralisation study. There were no dEB × phytase interactions for any of the growth performance and bone mineralisation responses. But an interaction ($P<0.05$) was observed for ileal digestibility and total tract retention for most of the nutrients. Increasing dEB from 214 to 266 mEq/Kg had no effect on growth performance or tibia bone mineralisation. Phytase supplementation increased ($P<0.05$) broilers growth performance and tibia bone mineralisation responses relative to the diets without phytase. Growth performance and bone mineralisation responses were greater ($P<0.05$) in PC compared with NC, except for feed intake. There were no differences in the response between PC and the diets containing equivalent dEB (234 mEq/Kg) but broilers fed the corresponding diets with phytase outperformed NC and PC broilers. Ileal and total tract P utilisation were greater ($P<0.05$) in birds fed NC and phytase-supplemented diets than the PC birds. Utilisation of DM, Ca, K, and P was also greater ($P<0.05$) in phytase-supplemented diets at 234 mEq/Kg but for P and K was greater in phytase-supplemented diets when dEB was 266 mEq/Kg. In Experiment 2, the factors included 3 levels of enzyme (no enzyme, phytase alone or combination of phytase and xylanase)

and 2 types of negative control (NC1 and NC2). All the birds and feed were weighed on day 0 and 21. After slaughter on day 21, the left tibia bones were collected and the pH of the gizzard, jejunum and caeca (left and right) were taken. Caecal content were collected into tubes to be analysed for volatile fatty acids content. There were no significant matrix × enzyme interactions for any of the responses. Reducing the dietary levels of P and Ca in both NC1 and NC2 reduced ($P<0.05$) gain:feed compared with PC but had no significant effect on the other growth performance responses. Phytase alone had no effect on growth performance compared to diets with no enzyme supplementation, but when phytase was combined with xylanase an increase ($P<0.01$) in gain:feed was observed. Tibia ash was lower ($P<0.01$) in NC1 and NC2 compared to PC and improved ($P<0.05$) with supplementation of phytase alone or combined with xylanase. The treatments had no effect on the digesta pH or caeca VFA. It was concluded from experiment 1 that phytase promoted growth and tibia bone mineralisation independently of the dEB levels and that the extent of phytase effect on nutrient utilisation differs depending on dEB content of the diets, which indicates that treatments effects seen in nutrient utilisation do not always reflect effects observed in growth performance. It was concluded from the second experiment that dietary reductions of Ca and P negatively affected tibia mineralisation and gain:feed which were counterbalanced by supplementation of phytase plus xylanase .

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Author's Declaration

The work, of which this thesis is a record, is original and all sources of information have been specifically noted by means of appropriate references. This thesis has been composed by the author and no part of the dissertation has been submitted for examination in any previous application for a degree.

Rita Pereira Gonçalves

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Abbreviations

AA	:	Amino acids
AME	:	Apparent Metabolisable Energy
BSE	:	Bovine spongiform encephalopathy
Ca	:	calcium
Cl	:	chloride
Cr	:	chromium
Cu	:	copper
dEB	:	dietary electrolyte balance
DM	:	dry matter
dUA	:	dietary undetermined anion
Fe	:	iron
g	:	gram
GE	:	gross energy
GIT	:	gastrointestinal tract
HDL	:	high-density lipoprotein
HMG-CoA	:	3-hydroxy-3-methylglutaryl coenzyme A
IDE	:	ileal digestible energy
K	:	potassium
Kcal	:	kilo calorie
Kg	:	kilo gram
ME	:	metabolisable energy
mEq/Kg	:	mill equivalents per Kilogram
Mg	:	magnesium
N	:	nitrogen
Na	:	sodium
NSP’s	:	non-starch polysaccharides
P	:	phosphorus
pH	:	potential of hydrogen
SEM	:	Standard Error of the Mean
Ti	:	titanium
TiO_2	:	titanium dioxide
VFA	:	volatile fatty acid
XAP	:	xylanase, amylase and protease
Zn	:	zinc

1. Introduction

In the animal production industry, feed is the biggest factor in production costs representing around 60 to 75% of total costs (Ligeiro, 2007) therefore, small improvements in the efficiency and utilisation of nutrients and the use of alternative ingredients might result in great savings (Ligeiro, 2007). Animals depend on nutrients from the diet to maintain normal physiological activities. To access these nutrients, animals have digestive systems that break down ingested feed into smaller particles for digestion and absorption into the body. The major purpose of the digestive system is the assimilation of nutrients required for maintenance, growth (including bone mineralization), reproduction and meat or egg production (Stevens & Hume, 2004). Due to the inferior diversity and amounts of certain microbial species in their gut compared to other species like cattle, the simplicity of poultry's digestive system in structure and function, demands that the diets formulated for poultry use must be high in quality and digestibility in order to expedite absorption. Exogenous enzymes have been available for use in the feed industry for a number of years but their use has increased in the last few years due to the increase of feed costs (Hahn, 2010) and the use of more unconventional feedstuffs. Exogenous enzymes will improve their nutrient and energy availability by reducing the action of anti-nutritive factors and by increasing their digestibility and improving their nutritive value (Ravindran, 2013).

Several studies have shown the importance of exogenous enzymes in reducing the effect of anti-nutritional factors (Meng & Slominski, 2005; Hruby & Pierson, 2009) and improving feed efficiency (Wyatt & Goodman, 1993) and these enzymes have proven to be valuable due to their lower cost, as well as environmental and productivity benefits. The global feed enzyme market can be broadly divided into phytase (approximately 60%) and non-phytase (40%) enzymes segments and is worth in excess of $550 million US dollars, saving the global feed market an estimated $3 to $5 billion per year (Adeola & Cowieson, 2011). Over two-thirds of phosphorus (P) in cereals and legumes, the main ingredients of poultry diets, are contained in chemical structures called phytic acid or its salts, collectively known as phytates. Phytate-P is not available for poultry unless at least one of the phosphate groups is removed from the inositol molecule, by intrinsic feed phytase, intestinal phytase or microbial phytase. Non-ruminant animals do not produce phytase in significant quantity and therefore exogenous phytases are routinely added to their feed to enable better utilisation of phytate-P. Phytase does not just increase the digestibility of phytate-P in poultry but may improve the digestibility of other minerals like calcium (Ca), as well as

other micro minerals and energy (Kornegay, 2001). Phytate is often considered an anti-nutrient because of its ability to chelate with dietary cations, rendering the chelated cations partially or completely unavailable to the animal. It has been shown that phytase and phytate influence the secretion patterns of sodium (Na) at the ileal level in broilers; whereas phytate increases but phytase reduces Na losses (Ravindran *et al.*, 2006; Cowieson *et al.*, 2004).

In relation to acid-base homeostasis, the physiological importance of the combined intake by broiler chickens of Na, potassium (K), and chloride (Cl), or the dietary electrolyte balance (dEB), has been recognized for some time and can be used as an indicator of the acid or base forming properties of a diet and also as a contributor to performance. By definition, Na affects the dEB levels and by compromising Na secretions into the gut lumen, it is implicit that phytate might be influencing the digesta electrolyte balance and also, might be compromising the Na-dependant transport mechanisms involved in the intestinal uptake of some nutrients, including amino acids (AA), which negative effect is counteracted by phytase. Because of the possibility for phytase to reduce endogenous Na losses, but also reduce dietary need for Na, Ca, P and other minerals, a matrix is usually included for these in diet formulation. Matrix is defined as the amount of inorganic-P or any other nutrient that can be produced by a given amount of added phytase in the diet (Shelton *et al.*, 2004). When nutritionists do not make use of such matrix values, there are potentials for imbalance in dEB.

Apart from phytate, the majority of poultry feed ingredients also contain considerable amounts of non-starch polysaccharides (NSP's). These are cell constituents mostly made of cellulose, hemicellulose and pectin (Smits & Annison, 1996) with varying degrees of water solubility, size and structure. They cannot be degraded by endogenous enzymes and reach the end of the digestive tract almost indigested (Caprita *et al.*, 2010). Ingestion of water soluble NSP increases digesta viscosity in broilers (Annison & Choct, 1991) but through the knowledge of their chemical structure, enzymes have been developed to overcome their anti-nutritional effects. Nutrient utilisation in poultry has been shown to increase with the supplementation of NSP-degrading enzymes due to elimination of the nutrient encapsulating effect of cell walls and reduction of digesta viscosity (Kim *et al.*, 2005). Debon & Tester (2001) observed in an *in vitro* study that NSP have the capacity to bind cations but that the components in the diet and the different physiological conditions in the gastro-intestinal tract (GIT) of the different animals can potentially interfere with this binding capacity.

This nutrient encapsulating effect of cell walls seems to also affect phytate-P release by phytase, since it has been shown that the amount of phytate-P released by phytase depends on the amount of phytase added to the feed but there is some of this phytate-P that remains undigested even when considerable amounts of phytase are used. This might be an indication that phytate might be "locked" away in intact cells (Karimi *et al.*, 2013). Therefore, the addition of NSP enzymes such as xylanase may increase the efficacy of phytase by breaking down the cell walls and releasing phytate-P for phytase hydrolysis and by eliminating the phytate chelating effects of NSP in both, feed stuff and digesta (Kim *et al.*, 2005).

Therefore, the present MSc study objective was the investigation of the impact of changes in dEB levels on phytase efficacy in growth performance, bone mineralisation and nutrient utilisation in broilers up to 21 days old, as well as to assess the effects of phytase supplementation, alone or in combination with xylanase, on growth performance, bone mineralisation, volatile fatty acids production and pH at different parts of the GIT in diets marginally deficient in dEB.

2. Literature Review

2.1 Phytic acid

Phytic acid ($C_6H_{18}O_{24}P_6$) is also known as inositol hexaphosphate (IP6) and consists of a *myo*-inositol ring associated with up to 6 phosphate ions (Figure 1) mainly present in grains and seeds as a mixed salt of mineral cations including zinc (Zn^{2+}) and iron (Fe^{3+}) known as phytate (Raboy, 1997). Phytate is present in cereal grains and oil seeds and its primary physiological role is to store nutrients, mainly P that will be released with the help of endogenous phytases when germination occurs (Ligeiro, 2007). Although the term phytate is the most frequently used in the literature to refer to phytase substrate, phytin can also be used and represents the deposited complex of IP6 with Ca, K and magnesium (Mg) present in plants (Khalid *et al.*, 2013).

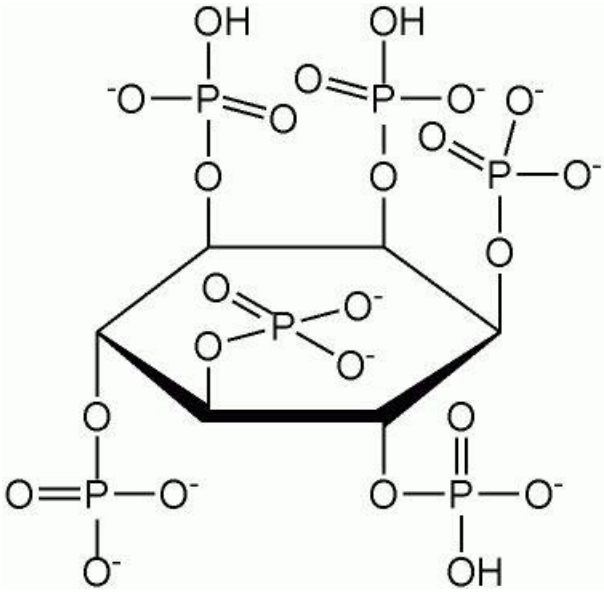

Figure 1 – Phytic acid molecule

The main ingredients used in poultry feeds are plant-based and for the animals to have access to the P from phytic acid it is necessary that they are able to hydrolyse the ester bonds between the phosphate groups and the inositol ring before P becomes available for absorption in the GIT (Cowieson *et al.*, 2004). Phytate stores up to 80% of the total P present in plant seeds (Ravindran *et al.*, 1995) but the P in the form of phytate-P is generally not available to monogastric animals due to a lack in the intestinal digestive

enzyme, phytase, needed to hydrolyse the ester chemical bonds and release P from the phytate molecule (Ravindran *et al.*, 1995).

It is known that the capacity of chelation that phytic acid possesses is far more encompassing than simply limiting the P and minerals availability. Because of their negative charges, phytate molecules can bind to positively charged molecules like nutritionally important minerals (e.g. Ca, Mg, Zn and Fe), proteins (including enzymes) and starch (Figure 2), making them less soluble or completely insoluble (De Boland *et al.*, 1975) and affecting their absorption and digestion. Therefore phytate has been described in animal nutrition as "an anti-nutritional factor and an indigestible nutrient" (Swick & Ivey, 1992).

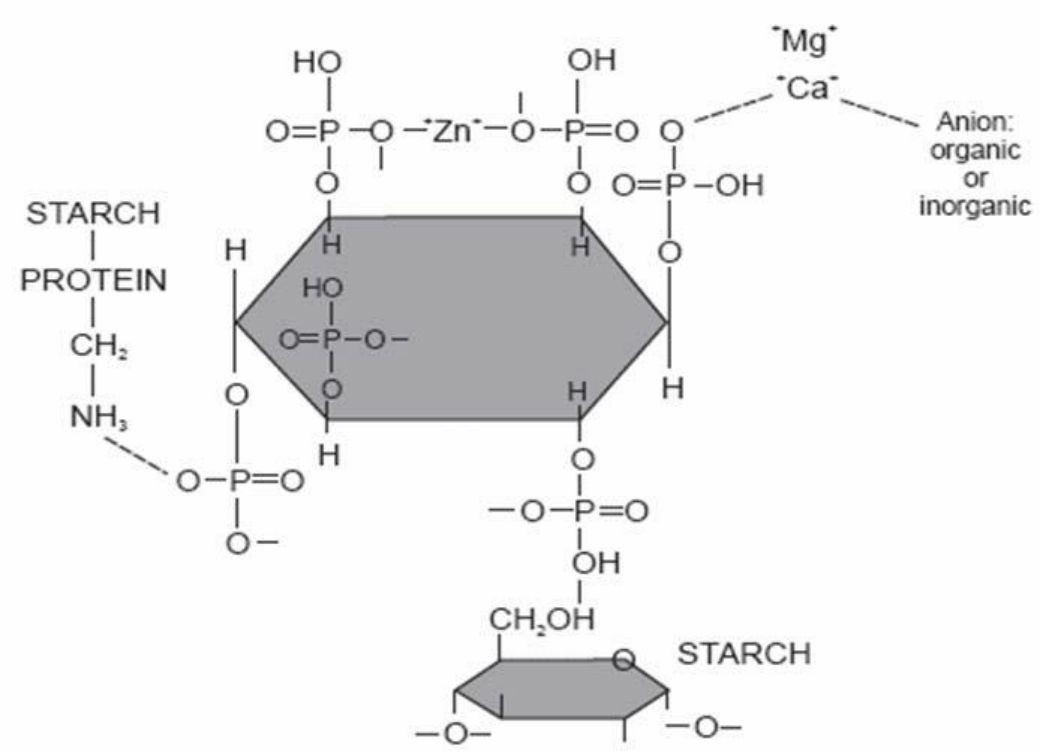

Figure 2 - Phytate-protein-starch complex molecule: a potential structure (Jongbloed *et al.*, 2000)

2.1.1 Phytate and P interactions

Phosphorus is an essential mineral for animals and it plays a critical role in cellular metabolism, cellular regulatory mechanisms, and in bone. Bone stores the majority of P present in the body containing 85% of the body's total P (McDonald *et al.*, 2011). The digestibility of phytate-P in broilers can be as low as 10% (Rutherfurd *et al.*, 2004) and

therefore, the poor digestion of phytate-P in monogastric animals, due to insufficient amount of phytase, can lead to an increase in P in excreted faeces and, as a consequence, lead to environment pollution (Knowlton *et al.*, 2004). The non-renewability of rock phosphate reserves can also be a problem in the near future when trying to fulfilling the high demand for inorganic-P (Abelson, 1999). Concerns with P and reduction of animal feed costs production without loss of their nutritional value have been concepts that, although not new, are still subject of study.

2.1.2 Phytate and minerals interactions

The digestibility of minerals can also be reduced by phytate. The absorption of minerals takes place in the upper part of the intestine (Khalid *et al.*, 2013). The phosphate groups present in the phytic acid molecule have one or two oxygen atoms that, by being negatively charged can bind to cations at neutral pH. This liaison can be stronger or weaker according to the number of phosphate groups involved from the same or different phytic acid molecule (Sebastian *et al.*, 1998) and can form insoluble complexes with these cations reducing their availability for absorption (Maenz *et al.*, 1999). A result of these insoluble complexes and interference in mineral absorption was observed by Sandberg *et al.* (1993) when feeding pigs a rapeseed diet supplemented with Ca. Zinc is another mineral that can also form an extremely insoluble complex with phytate in the upper part of the intestine where the pH is around 6, leaving broiler with a Zn deficiency when they are fed diets high in phytate (Maddaiah *et al.*, 1964). On the other hand, in alkaline pH, phytate will form complexes with proteins in the presence of divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}) which will function as a bridge between the carboxylic group charged negatively and phytate (Cousins, 1999). Furthermore, Woyengo *et al.* (2009) reported a 2% reduction in apparent ileal digestibility of Ca, Mg, Na and K when feeding piglets a casein maize starch base diet with phytate (as sodium phytate). Ravindran *et al.* (2006) also observed a reduction in Ca and Fe ileal digestibility when broilers were fed a corn-soybean meal diet with phytic acid (as rice bran) supplementation. Apart from high amounts of phytate, rice bran also contains high amounts of NSP's. These increase the viscosity of digesta and intestinal contents, and can have a negative effect on the digestion of some dietary components due to a reduction in enzyme activity and nutrient absorption (Smits & Annison, 1996). They can also enhances bacterial overgrowth (Salih *et al.*, 1991) and bacterial fermentation due to undigested material accumulation in small intestine (Smits *et al.*, 1997).

2.1.3 Phytate and amino acids interactions

Phytate chelating effect depends on pH conditions to exist. At low pH, phytate can bind to basic residues like arginine, lysine and histidine resulting in an insoluble complex (Kumar *et al.*, 2010). Ravindran *et al.* (2000) observed a decrease in ileal digestibility of all essential AA when broilers were fed a wheat-sorghum-soybean meal based diet with phytate at 3 different levels (manipulated by the inclusion of rice pollards). Later on, Ravindran *et al.* (2006) also observed a decrease in apparent ileal digestibility of AA in broilers fed a corn-soybean meal based diet due to an increase in dietary phytate concentration. Similarly, in a precision feeding study, Cowieson *et al.* (2006) observed a compromise in growing broilers ileal AA digestibility when fed with phytic acid and casein supplementation. As for minerals, the reduction of apparent AA digestibility and absorption can possibly be explained by the reduction in dietary AA availability from phytic acid which is, in its natural state, complexed with AA in protein bodies (Joyce *et al.*, 2005; Lin *et al.*, 2005) or it could bind to dietary proteins in the stomach and small intestine and reduce their digestion and increase endogenous losses (Liu *et al.*, 2009).

In poultry, the protein-phytate complexes are more likely to occur in the proventriculus due to the low pH observed in this part of the GIT (Selle *et al.*, 2012). Because exogenous phytase is mainly active in the crop (Liebert *et al.*, 1993), phytase prevents the formation of protein-phytate complexes by hydrolysis of phytate. On the other hand, the high pH in the crop can also lead to an increase in the formation of mineral-phytate complexes which will reduce phytase capacity to degrade phytate (Maenz *et al.*, 1999). Therefore, the pH changes observed in different parts of the gut can have trade off effects on phytate and are not necessarily indicative of the hydrolysis of phytate (Campbell & Bedford, 1992).

2.2 Phytase

Enzymes are one of the many types of proteins that are produced by an organism. They have a three-dimensional conformation made from one or more strings of AA and it is the specificity of this three dimensional form that makes enzymes so unique (Bregendahl, 2007). Enzymes can break down products such as nutrients that can then be easily absorbed. Enzymes are biological catalysts and are some of the most notable biomolecules due to their extraordinary specificity and catalytic power (Rastogi, 2006). They are considered the functional units of the cellular metabolism since they are able to accelerate the velocity of a reaction without participating in the reaction itself as a substrate or

product. When they catalyse, they are not altered but they chemically modify the substances (substrate) that are affected by their action (Khattak *et al.*, 2006). Each enzyme has an optimal temperature and pH and the structure can be altered, sometimes irreversibly, by excessive heat (Bregendahl, 2007).

Because certain animals do not have the necessary enzymes to degrade some of the plant compounds, nutritionists have been able to identify these indigestible compounds and feed a suitable enzyme or a combination of enzymes to the animals. These feed added exogenous enzymes come from carefully selected microorganisms and are grown under controlled conditions (Wallis, 1996).

Exogenous feed enzyme usage has been studied and reported since the 1950s (Jensen *et al.*, 1957; Arscott & Rose, 1960). Their effects on animals are assessed through growth performance responses, nutrient utilisation, evaluation of plasma composition, analysis of whole-body nutrient utilisation and carcass quality. Although the use of exogenous enzymes in the animal industry has grown considerably and with success, the wide range of published work about animal nutrition and other relative areas brings vast product choices and also formulation strategies that the normal end user can find challenging (Adeola & Cowieson, 2011).

One of these exogenous enzymes that is widely used in animal feeds is phytase. Phytase is vital in feed formulation for non-ruminant animals like poultry because of the reasons mentioned before; namely lack of phytase to hydrolyse phytate and release P for absorption; scarcity of and non-renewable nature of environmental sources of inorganic-P which together, make P an expensive ingredient in the feed industry.

Phosphorus is also an environmental concern by being unavailable to monogastric animals and excreted in large amounts in their faeces. Excessive P concentrations can occur either by extra supplementation of inorganic-P in poultry and pig diets or by phytate-P that can run off to the environment creating an environment P burden and leading to soil pollution and eutrophication of rivers and lakes (Correll, 1999) which may result in appearance of toxic algae and lead to fish kills (Sharpley, 1999). Therefore, by including exogenous phytases into poultry and pig diets, a reduction in P excretion is of benefit to both the environment and sustainable production.

2.2.1 Phytase activity

Phytase activity was first detected in 1907 but it was just in 1962 that first attempts to develop a phytase feed enzyme were made and only in 1991 that it first became commercially available (Selle & Ravindran, 2007). Phytase activity is defined as phytase units (FTU). One FTU will release 1µmol of inorganic orthophosphate/min from 0.00512 mole per litre of sodium phytate at pH 5.5 and at 37˚C of temperature (Selle & Ravindran, 2007).

2.2.2 Sources of phytase

For poultry to use P or any other minerals bound to phytate complexes, phytate needs to be hydrolysed by the enzyme phytase. Phytase is present in nature in several microorganisms, plants and animals (intrinsic phytase) (Ravindran *et al.*, 1995; Lei *et al.*, 2007).

2.2.2.1 Microbial phytase

There are several of these phytases that can be commercialised and used as feed additives and they have been isolated from fungi, yeast and bacteria. Fungi and bacteria are the most important sources but the different sources of phytase have different physical and chemical properties, which give them different enzyme activities and result effects when used in monogastric animals. These commercial products are added to the feed of monogastric animals to hydrolyse phytate within their digestive tract and improve the utilisation of dietary phytate-P (Beutler, 2009).

Aspergillus species are fungi (Konietzny & Greiner, 2002) and *A. niger*, *A. fumigatus* and *A. ficuum* are the most commonly used in genetics to further improve these *Aspergillus* enzymes (Wyss *et al.*, 1999). *Saccharomyces cerevisiae* is yeast phytases for bread making. *Pichia anomala* phytase have potential application in food processing because they remain stable even at high temperatures and acidity (Vohra & Satyanarayana, 2002). Among bacteria sources, enzymes from *Bacillus* (Kim et al., 1998) and *Escherichia coli* (Greiner et al., 1993) have been characterized and can degrade phytate during growth through production of extracellular phytases. The way fungal and bacterial phytases are produced is also different. Fungal phytases are produced extracellularly while bacterial phytases are produced intracellularly (Rao *et al.*, 2009).

2.2.2.2 Plant phytase

Phytase activity has been found and phytase enzymes have been isolated from cereals, legumes, and oil seeds (Viveros *et al.*, 2000) such as corn (Maugenest *et al.*, 1999), barley (Greiner *et al.*, 2000), rice (Hayakawa *et al.*, 1989), canola seed (Houde *et al.*, 1990) and soybean (Hamada, 1996). The ability to hydrolyse phytate and the level of phytase varies between plants (Eeckhout & De Paepe, 1994). While soybean meal have been found to have low phytase levels (Eeckhout & De Paepe, 1994), cereal grains like wheat, barley and triticale can reach phytase activity levels of 5,000 units/kg and by-products from these cereals have been used in animal feeds as a source of plant phytase. A rapid increase of phytase activity was observed during corn germination (Chang, 1967) and fermentation. Soaking processes have also shown to be efficient in improving intrinsic phytase activity present in plant foods (Lei *et al.*, 2007).

2.2.2.3 Animal phytase

Phytate-degrading enzymes have been isolated from tissues of several monogastric animal species and ruminants (Bitar & Reinhold, 1972) but in comparison with the investigation of phytases in plants and microorganisms, animal phytase investigation is limited (Konietzny & Greiner, 2002). Phytase is present in the digestive tract of chickens but the levels of this enzyme are not enough to effectively hydrolyse phytate (Maenz & Classen, 1998). In pigs, the mucosal production of this enzyme is also minimal however, endogenous phytases might complement the use of exogenous phytase (Selle & Ravindran, 2008). An increase in intestinal phytase activity has been observed in chickens fed P deficient diets (Davies & Motzok, 1972). The phytase found in the large intestine or rumen is mainly of microbial origin (Yanke *et al.*, 1998) and a phytate-degrading enzyme has also been purified and characterized from the protozoan *Paramecium* (Freund *et al.*, 1992).

2.2.3 Classification of phytase

Depending on where the hydrolysis of phytate begins, phytases can be classified into 3-phytase (EC 3.1.3.8 myo-inositol hexakisphosphate 3-phosphohydrolase) and 6-phytase (EC 3.1.3.26 myo-inositol hexakisphosphate 6-phosphohydrolase). Enzymes are classified according to the chemical reactions they catalyse. Each enzyme has a set of four numbers, called an EC (Enzyme Commission) number that define the classes and sub-classes of enzyme it belongs to. Both of them remove the phosphate groups, what differs in the

starting place of the de-phosphorylation (carbon 3 or 6 respectively) (Selle & Ravindran, 2007). The most commonly used phytase feed enzymes are derived from *A. niger*, which shows 3-phytase activity and *Peniophora lycii* and *E. coli*, which shows 6-phytase activity (Selle *et al.*, 2007a).

The same category of phytases can present optimum activity at different pH (Bohn *et al.*, 2008) and can also present different substrate specificity. Phytate degrading enzymes can be divided into alkaline phytases (maximum activity at pH around 8) and acid phytases (maximum activity at pH around 5) (Baruah *et al.*, 2007). Most microbial phytases are acid phytases with the exception of the ones from *Bacillus* group which are alkaline phytases (Selle *et al.*, 2007a). Fungal phytases seem to have a broad substrate specificity while bacterial phytases exhibit high substrate specificity (Rao *et al.*, 2009). Nevertheless, the effectiveness of both fungal and bacterial phytase on improving P and Ca digestibility has been demonstrated (Guggenbuhl *et al.*, 2007).

When comparing a *A. niger* 3- phytase with a *E. coli* 6-phytase (Rodriguez *et al.*, 1999) noted that *A. niger* phytase was more resistant to trypsin while *E. coli* phytase was more resistant to pepsin which showed that the *E. coli* phytase had the potential of being more resistant to pepsin activity in the stomach and therefore more likely to survive longer in the digestive tract than *A. niger* phytase.

2.3 The use of phytase in the feed industry

The usage of phytase feed enzymes was initially confined to the Netherlands due to a demanding anti-pollution legislation but, since 2000 that phytase has been globally accepted in the majority of poultry and pig feeds due to several factors mainly; the prohibition of meat-and-bone meal in pig and poultry diets; the declining costs of exogenous phytases together with increasing prices of feed ingredients; the introduction of phytases from bacterial origin which proved to be more effective than the original fungal phytases, and the high costs of inorganic-P supplements (Selle *et al.*, 2012).

The poor utilisation of phytate P by monogastric animals is of importance because inorganic-P is an expensive ingredient in poultry industry that is facing a supply crisis in the near future (Selle & Ravindran, 2007). Crop seeds can incorporate, worldwide, around 14 million tonnes of phytate-P (Lott *et al.*, 2000) and on average, commercial poultry feed contains 2.5 to 4 g/Kg of phytate-P (Ravindran, 1995). Phytase is able to break down the

phytic acid molecule and release P for absorption. This will reduce the need of inorganic-P in the diet and reduce the amount of P being released to the environment. Phytase releases not just P but also other cations or AA that are bound to phytic acid. Phytase can increase the digestibility of phytate in poultry from around 25% to 70% and can also improve the digestibility of other nutrients as well as energy (Ravindran *et al.*, 1999a; Kornegay, 2001).

2.4 Benefits of the use of phytase

Although phytase is mainly used to increase the availability of P and by that, reduce the addition of inorganic-P to the feed which have positive effects on the environment, phytase supplementation can be also beneficial in other areas. Some of these benefits are described below.

2.4.1 Improvement in growth responses

Phosphorus is an essential mineral for animal performance since it is one of the most abundant elements in bone composition and therefore, one of the most important elements for body growth. Consequently, if animals are fed a P deficient diet their growth and performance might be compromised. Phosphorus is a component that is present in the vast majority of poultry diets products as phytate–P (Selle *et al.*, 2007b) and exogenous phytases are normally added to diets in order to increase the availability of phytate-P. Feed addition of phytases has shown the same or better results in restoring the production performance criteria and growth of birds than the simple feed supplementation of P (Simons *et al.*, 1990). This positive response to phytase on growth and feed conversion ratio might be explained by a release of minerals from complexes with phytic acid and/or by the use of inositol after phytic acid hydrolysis and/or by increased starch digestibility (Simons *et al.*, 1990). Adeola *et al.* (1995) also suggested that phytase influence in pig growth might be related to an increase in the availability of minerals. A study performed by Hussein (2006) showed that the supplementation of phytase to broiler diets significantly improved growth performance and bone mineralization of birds when they were fed a balanced starter diet with no phytase supplementation and a low-P finisher diet supplemented with phytase enzyme. In general, feed intake and weight gain responses to phytase are more robust and consistent than feed efficiency responses (Selle & Ravindran, 2007) which is probably attributed to the fact that broilers strains are under constant genetic improvement as are their management techniques and feeds (Rosen, 2003). Shirley & Edwards (2003) observed that, when feeding broilers with a maize-soybean

meal diet, increasing phytase inclusions from 0 FTU/Kg to a maximum of 12000 FTU/Kg increased the total tract phytate degradation (from 40.3% to 94.8% respectively). Moreover, this increase in phytase inclusion levels increased P retention, tibia ash, weight gain, feed intake, nitrogen (N) retention, feed efficiency and Ca retention. It is likely that increased bone mineralisation in phytase-supplemented diet makes birds legs stronger thus enabling them to stand and feed or that positive effects on electrolyte balance improves birds appetite (Olukosi *et al.*, 2013).

Olukosi *et al.* (2007b) reported positive effects on broiler growth when phytase was added to diets that were marginally deficient in P. Similarly, Woyengo *et al.* (2010) showed that phytase alone can improve growth performance of broilers fed a P-deficient diet. However, the growth responses obtained for the supplemented diets were lower than the ones obtained in the diets adequate in P (positive control (PC) diets), in comparison to Olukosi *et al.* (2007b) study that observed a similar response between PC and P deficient phytase supplemented diets. This could be due to the fact that the levels of P between both studies were different; 0.1% non-phytate P deficiency in Olukosi *et al.* (2007b) study compared with 0.18% non-phytate P deficiency in Woyengo *et al.* (2010). Moreover, Denbow *et al.* (1995) who used seven different levels of phytase in soybean meal diets with three levels (0.2, 0.27 and 0.34%) of non-phytate P deficiency, observed an improvement in body weight gain and feed intake at all non-phytate P levels but with a better response observed for the diets with lower non-phytate P. Pillai *et al.* (2006) also reported growth performance improvements in broilers when fed P deficient phytase supplemented diets and same improvements in growth were observed by Baker *et al.* (2007) when fed broiler chicks a low-phytate corn diet supplemented with phytase. These growth performance improvements might be explained by phytase activity that will release and increase the utilisation of P from phytate-mineral complexes (Qian *et al.*, 1996b) or by an increase in the utilisation of protein and AA (Ravindran *et al.*, 2000) and, as a result, originate an increase in feed intake and feed efficiency.

However, a study performed by Perney *et al.* (1993) showed that the addition of phytase to a corn-soybean meal diet with low P did not make any significant improvements on weight gain, feed intake or feed conversion of broiler chicks. This was attributed to the level of Ca and phytase used (1% and 750 FTU/Kg respectively) in the study that could have resulted in a lower phytase effect on P retention and consequent improvement of growth performance.

Therefore, the results from these studies suggest that phytase supplementation can reduce the adverse effect of phytic acid (Ravindran *et al.*, 2000) and that the increase in phytase supplementation levels can improve growth and weight gain rate in broilers (Khan *et al.*, 2013).

2.4.2 Effects of phytase on nutrients utilisation

Phosphorus is an important mineral in bone composition and therefore, excessive P levels are normally incorporated (inorganic-P) in poultry diets to guarantee skeletal integrity and growth performance (Waldroup, 1999). Nelson *et al.* (1968) observed that hydrolysed phytate-P is as efficient as P from inorganic sources when, in the referred study, he incubated soybean meal with phytase before feeding chicks. A lot of studies have been performed to improve the nutrient utilisation of nutrients bound to phytate and some of these studies have demonstrated that exogenous phytase improved P utilisation in broilers and pigs (Simons *et al.*, 1990; Selle & Ravindran, 2008). As a consequence, P reduction in excreta will benefit the environment and a more sustainable animal production. Phytase supplementation benefits to P availability are normally in the range of 20 to 40%. In a study with broilers Simons *et al.* (1990) observed an increase of 60% in the bioavailability of P and a decrease of 50% in the levels of P in broilers excreta in a low-P corn-soybean diet supplemented with phytase. Nevertheless, the P amount released from phytate when hydrolysed by phytase can depend on the phytase source and concentration (Simons *et al.*, 1990; Yi *et al.*, 1996) and also from the source of phytate (Ravindran *et al.*, 1994). Waldroup *et al.* (2000), based on tibia ash results, reported that broiler diets based on normal corn had a requirement of 3.9g/Kg of non-phytate-P while, if supplemented with phytase, the non-phytate-P requirement was reduced to 2.9g/Kg. Phosphorus retention in broiler chickens was increased when fed a low-P corn-soybean meal diet supplemented with phytase (Ahmad *et al.*, 2000) as well as P availability in broilers was increased in wheat-sorghum-soybean meal diets with low, medium or high phytate-P levels in diets supplemented with phytase (Ravindran *et al.*, 2000).

Calcium is another important mineral in bone composition and, therefore, is one of the biggest concerns in animal nutrition because, even if it is not one of the strongest inorganic elements that can bind to phytate (e.g. cations like Zn), due to the high amounts of Ca present in common diets, this element can easily chelate with phytate and precipitate in the GIT. When phytate hydrolysis by phytase occurs, phytate capacity to chelate with Ca reduces, leaving Ca available for absorption in the small intestine (Selle *et al.*, 2009a).

A few studies have been developed and can corroborate the idea that phytase has the capacity to improve Ca digestibility. When using a corn-soybean meal base diet with low, medium and high concentrations of phytic acid, Ravindran *et al.* (2006) observed that increasing phytase inclusion levels (0 to 1000 FTU/Kg) increased Ca ileal digestibility in broilers. Calcium and P total tract digestibility was also improved and Ca excretion reduced in weaned piglets when Guggenbuhl *et al.* (2007) included three different phytases into the diets containing P exclusively from plant origin. Later on, Ravindran *et al.* (2008) reported an increase in broilers ileal digestibility of Ca when phytase was added to a corn-soybean meal and canola meal based diet. Therefore, the referred and other studies demonstrate that phytase can enhance Ca digestibility in poultry and pigs.

Moreover, not just Ca but also endogenous Na excretion was affected with the ingestion of phytate in a study with broilers (Cowieson *et al.*, 2004) suggesting that Na levels are also affected by phytate ingestion and which will, as a consequence, compromise the Na-dependant transport systems and the Na pump activity (Selle *et al.*, 2012). Therefore, phytate might be limiting intestinal absorption of Ca by compromising the Na-dependant transport system and phytase has shown to be able to counterbalance this effect by increased Na digestibility in broilers (Ravindran *et al.*, 2006; Ravindran *et al.*, 2008; Selle *et al.*, 2009b) and by ameliorating Na secretion into the gut which can improve Ca absorption. By compromising the Na-dependant transport systems into the gut, this might reduce the uptake of dietary AA and endogenous AA by the animal (Selle *et al.*, 2012) as Cowieson *et al.* (2004) reported in his study when he observed that broilers excretion of total endogenous AA was increased by phytate dietary inclusion and decreased by phytase supplementation.

Phytase has shown to be effective in altering AA digestibility (Ravindran *et al.*, 1999a; Ravindran *et al.*, 2006) even if this effect can be variable and dependent on factors such as the type of phytate complexes, the type of ingredients in the diet and the location of phytate in those ingredients as well as species and age of the animals (Cowieson *et al.*, 2004). Discrepancies in results might also be attributed to the type of marker used in the study (Selle & Ravindran, 2007) as observed by Olukosi *et al.* (2012) whose results showed that, independently of the level of phytase used, ileal AA digestibility in broilers can be improved when using Titanium (Ti) instead of Chromium (Cr) as a dietary marker. Moreover Cowieson *et al.* (2008) reported that the use of two different sources of phytase (bacterial and fungal) reduced the flow of N and AA in broilers. Although, there were differences in flow values between the sources of phytase suggesting that the capacity of

counteracting phytate anti nutritive properties can vary according to the source of phytase used.

When broilers were fed diets rich in phytates it was observed that the absorption of Zn and other minerals like Mg and Copper (Cu) was decreased (Maddaiah *et al.*, 1964; Davies & Reid, 1979). At the upper part of the intestine Zn can form an insoluble complex with phytate which will lead to a deficiency in Zn if broilers are fed a diet rich in phytate. When Kornegay *et al.* (1998) fed a low-protein corn-soybean meal diet supplemented with phytase to broilers, he observed an increase in protein utilisation and an improvement on breast weight probably due to an effect of phytase in increasing the AA availability for lean tissue deposition. Later on, when using a wheat based diet deficient in lysine and with different levels of phytase supplementation, Ravindran *et al.* (2001) observed a significant effect on ileal AA digestibility. More recently, Cowieson & Ravindran (2007) also reported an improvement in AA digestibility upon phytase supplementation which corroborates the findings in the previously mentioned studies and indicates that phytase can improve AA digestibility and protein utilisation in broilers by counteracting the anti-nutritive effect of phytate.

2.4.3 Effects of phytase on energy availability

Selle & Ravindran (2007) reported on the influence of exogenous phytase on dietary apparent metabolisable energy (AME) in a number of studies. In general, it was observed in these studies that phytase supplementation increased AME by 2.8% when compared to non-supplemented diets. Moreover, the dietary AME energy was improved when phytase was supplemented to wheat based diets (Selle *et al.*, 2006b).

Ravindran *et al.* (2000) supplemented phytase to wheat-sorghum-soybean meal diet and observed an increase in dietary AME. This increase was greater for diets adequate in non-phytate P and phytase also improved digestibility of other nutrients. Moreover, when the non-phytate P in the diet content was increased with the addition of di-calcium phosphate, the levels of dietary AME reduced significantly and this effect was overcome by phytase supplementation. This suggests that the high molar ratio of Ca to phytate in diets adequate in non-phytate-P leads to the formation of Ca-phytate complexes (Ravindran *et al.*, 2000) and that these, with lipids, might be forming insoluble metallic soaps in the gut lumen limiting the utilisation of energy derived from fat (Atteh & Leeson, 1983; Atteh & Leeson, 1984). While feeding broilers a basal diet supplemented with a source of fat (oleic acid,

palmitic acid or a combination of both) at different levels of Ca, Atteh & Leeson (1984) reported an increase in birds feed intake and weight gain. Mineral metabolism can be compromised during digestion since the fat source can form insoluble soaps of fatty acids and minerals and therefore having a detrimental effect on mineral absorption (Atteh & Leeson, 1983).

When using a corn-soybean meal diet supplemented with phytase, Camden *et al.* (2001) reported an increase in fat ileal digestibility and also an increase in AME and apparent ileal digestibility in broilers. Santos *et al.* (2008) observed the effects of phytase on corn-soybean diets low in AME, Ca and P where a constant 5.5% of rice bran was added to increase the levels of phytate-P. In Santos *et al.* (2008) study, phytase increased AME, mineral absorption and ileal AA and crude protein digestibility. Phytase also improved nutrient digestibility and body weight and improved bone strength. Overall, phytase activity improved performance and bone mineralization. Moreover, Olukosi *et al.* (2008b) reported an increase in broiler performance and energy utilisation when phytase was supplemented to a corn and soybean based diet deficient in energy and P.

As reviewed by Selle *et al.* (2000), there is an increase on energy utilisation when phytase is added to poultry diets and this increase is probably related with an increase in protein digestibility. Although, it might be possible that, not just the concentration of phytate is relevant for phytase activity, but also the source of phytate and protein and their structural end chemical properties might be contributing to the observed differences in energy responses form phytase use.

2.4.4 Whole body nutrient accretion or nutrient partitioning

Another positive effect of phytase is the diminution of the gastrointestinal system which augments the partitioning of the nutrients in the gut. Their use also alters the microbial fermentation affecting the availability of nutrients and the health status of the animal and increases energy digestion (Bedford & Partridge, 2001).

Weight gain is a product of an increase of fat and protein and is a good indicator of phytase efficacy. However, because weight gain is a consequence of fat and protein accretion, there is the need to partition weight gain into accretions of fat and protein because these have different efficiencies.

A combination of phytase with other enzymes like xylanase has been shown to reduce relative weight and length of small intestine and benefit nutrient digestibility and retention in broilers (Wu *et al.*, 2004). Olukosi *et al.* (2008a), reported an increase in carcass nutrient accretion with age (0 to 21 days) for broilers fed diets supplemented with phytase or a cocktail of xylanase, amylase and protease (XAP), individually or in combination. This increase was observed for dry matter, fat, protein, ash, P and Ca and there was an inverse relationship on fat gain and protein use according to age. There was also a decrease in whole body ash, P and Ca with age observed in Olukosi *et al.* (2008a) study that possibly occurred because of a decrease in the contribution of these minerals to the whole body nutrient accretion instead of a decrease in the minerals accreted while broilers grew.

Later on Olukosi & Adeola (2008a) reported an improvement in birds growth performance when fed a wheat based diet supplemented with phytase and xylanase. There was also an improvement in whole body dry matter accretion that revealed to be mainly composed by protein (58%) rather than fat (32%) for the phytase supplemented treatments. Pillai *et al.* (2006) also reported that broiler carcasses characteristics and yields were improved with phytase supplementation. These observations might suggest that, when supplemented with phytase, the increase in broiler weight could maybe be more related to an improvement in protein deposition rather than fat deposition.

2.4.5 Economical and environmental impact of phytase

The economic benefits from the use of phytase includes feed costs reduction, allowing flexibility in diets formulation a more optimum growth performance as well as better litter quality and bird health (Costa *et al.*, 2008).

The cost of supplementing P is the third largest cost in poultry feeds after the cost of providing protein and energy (Ligeiro, 2007; Teichmann *et al.*, 1998). The high price of inorganic-P is due to the fact that it is a non-renewable mineral in nature and, in a long term, its sources might all be used up (Selle & Ravindran, 2007). Apart from this, there is also an environmental aspect added to the economical one which is the fact that, when P is added to feeds, a big part of this P can contaminate the water and soil when the excreta are added to fertilisers (Ligeiro, 2007). Therefore, animal production is suffering an increasing pressure when it comes to the control of their effects on the environment.

There has been a profound change in the management of feed P since the dietary supplementation of meat and bone meal (as a cheap source of P) was banned in Europe to prevent possible transfer of diseases such as the bovine spongiform encephalopathy (BSE). This, together with the low availability of phytate by monogastric animals, has also given phytase a new social-economic impact. Phytase is a cost effective alternative that can be used to ensure animals obtain adequate available P from the plant based diets (Lei *et al.*, 2007).

2.5 Factors affecting phytase efficacy

The phytate content of the raw materials used in the feeds can vary and, as a consequence, so does its availability after being hydrolysed by phytase. This and other factors such as the source of phytase; the age and species of the animals; the level of intrinsic phytase activity present in the ingredients used; temperature; pH; humidity and the dietary content of Ca, P and vitamin D can influence phytase activity and therefore give variable results.

Several studies have shown that phytase is more efficient in diets that contain low concentrations of inorganic-P (Qian *et al.*, 1996b; Kornegay *et al.*, 1996; Ahmad *et al.*, 2000; Kornegay, 2001). This might be due to the fact that higher concentrations of inorganic-P might make phytase activity less pronounced because the animal's requirements for P have been met (Beutler, 2009).

2.5.1 Level of anti-nutritive factors in the cereal

Phytate predisposition to form complexes with proteins at different pH can affect the protein structure and can decrease phytase activity, the protein solubility and digestibility (Greiner & Konietzny, 2006). At low pH, phytate can bind to proteins through electrostatic charges and at high pH the binding is made through salt bridges (Graf, 1986). Because phytate function at abroad range of pH and because of its strong negative charge it can easily chelate to positively charged components such as minerals which will compromise the nutritional value of the feed ingredients (Greiner & Konietzny, 2006). Therefore, the degree of ionization of the phytate-minerals complex can potentially change the efficacy of different phytases (Angel *et al.*, 2002).

2.5.2 Dietary calcium level and Ca:P ratio

It was previously mentioned that phytate has the capacity to strongly bind to cations due to the compound being a polyanionic molecule. Within these possible bounds, calcium is no exception and these phytate-Ca complexes might be detrimental to phytate-P utilisation and to phytase activity (Taylor, 1965; Wise, 1983). One phytate molecule can bind with up to five Ca atoms thus forming a complex, when such complexes formed along the GIT, they might bind up large amounts of Ca from the diet which makes phytate a limiting factor for not just P but also Ca (Selle *et al.*, 2009a).

Several studies in broilers, turkeys and pigs have demonstrated that high levels of Ca in diets can inhibit phytase activity (Lei *et al.*, 1994; Qian *et al.*, 1996a; Qian *et al.*, 1997; Tamim *et al.*, 2004). In a study with turkeys Qian *et al.* (1996b) reported that when feeding a corn-soybean meal diet with two levels of non-phytate-P (0.27 and 0.36%) and with phytase supplementation, the increase of Ca:P ratio from 1.4:1 to 2:1 decreased phytase activity by 4.9 and 7.4% respectively, for 0.27 and 0.36% non-phytate-P diets. This is an indication that the dietary Ca concentration and the overall Ca:P ratio are important factors affecting the efficacy of phytase in the digestive tract (Beutler, 2009). In another study with broilers fed a corn-soybean meal diet supplemented with 4 different levels of phytase, Qian *et al.* (1997) observed improvements in Ca and P retention, weight gain and feed intake in broilers fed diets with phytase supplementation but, as the ratio of Ca:P increased from 1.4:1 to 2:1, all these measurements were negatively influenced. Moreover, when pigs were fed a corn-soybean meal diet supplemented with phytase and with high Ca:P ratios (1.5:1 to 2:1) the utilisation of P in weanling (Qian *et al.*, 1996a) and growing/finishing pigs (Liu *et al.*, 1998; Liu *et al.*, 2000) decreased.

Despite the results obtained in these studies, several conflicting or inconsistent results from different studies on Ca influence on the efficacy of exogenous phytases have been obtained through the years. Scott *et al.* (1999) investigated the effect of Ca:P in layers and reported that phytase compensated for reduced available P levels in the diets with high Ca levels resulting in an improvement in egg production, egg weight and feed intake. However, when the Ca levels were low, the effect of phytase and available P on the referred parameters was lower. Moreover, when broilers were fed diets with Ca:P ratios from 1:1 to 2:1 and suplemented with phytase, Singh & Sikka (2006) reported that weight gain and feed intake responses to phytase were not affected by Ca:P ratios and that there was also no affect on the retention of Ca, P and N. In a study on weanling pigs,

Adeola *et al.* (2006) observed that weight gain and feed efficiency increased when Ca:P ratio was decreased from 1.8 to 1.2. However, there were no significant interactions between Ca:P and phytase for weight gain and feed efficiency responses, but there was an enhanced growth performance with phytase supplementation when Ca:P ratio values were reduced. Driver *et al.* (2005) also observed that broilers growth and bone quality responses were higher for diets supplemented with phytase, with higher Ca levels and low non-phytate-P. Also, growth performance and bone quality responses to phytase decreased when Ca level was reduced and non-phytate-P levels increased.

These results suggest that phytase efficacy at different Ca:P levels can be variable and are still not very clear. Although, when phytase is supplemented in diets, it might be important to consider a narrow Ca:P ratio. Phosphorus absorption and availability might be compromised when high dietary Ca levels might form an insoluble complex with P and/or phytate-P, leaving phytate-P less available for degradation by phytase. This might lead to a limit or decrease in P bioavailability and absorption (Wise, 1983; Fisher, 1992), especially if the dietary P used is below the levels recommended by the NRC (National Research Council, 1994). Moreover, the use of limestone as dietary Ca supplement can increase the pH in the crop (Shafey *et al.*, 1991) which is the main site for phytate degradation by phytase (Liebert *et al.*, 1993). The high crop pH can also lead to an increase in the formation of mineral-phytate complexes (including Ca) which will reduce phytase capacity to degrade phytate (Maenz *et al.*, 1999).

2.5.3 *Effect of other exogenous enzymes*

Combination of exogenous enzymes has been used in monogastric animals to increase nutrient digestibility and bird performance and also to decrease nutrients excretion as well as cost of production. These enzymes have a variety of nutritional benefits which include, apart from releasing of P from phytate, the hydrolysis of plant cell walls non-starch polysaccharides (NSPs) and the elimination of certain anti-nutrients like NSPs and phytate from the diets (Costa *et al.*, 2008; Slominski, 2011).

Certain types of grains are prompt to induce more viscosity of the intestinal contents which will reduce the nutrient diffusion and absorption in the gut and compromise birds performance (Bedford, 1996) by altering endogenous enzyme synthesis rates, microfloral and coccidial populations and litter quality. Non-starch polysaccharides are present in many plant foods but are normally not degraded by monogastric animals. The presence of

NSP's in the intestinal lumen promotes the viscosity of digesta due to the formation of polymers or gels with water. This compromises the digestion and absorption of nutrients because these polymers restrict the action of the digestive enzymes and the diffusion of the substances related with the digestion and absorption. Increase in the viscosity of the intestine can affect the starch, the protein and the lipids digestibility (Choct *et al.*, 2001). Moreover, they can also increase litter moisture and thickness of excreta (Hayat *et al.*, 2005; Buchanan *et al.*, 2007). To reduce the digest viscosity it's necessary that the NSP's are degraded by NSPs degrading enzymes in small parts, losing the capacity of water retention. With reduction in the digesta viscosity and by releasing nutrients encapsulated in the cell walls, the enzyme activity is improved which will lead to improvement in nutrient digestibility, increase on intestinal transit rate and reduction of water in the faeces (better litter quality) (Opalinski, 2006). These enzymes however, can also improve release of P from phytate by phytase (Slominski, 2011) by exposing phytate and making it available for phytase hydrolysis (Bedford, 2000). This has been demonstrated with the combination of phytase and xylanase in broilers (Ravindran *et al.*, 1999b; Selle *et al.*, 2003).

Glycanases are the type of enzymes normally used to break the NSP's. They have shown to be able to reduce the digesta viscosity up to 50% and improve the apparent metabolisable energy (AME) and feed conversion ratio by 24% and 31% respectively (Choct, 1997). It has also been observed that different types of glycanases can give different results when it comes to excreta moisture. Three different glycanases commercial products all proved efficiency in improving birds performance but they all showed, although positive, different results for moisture levels of excreta (10 to 29%) which gives thought that the breakdown site of the NSP's might be different (Choct, 1997).

Juanpere *et al.* (2005) observed that phytase can increase intestinal viscosity in corn diets and that glycosidases decreases intestinal viscosity in corn, wheat and barley diets. When combined, phytase and glycosidases reduced the excretion of Ca and P and increased the retention of these two minerals. The combination of these two types of enzymes was, in general, positive and beneficial for the broilers since they improve energy values and nutrient bioavailability, resulting in better growth performance and intestinal viscosity. Moreover, the combination of multiple carbohydrases: xylanase, amylase, and protease (XAP) have shown to be more beneficial than these enzymes acting individually (Olukosi *et al.*, 2007b). Cowieson & Adeola, (2005), using a corn-soybean meal based diet, nutritionally marginal in Ca and P, observed that phytase combined with XAP was effective in improving birds performance. Olukosi *et al.* (2007b) also showed that phytase

and XAP, when added to a maize-soybean meal diet, improved growth performance in broilers but the same results were not observed in another study (Olukosi *et al.*, 2010) when phytase and xylanase were added to a diet with the same basal composition. Woyengo *et al.* (2010) study reported that phytase alone improved growth performance and tibia ash in a P-deficient diet for broilers and that there was a significant improvement in growth performance and nutrient digestibility and retention when phytase was combined with multi-carbohydrase enzyme. In contrast, in a previous study, Wu *et al.* (2004) added phytase and xylanase, individually or in combination, to a wheat-soybean meal diets and observed that phytase (500 FTU/Kg) improved broiler performance by 17.5% when added alone but that there was no improvement in birds performance when phytase was added in combination with xylanase. The authors explained these results with the fact that the production of phytase was made through solid state fermentation that contained significant amounts of xylanase and glucanase. Moreover, Wu *et al.* (2004) also reported that the addition of phytase combined with xylanase to the wheat basal diet reduced significantly the viscosity of the digesta at the duodenum, ileum and jejunum level and that phytase individual addition reduced viscosity at the duodenum and ileum but not at the jejunum. A combination of phytase and xylanase might be beneficial since xylanases can increase the permeability of the aleurone layer of wheat which is the site of phytic acid storage (Adeola & Cowieson, 2011).

Exogenous enzymes, including phytases, can increase and retain the availability of nutrients in the diets such as P. Nevertheles, if what is limiting the animal performance are not these "stored" nutrients then the response to enzyme supplementation will be small or null (Wyatt *et al.*, 2008).

2.5.4 Dietary Electrolyte Balance (dEB)

Minerals have important roles in biological functions since they play a role in cellular functions, osmotic balance and acid base balance. They are also involved in the expression and regulation of genes, detoxification systems and enzyme systems and structurally in bone metabolism. All these physiological processes operate in different conditions and are sensitive to pH (McDonald *et al.*, 2011).

To keep the acid base homeostasis close to normal, the animal has to regulate the input or the output of acidity or both (Mongin, 1981) otherwise the metabolic and biochemical pathways will be compromised and the animal resources will be diverted towards

homeostasis balance instead of growth (McDonald *et al.*, 2011). The diet is extremely important in the maintenance of cellular acidity or alkalinity in view of the diet levels of anions and cations that are consumed or produced during metabolism (McDonald *et al.*, 2011).

Consumption of diets with high anion content decreases blood pH and causes academia in broilers whereas diets with high cations content increases blood pH and causes alkalemia. The performance of broilers under thermo neutral environments can be affected in any of these situations (Ahmad & Sarwar, 2006). When the intake of acid and the endogenous acid production equal the acidic values of urine excreted, the animal is said to be in a steady state and the blood pH under this condition is in the range of 7.3-7.5 (Mongin, 1981). If pH deviates from this range, the bird corrects the imbalance by excreting bicarbonate in the urine and retaining H^+ (Ahmad & Sarwar, 2006).

Minerals with electrolytic properties are considered functionally as separate entities (McDonald *et al.*, 2011). Electrolytes are chemical compounds that dissolve into positive (cations) and negative (anion) compounds in solution and have the inherent ability to conduct electric current (Mushtaq *et al.*, 2013). Sodium (Na^+), Potassium (K^+) and Chloride (Cl^-) are the main ions that are fundamental in the maintenance of osmotic pressure and acid-base equilibrium of the body fluids (Borges *et al.*, 2003c). The dietary amount and ratio of these monovalent minerals summarizes the dietary electrolyte balance (dEB) which has been recognized for some time (Nesheim *et al.*, 1964; Mongin, 1980) and is calculated using the formula: $dEB=Na^+ + K^+ - Cl^-$ (McDonald *et al.*, 2011). These minerals are essential for synthesis of tissue protein, maintenance of cellular acid-base balance and cellular homeostasis (Shahsavari *et al.*, 2012) and need to be supplied consistently to meet the desired production levels in all classes of poultry (Mushtaq *et al.*, 2013). A dEB level of 250 mEq/Kg has been suggested as satisfactory for optimal broiler growth and litter quality (Mongin, 1981) but some authors suggested a range between 100-250 mEq/Kg (Hooge, 2003) or even 290-330 mEq/Kg (Borgatti *et al.*, 2004) as also beneficial for broilers performance. It has been suggested that each individual electrolyte can have its own individual role but its function can be changed by the cation or anion present in its supplemental salt (Mongin, 1981).

2.5.5 Role of Na^+, K^+ and Cl^- in the body

Sodium (Na^+), potassium (K^+) and chloride (Cl^-) play important roles in nutrition due to their physiological functions namely maintenance of acid-base balance, osmotic control of water distribution within the body, synthesis of tissue protein, maintenance of intracellular and extracellular homeostasis (Borges *et al.*, 2003a; Kurtoglu *et al.*, 2007). Broilers have a minimum requirement for these minerals and their supplement is necessary at balanced and adequate levels to acquire optimum growth, bone development, and good litter quality (Murakami *et al.*, 2001).

Sodium is present in the soft tissues and body fluids and is the main cation present in the plasma. Sodium concentration in the blood is regulated by the kidneys via elimination in the urine and perspiration. A deficiency in Na in the diets can lead to adrenal malfunction and into an increase in uric acid levels which can lead to a physiological shock and even death (Mushtaq *et al.*, 2013). The main role for Na is in controlling broilers basal metabolism and the distribution of water throughout the body and maintenance of a normal fluid balance (Mongin, 1981; Ahmad, 2004). If the balance is not achieved the cell can become dehydrated or there may be fluid retention. Sodium also plays an important role in the transmission of nerve impulses and in the absorption of sugars and AA from the digestive tract (McDonald *et al.*, 2011). Therefore, inadequate Na levels in the diets can compromise the utilisation of digested protein and carbohydrates and influence the absorption of AA and sugars in the small intestine (Mushtaq *et al.*, 2013). Sodium levels for broilers diets are recommended to be used at 0.2% for the starter phase (1 to 3 weeks) and 0.15% for the finisher phase (4 to 6 weeks) (NRC, 1994).

Just as Na is the main cation of the extracellular fluids, K is the main cation of the intracellular fluids. It is one of the main electrolytes in the body as it plays an important role in the nerve impulses and is essential to the body homeostasis such as acid-base balance, osmotic pressure regulation, glucose and AA absorption and transport (Leeson & Summers, 2001). Potassium is essential for the proper functioning of the heart, kidneys, muscle contraction, and digestive system (Ahmad, 2004).

Most cells in the body need to have a high concentration of K^+ and a low concentration of Na^+ than their surroundings. The balance between these two ions is extremely important and in order to achieve this balance, cells constantly pump these ions in and out of the cells through a biological mechanism called the “sodium and potassium pump”.

Because the transport of the ions is made against the concentration gradient of the cell, energy is requires to help the transport of Na^+ out of the cell and the K^+ into the cell (active transport). This transport is made by a heterodimeric membrane protein (ATPase) that uses the energy from ATP hydrolysis to transport the ions through the cell membrane. The ATP hydrolysis and the phosphorylation of "the pump" promotes the change of the transporter protein and facilitates the transport of the ions in and out of the cell (Janovska, 2010). Sodium is a cation that has strong affinity for water and is normally found in the extracellular fluid while K^+ is, in its majority in the intracellular fluid. If, for some reason there is an increase in the intracellular fluid, Na^+ can transport the excess water out of the cell and thus reduce the pressure inside the cell. If on the contrary, the cell is dehydrated, Na^+ can transport water into the cell thus rehydrating the cell (Campbell & Reece, 2005). In combination with Na and Cl, K is necessary to maintain growth performance, nutrient utilisation and bone development (NRC, 1994) and is recommended for poultry diets at a level of 0.3% (NRC, 1994) but studies using broilers from 7 to 21 d estimated 0.824% K as the requirement for optimum broiler weight gain. This suggests that the K levels recommended by the NRC (1994) is lower than the levels normally present in commercial diets (Hooge & Cummings, 1995).

Chloride is the main anion found in the extracellular tissue and, associated with Na^+ and K^+, plays a critical role in maintaining the body's acid balance and osmotic regulation (McDonald *et al.*, 2011). By travelling primarily with Na and water, Cl helps generate the osmotic pressure of body fluids and also helps in maintaining the body acid-base balance. Like Na, the excretion or retention of Cl takes place at the kidney level depending on the body need to increase or decrease acid levels. If Cl^- increases, HCO_3^- reabsorption decreases resulting in metabolic acidosis associated with hyperchloremia. The inverse process results in a decreased reabsorption of Cl^- and Na^+ which can result in hypochloremic metabolic alkalosis (Powers, 2001). Chloride also plays an important role in gastric secretion and is excreted from the body in the urine and also in perspiration along with Na and K (McDonald *et al.*, 2011). Chloride levels for broilers diets are recommended to be at 0.2% for the starter phase (1 to 3 weeks) and 0.15% for the finisher phase (4 to 6 weeks) (1994).

2.5.6 dEB and growth performance

The acid-base balance can be influenced by the environment and by the diet as well as by the animal metabolism (Olanrewaju *et al.*, 2007). The proportion of certain added ions (Na^+, K^+ and Cl^-) to the diet will maintain this equilibrium and the input of these cations and anions will prevent an electrolyte unbalance and therefore, contribute for a better bird performance (Mongin, 1981). The growth performance can be affected if there is a change in blood pH, either by acidemia (high content of Cl^-) or alkalemia (high content of Na^+ and/or K^+) in the diet (Ahmad & Sarwar, 2006).

Melliere & Forbes (1966) showed that the anion to cation ratio of the diet is important for the growth and feed consumption of young chickens. (Johnson & Karunajeewa (1985) observed that a dEB lower than 180 mEq/Kg and higher than 300 mEq/Kg depressed birds weight at 42 days of age and that dEB levels between 250 mEq/Kg to 300 mEq/Kg were within the range for maximum growth of broilers. Murakami *et al.* (2001) also established that the ideal dEB value for broilers between 21-42 d of age was between 249 and 261 mEq/Kg. In addition, Borges *et al*, (2002) evaluated dEB in broilers pre-starter diets based on corn and soybean meal and observed that dEB interferes with chick performance during the first week of life and that the ideal dEB obtained by level manipulation of Na and Cl was 246 and 277 mEq/Kg for feed:gain ratio and weight gain, respectively. The authors also showed that there is the need to avoid high concentrations of Cl (0.77%) and K (1.05%) when manipulating dEB. Creating dEB higher or lower than 240 mEq/Kg decreased growth performance of birds which suggests that the metabolic pathways might be altered when acid-base balance deviates from homeostasis and conditions of alkalosis or acidosis is precipitated and that the ions are likely more involved in the regulation of homeostasis than in animal growth (Mongin, 1981). In Borges *et al.* (2004), a dEB of 40 mEq/Kg reduced feed intake and weight gain in broilers aged 21 to 42 d old and this loss was attributed to an imbalanced Na^+ and Cl^- ratio (0.15:0.7 respectively). Feed efficiency and weight gain were higher for diets at dEB between 202 and 235 mEq/Kg and with manipulation of Na and Cl whereas feed intake was highest at 264 mEq/Kg when Na was increased in the diet and at 213 mEq/Kg when Na and K were increased in the diet which indicates that broilers are sensitive to the association of high levels of Na and K in the diets.

Borges *et al.* (2003a) recommended dEB in the range of 240-250 mEq/Kg as the optimum level for promoting growth performance in broilers under hot summer conditions and

levels between 200-235 mEq/Kg for broilers kept under moderate temperatures (Borges *et al.*, 2004). Moreover, a study by Borgatti *et al.* (2004) on broiler performance under summer conditions observed that the greatest rate of body weight gain for broilers aged 1 to 21 days old was obtained at a dEB of 290 and 330 mEq/Kg. This dEB value contradicts the one advised by Borges *et al.* (2003a) but it is in the range of the value of dEB advised by Johnson & Karunajeewa (1985). Later on, (Ahmad *et al.*, 2009) reported levels between 150 and 250 mEq/Kg for better performance in broiler chicks kept during a 6 week feeding trial under tropical summer conditions (26 to 37.5ºC). Therefore, it is suggested, form the different results observed in these referred studies, that a range of different dEB levels is recommended for broilers under heat stress conditions. Moreover, levels between 200 to 300 mEq/Kg of dEB are suggested as adequate in situations that cover a broad range of high temperatures and the use of high levels of electrolytes, particularly cations, are also recommended under these circumstances (Mushtaq *et al.*, 2013).

2.5.7 Influence of dEB on phytase efficacy in poultry

Exogenous phytase is known to release not just P but also other nutrients such as AA and minerals from phytic acid (Ravindran *et al.*, 2006). More recently it has also been mentioned that phytase might have an effect on Na metabolism (Cowieson *et al.*, 2004; Ravindran *et al.*, 2008; Goodgame *et al.*, 2011). Because of the effects of phytase and phytate on Na secretion in the gut (phytate increases Na loses, whereas phytase reduces it) it can be reasoned that the digesta dEB might be influenced by phytate. By compromising the Na-dependent transport mechanisms, phytate might be also compromising the intestinal uptake of some nutrients, including AA, which can be counterbalanced by phytase (Ravindran *et al.*, 2008). Moreover, the impact of phytate and phytase on Na might also influence acid-base homeostasis (Selle & Ravindran, 2007).

A study with pigs performed by Haydon & West (1990) reported that AA digestibility was increased with an increase in dEB (from -50 to 400 mEq/Kg). Moreover, Selle & Ravindran, (2007) suggested that dEB may influence the magnitude of AA response to exogenous phytase while Ravindran *et al.* (2008) demonstrated that phytase has a greater capacity to enhance AA digestibility in diets with low Na concentration and/or dEB due to its Na sparing effect. These findings suggest the possibility that phytate might be compromising the "sodium and potassium pump" by limiting Na-transport systems which will, in turn, limit the intestinal uptakes of nutrients and AA (Selle & Ravindran, 2007).

Acid base balance can have an influence in pig growth (Patience *et al.*, 1987), broilers response to heat stress (Ahmad & Sarwar, 2006) as well as the metabolism of certain nutrients such as AA (Brake, 1998; Adedokun & Applegate, 2013). The effect of changing animal's acid base balance through the diet is of importance due to its influence and repercussion in animal performance and production. Austic & Calvert, (1981) suggested that lysine can contribute to the regulation of acid-base homeostasis and Selle *et al.* (2005) reported that lysine increased the digestibility of certain AA from the gut. The dEB used in the later study was 155 mEq/Kg, which is lower than the recommended (250 to 300 mEq/Kg) by Johnson & Karunajeewa, (1985), and phytase supplementation of lysine-deficient diets increased AA digestibility. The interactions observed in the referred study on the AA digestibility could have been explained by an influence of phytase on the acid-base balance. Through an impact of phytase on the Na movements into the gut (Cowieson *et al.*, 2004), the intestinal uptake of AA via the Na-dependant transport systems could have been influenced. Therefore, it is suggested that phytase supplementation might influence dEB since phytate and phytase influence Na secretions in the gut (Cowieson *et al.*, 2004; Ravindran *et al.*, 2006) and these changes to acid–base homeostasis and/or Na-dependent transport systems can influence intestinal uptakes of glucose and certain AA (Selle & Ravindran, 2007; Ravindran *et al.*, 2008).

There are, however, inconsistent findings about the influence of dEB in AA digestibility. A study by Ravindran *et al.* (2008) reported that dEB improved the ileal digestibility of 13 AA out of the 17 AA assessed, but a study by Balnave & Oliva, (1991), reported that by increasing dEB from 180 to 380 mEq/Kg, no effect on AA ileal digestibility was observed. Moreover, Blank *et al.* (1999) reported a decrease in AA ileal digestibility in weanling pigs upon an increase on dEB (225 to 640 mEq/Kg) while Haydon & West, (1990) had reported an increase in AA ileal digestibility in weanling pigs as a response to an increase in dEB (-50 to 400 mEq/Kg). Reasons for this discrepancy in the results have been discussed (Selle *et al.*, 2006a) and include, among others; marker used, ingredient type, dietary levels of Ca, non-phytate-P, and phytate and inclusion levels and source of phytase. Phytase influence on AA digestibility has also been inconsistent (Selle *et al.*, 2006a).

As mentioned earlier, Na plays an important role in the intestinal uptake of AA due to its participation in the Na-dependent transport systems and in the Na/K Pump. Therefore, Na dietary deficiencies can have a detrimental effect on the animal's wellbeing and performance since it will limit their nutrient uptake (Sklan & Noy, 2000). Although not clear how phytate draws Na into the gut lumen, it has been shown that phytate depresses

Na availability. A reduction of broilers ileal Na availability was observed by Ravindran *et al.* (2006) upon an increase in dietary phytic acid but this reduction was counterbalanced with the supplementation of phytase (1000 FTU/Kg). Similar results were observed by (Ravindran *et al.*, 2008) suggesting that, if Na can affect dEB levels, phytase can modify the dEB. Moreover, if phytate draws Na into the gut lumen, the dEB of the digesta is being influenced by phytate. Phytase supplementation increased ileal Na availability in Ravindran *et al.* (2008) study and these results were higher at lower dEB levels (150 and 225 mEq/Kg) and less pronounced at the higher dEB level (375 mEq/Kg), suggesting that phytase is more likely to improve broilers performance in diets with low dEB. Similar finding were made by (Shahsavari *et al.*, 2012) when he observed that phytase (500 FTU/Kg) was more effective in promoting growth performance in broilers at a dEB of 200 mEq/Kg. Moreover, in the later study, an interaction between phytase and dEB was observed for all parameters tested.

The overall objective of this study was to investigate nutritional factors that can improve the performance of broilers. Manipulations of dEB have been shown to influence broilers growth with Na levels playing an important role. Because phytase and phytate compromise Na secretions into the gut lumen it is implicit that this might influence the dEB levels. Moreover, there seems to be a considerable amount of phytate-P that remains "encapsulated" in the cell walls and that is undigested, even when high amounts of phytase are used. Supplementation of NSP degrading enzymes like xylanase may increase the efficacy of phytase.

2.6 Objectives

The main objectives of this MSc study were:

- Assess the impact of changes in dEB levels on phytase efficacy in growth performance, bone mineralisation and nutrient utilisation in broilers up to 21 days old.
- Assess the effects of phytase supplementation, alone or in combination with xylanase, on growth performance, bone mineralisation, volatile fatty acids production and pH in different parts of the GIT of broilers at 21 days of age.

The study specific hypotheses are mentioned in each chapter.

3. Assessment of the effect of dietary electrolyte balance on the efficacy of a microbial phytase

3.1 Introduction

Phytic acid or phytate is a naturally occurring organic complex that is found in plants. The majority of the P found in cereal grains and oilseeds exists as phytic acid (phytate-P) (Simons *et al.*, 1990). P is an essential mineral for animal's cellular metabolism and regulatory mechanisms and for skeletal development (McDonald *et al.*, 2011).

The use of plant based ingredients in poultry feeds is very common and for the animals to have access to the P from phytate –P it is necessary that they possess endogenous phytases that hydrolyse phytate. Due to the insufficient amount of phytase present in monogastric animals, the ability to use the P present in the grains and seeds is limited for these animals (Cowieson *et al.*, 2004; Selle *et al.*, 2000; Denbow *et al.*, 1995) which leads to the need for adding phytase to the feeds in order to increase P availability. The phytic acid molecule is broken down by phytase and the P is released for absorption, reducing the need for inorganic-P supplementation in the diet and reducing the amount of P being released to the environment. The partial availability of phytate P to monogastric animals assumes importance due to the fact that inorganic-P is an expensive ingredient in poultry feed and it might be facing a supply crisis in the near future (Selle & Ravindran, 2007). Apart from P, phytase can also release any other cation or AA that is bound to phytic acid. Phytase increases the digestibility of phytate in poultry from around 25% to 50-70% and can improve the digestibility of other nutrients as well as energy (Ravindran *et al.*, 1999a; Kornegay, 2001).

A combined intake of minerals is important for the physiological functions of broilers (Ravindran *et al.*, 2008; Shahsavari *et al.*, 2012) as it constitutes the acid-base balance of the diet and body. Because cells require a specific balance of anions and cations to function efficiently, minerals with electrolytic properties are important and considered functionally as separate entities because, cells are able to maintain voltages across their membranes and to carry electrical impulses (nerve impulses, muscle contractions) across themselves and to other cells (McDonald *et al.*, 2011).

The amount and ratio of the monovalent minerals Na, K and Cl is summarized as dEB and are essential for synthesis of tissue protein, acid-base balance and cellular homeostasis (Shahsavari *et al.*, 2012).

Several studies involving dEB manipulation showed that it can influence growth performance (Ahmad & Sarwar, 2006; Ravindran *et al.*, 2008) but Johnson & Karunajeewa (1985), demonstrated that there is a specific cation (Na^+ and K^+) effect in growth regardless of the dEB levels.

In more recent studies (Cowieson *et al.*, 2004; Ravindran *et al.*, 2006) it was shown that phytase and phytate influence the secretion patterns of Na at the ileal level in broiler chicks (phytate increases Na losses but phytase reduces Na losses). By definition, Na affects the dEB levels and phytate and phytase influence the Na status in the GIT. Because phytate may compromise Na secretions into the gut lumen it is implicit that it may influence the digesta dEB and also, consequently might be compromising the Na-dependant transport mechanisms involved in the intestinal uptake of some nutrients, including AA, but these negative effects are countered by phytase (Ravindran *et al.*, 2008). A study with pigs (Haydon & West, 1990) showed that an increased dEB (450 mEq Kg^{-1}) increases AA digestibility. Consequently Selle and Ravindran (2007) proposed that dEB may influence the magnitude of AA response to exogenous phytase.

3.1.1 Objective

The present study was undertaken to more fully investigate the possible effects of dEB on phytase activity with a focus on animal growth performance and nutrient utilisation.

3.1.2 Hypothesis

It is hypothesised that lowering or increasing the levels of dEB from the recommended level (250 mEq/Kg) will reduce the efficacy of phytase in improving growth performance and nutrient utilisation and will increase water content of excreta.

3.2 Material and Methods

3.2.1 Experiment Design

A total of 336 day-old male Ross 308 broilers were used in this study. On the day of arrival (day 0), all birds were allocated to 7 treatments in a randomised complete block design (diets were randomly allocated within blocks) with a 3x2 + 1 factorial arrangement, using initial body weight as the blocking criterion to ensure that the treatments had the same average body weight at day 0. A positive control diet (PC) was formulated to meet the NRC nutrient requirements and was designed to meet the dEB recommended level of 250 mEq/Kg. The remaining negative control (NC) diets were formulated to have 3 levels of dEB (214, 234 and 266 mEq/Kg) and 2 levels of phytase (0 FTU/Kg and 1500 FTU/Kg). Each treatment had 6 replicate modified raised floor pens and 8 birds per replicate pen. Titanium dioxide (TiO_2) was added as an indigestible marker to the diets to enable determination of energy and nutrient digestibility by the index method.

3.2.2 Diets

The diets used in this experiment were corn-soybean meal based and were fed in mash form. A PC diet that was adequate in all nutrients and was designed to meet the dEB recommendation level of 250 mEq/Kg. The NC diets were formulated to have 0.30% non-phytate P, 0.85% Ca and 0.3% less Na. These reductions were made to make room for the supplemented phytase which is expected to release the amount of P, Ca and Na that were reduced from the positive control diet. The diet formulas are presented in Table 3.1.

Table 3.1 - Ingredient composition (g/Kg) of the experimental diets

Description of diets	*PC*	*NC_1*	*NC_2*	*NC_3*	*NC_1 + phytase*	*NC_2 + phytase*	*NC_3 + phytase*
Phytase (FTU/Kg)	0	0	0	0	1500	1500	1500
Ingredients g/kg / **Diet n° =>**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Corn	451	462	459	453	462	459	453
Wheat	63	63	63	63	63	63	63
Soybean meal	350	350	350	350	350	350	350
Methionine	5	5	5	5	5	5	5
Lysine	4	4	4	4	4	4	4
Soybean oil	50	50	50	50	50	50	50
DCP(A)	22.0	12.9	12.9	12.9	12.9	12.9	12.9
Limestone (B)	9	8	11	15	8	11	15
Titanium dioxide premix (C)	25	25	25	25	25	25	25
Corn gluten meal	10	10	10	10	0	0	0
Enzyme premix (D)	0	0	0	0	10	10	10
Vitamin-mineral premix (E)	5	5	5	5	5	5	5
$NaHCO_3$	2.4	1.0	1.5	4.0	1.0	1.5	4.0
NH_4Cl	0.2	1.0	0.2	0.0	1.0	0.2	0.0
Salt NaCl	3	3	3	3	3	3	3
Total	1000	1000	1000	1000	1000	1000	1000
Calculated Nutrients & Energy							
Protein, g/kg	232	233	232	232	233	232	232
ME, kcal/kg	3007	3043	3034	3013	3043	3034	3013
Ca, g/kg	10.0	7.2	8.4	10.0	7.2	8.4	10.0
P, g/kg	7.0	5.4	5.4	5.4	5.4	5.4	5.4
Available P, g/kg	5.1	3.5	3.5	3.5	3.5	3.5	3.5
Na	2.0	1.6	1.7	2.4	1.6	1.7	2.4
K	8.6	8.6	8.6	8.6	8.6	8.6	8.6
Cl	2.2	2.7	2.2	2.1	2.7	2.2	2.1
Mg	1.4	1.4	1.4	1.4	1.4	1.4	1.4
S	1.4	1.4	1.4	1.4	1.4	1.4	1.4
dEB (expected), mEq/Kg	243.7	213.5	233.5	266.2	213.5	233.5	266.2
dEB (analysed), mEq/Kg	206.0	164.3	238.3	246.2	190.6	221.1	254.4

PC – Positive Control diet - adequate in all nutrients and designed to meet the dEB recommendation level of 250mEq/Kg; NC – Negative Control diet - formulated to have 0.30% non-phytate P, 0.85% Ca and 0.3% less sodium (Na); A. 16% Ca, 21% P; B. 38% Ca;

C. Prepared as 1.1 Kg titanium dioxide (TiO_2) added to 4.4 Kg corn gluten meal;

D. Enzyme premix to be made to desired enzyme activity unit (1500FTU/Kg) and added at the rate of 10 g/kg;

E. Supplies the following per kg DIET: Vit. A, 5484 IU; Vit. D3, 2643 ICU; Vit E,11 IU; Menadione sodium bisulfite,4.38 mg; Riboflavin, 5.49 mg; d-pantothenic acid, 11 mg; Niacin, 44.1 mg; Choline chloride, 771 mg; Vit B12, 13.2 ug; Biotin, 55.2 ug; Thiamine mononitrate, 2.2 mg; Folic acid, 990 ug; Pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug. Also contains per g of premix: Vit. A, 1828 IU; Vit. D3, 881 ICU; Vit E,3.67 IU; Menadione sodium bisulfite,1.46 mg; Riboflavin, 1.83 mg; d-pantothenic acid, 3.67 mg; Niacin, 14.69 mg; Choline chloride, 257 mg; Vit B12, 4.4 ug; Biotin, 18.4 ug; Thiamine mononitrate, 735 ug; Folic acid, 330 ug; Pyridoxine hydrochloride, 1.1 mg; I, 370 ug; Mn, 22.02 mg; Cu, 1.48 mg; Fe, 14.69 mg; Zn, 14.69 mg; Se, 100 ug.

3.2.3 Husbandry

On the day of arrival (day 0), the birds were housed in modified raised floor pens where they remained until the end of the experiment, 21 days. Water and experimental diets were provided *ad libitum* during the experimental period. The birds were housed in an environmentally controlled room. The lighting regimen followed the breed specification and the temperature was controlled using automated temperature regulation. The temperature was maintained at 31°C on day 1 and then gradually reduced to 22°C by day 21. The relative humidity was kept below 70% during the 21-day trial.

3.2.4 Sample collection

Samples of each of the 7 diets and of the enzyme premix were collected and stored in a refrigerator until chemically analysed. Birds were weighed per pen on day 0 and individually on day 21 and feed weight data were recorded on days 0 and 21 for measurement of growth performance responses. Excreta were collected from each raised floor pen on days 19 and 20 to determine total tract nutrient retention. On day 21 one randomly selected bird from each pen was stun-killed and blood was collected immediately after from the jugular vein to heparinised, blood pH readings were taken and tubes were then stored in the freezer. The remaining birds in each pen were killed and ileal digesta samples were collected from all the remaining birds by gently flushing the ileum with water for subsequent determination of ileal nutrient digestibility. The left tibia bones from two randomly selected birds per pen were collected (pooled per pen) and frozen prior to ashing for determination of bone ash and bone mineralisation. Ileal and excreta samples were pooled within a pen and oven dried in forced air oven at 80°C after collection and prior to chemical analysis for minerals, Ti, N, phytate-P, dry matter and energy.

3.2.5 Chemical analysis

Prior to analysis, all diets, ileal and excreta samples were ground to pass through 0.5 mm sieve using a mill grinder. The diets, excreta and ileal digesta samples were analysed for Ti, dry matter, gross energy, N, phytate-P and minerals as described by Olukosi *et al.* (2008a) for digestibility and total tract nutrient retention determination. Bone ash was determined and the bones were analysed for Ca and P contents.

3.2.5.1 Ti analysis

Determination of titanium concentration in all samples was performed as described by Short *et al.* (1996).

3.2.5.2 Dry matter

Dry matter content was determined as described by Olukosi *et al.* (2007a) on all dried and ground diets, ileal digesta and excreta samples.

3.2.5.3 Gross energy

Gross energy content of diets, ileum digesta and excreta samples were determined in duplicates using an adiabatic oxygen bomb calorimeter (Parr Instruments, Moline IL) using benzoic acid as a calibration standard.

3.2.5.4 N analysis

Diets, excreta and ileal digesta were analysed for N using the combustion method (method 968.06; AOAC, 2006).

3.2.5.5 Mineral analysis

Minerals content was determined using Inductively Coupled Plasma – Optical Emission Spectroscopy (method 990.08; AOAC, 2006).

3.2.5.6 pH

Blood pH was measured using a sterile glass pH electrode (HI 99163, HANNA Instruments, Romania).

3.2.5.7 Bone mineralisation

The procedure for bone mineralisation was as described by Olukosi *et al.* (2008b). The left tibia was collected from two randomly selected birds from each metabolism cage and all attached muscle, tendon and cartilages was removed. The bones were then dried in an oven set to 80-100^{o}C overnight, to allow the bone to be defatted by ether extraction and dried

again. Following this, samples were placed in a Muffle furnace for 24 hours at 500-600^{o}C and weighed and analysed for DM, P, Ca and ash content.

3.2.6 Calculations

Using the excreta, ileal and feed Ti content analysis, the apparent dry matter digestibility (or total tract retention) were calculated using the following formula:

$$DMD = [1 - (Ti_{feed} / Ti_{excreta})]$$

Where DMD is coefficient of dry matter digestibility; Ti_{feed} is the concentration of the titanium dioxide (TiO_2) in the feed and $Ti_{excreta}$ is the concentration of the marker in the excreta.

Coefficients of apparent nutrient digestibility or total tract retention were calculated using the following formula:

$$ND = \{1 - [(Ti_{feed} / Ti_{excreta}) \times (N_{excreta} / N_{feed})]\}$$

Where ND is coefficient of nutrient digestibility; N_{feed} is the concentration of the nutrient in the feed and $N_{excreta}$ is the concentration of the nutrient in the faeces.

3.2.7 Statistical analysis

The diets were analysed as a 3×2 factorial plus 1. The data on the growth performance and nutrient utilisation were analysed using the generalized linear model of GenStat program (11th edition, VSN International, 2008). The model included block and diet to test for the effect of the diets on the response criteria. Factorial analysis was used on the six diets to test for main effects (effect of dEB and phytase separately) and interactions. The reference diet (PC) was not included in this factorial arrangement. Because of the hierarchical order of factorial arrangement, only the main effects (i.e. of dEB levels (3) or phytase levels (2)) are discussed below when there were no interactions whereas simple effects (effects of individual treatments) are discussed when there were factor interactions. Tukey's test (Snedecor & Cochran, 1989) was used to separate significantly different means for the main effect of dEB whereas pre-planned pair-wise orthogonal contrasts were used to separate the means for the simple effects. Significance was set at $P \leq 0.05$.

3.3 Results

Based on the results of the diets analysis for Na, K, Cl, Mg and S the dEB calculated values varied from the expected (23% lower than expected). In spite of the differences between analysed and formulated values, the anticipated variability in dEB values were observed and the expected values for dEB are used in consequent writing below.

3.3.1 Growth performance and bone mineralisation

The data on the growth performance and blood pH responses of broilers receiving the experimental diets are presented in Table 3.2. As expected, initial weights of the birds were not statistically different among all treatments. There were no dEB × phytase interactions for any of the responses. Reducing the dietary levels of P reduced ($P<0.05$) weight gain, gain:feed and final body weight. Increasing dEB from 214 to 266 mEq/Kg had no significant effect on growth performance but increased ($P<0.05$) blood pH. Phytase supplementation increased ($P<0.05$) weight gain, gain:feed and final body weight but had no effect on blood pH.

Table 3.3 shows the effect of the dietary treatments on bone mineralisation. Reducing the level of dietary P and Ca reduced ($P<0.05$) all the tibia bone mineralisation responses. There were no dEB × phytase interaction on bone mineralisation responses. Increasing the dEB from 214 to 234 mEq/Kg had no effect on bone ash but further increase to 266 mEq/Kg tended ($P<0.10$) to reduce bone ash but dEB had no effect on the other bone mineralisation criteria. Phytase supplementation increased ($P<0.05$) all the tibia bone mineralisation responses relative to the diets without phytase.

Table 3.2 - Growth responses of broilers to dietary phytase supplementation at different dEB levels

Diets	WG, g	FI, g	Gain:feed g/kg	FBW, g	Blood pH
Main effect means: dEB (mEq/Kg)					
214	921	1292	716	960	7.5[a]
234	897	1357	666	935	7.6[ab]
266	861	1323	652	899	7.6[b]
SEM	31.0	27.0	26.3	31.0	0.03
P-value for dEB effect	0.396	0.253	0.215	0.396	0.011
Main effect means: Phytase (FTU/Kg)					
0 (NC)	804	1334	606	843	7.6
1500	981	1314	749	1020	7.6
SEM	25.3	22.0	21.5	25.3	0.02
Main effect of phytase	<.001	0.510	<.001	<.001	0.607
dEB × phytase interaction	0.560	0.756	0.395	0.559	0.727
Simple effects means*					
1 Positive control (PC)	929	1319	712	968	7.5
2 Negative Control 1(NC1)	857	1286	672	896	7.5
3 NC2	807	1371	591	846	7.6
4 NC3	749	1346	556	787	7.7
5 NC1 + Phytase	985	1297	760	1024	7.5
6 NC2 + Phytase	986	1343	740	1025	7.6
7 NC3 + Phytase	973	1301	748	1012	7.6
Pooled SEM	41.2	39.3	36.4	41.2	0.04
P-values for contrasts					
PC vs NC (1 vs 3)	0.045	0.356	0.026	0.045	0.547
2 vs 5 (phytase)	0.035	0.847	0.095	0.035	0.717
3 vs 6 (phytase)	0.005	0.618	0.007	0.005	0.763
4 vs 7 (phytase)	<.001	0.422	<.001	<.001	0.384
2 vs 4 (dEB extremes)	0.074	0.291	0.033	0.073	0.012
5 vs 7 (dEB extremes)	0.829	0.948	0.812	0.830	0.039

*Note – data represents average of 6 replicate pens per treatment
WG – weight gain; FI – feed intake; Gain:Feed – body weight gain to feed intake; FBW – final body weight; PC – positive control; NC – negative control; NC1, 2 and 3 had dEB levels of 214, 234 and 266 mEq/Kg, respectively

Table 3.3 - Bone mineralisation responses of broilers to dietary phytase supplementation at different dEB levels

Diets	Ash, g	P, g	Ca, g	Zn, mg	Weight, g
Main effect means: dEB (mEq/Kg)					
214	1.906	0.340	0.710	0.787	3.832
234	2.004	0.360	0.750	0.811	3.875
266	1.771	0.310	0.640	0.743	3.519
SEM	0.07	0.02	0.03	0.03	0.14
P-value for dEB effect	0.078	0.105	0.108	0.283	0.165
Main effect means: Phytase (FTU/Kg)					
0 (NC)	1.526	0.260	0.560	0.635	3.126
1500	2.261	0.410	0.840	0.925	4.358
SEM	0.06	0.01	0.03	0.02	0.11
Main effect of phytase	<.001	<.001	<.001	<.001	<.001
dEB × phytase interaction	0.306	0.691	0.702	0.819	0.410
Simple effects means*					
1 Positive control (PC)	2.053	0.370	0.770	0.814	3.900
2 Negative Control 1 (NC1)	1.628	0.280	0.590	0.656	3.370
3 NC2	1.596	0.280	0.590	0.654	3.199
4 NC3	1.354	0.230	0.490	0.595	2.811
5 NC1 + Phytase	2.183	0.410	0.830	0.917	4.294
6 NC2 + Phytase	2.413	0.430	0.900	0.968	4.551
7 NC3 + Phytase	2.187	0.380	0.800	0.890	4.228
Pooled SEM	0.11	0.02	0.05	0.05	0.21
P-values for contrasts					
PC vs NC (1 vs 3)	0.005	0.007	0.012	0.020	0.022
2 vs 5 (phytase)	<.001	<.001	0.002	<.001	0.003
3 vs 6 (phytase)	<.001	<.001	<.001	<.001	<.001
4 vs 7 (phytase)	<.001	<.001	<.001	<.001	<.001
2 vs 4 (dEB extremes)	0.080	0.123	0.141	0.352	0.065
5 vs 7 (dEB extremes)	0.981	0.523	0.584	0.685	0.822

*Note – data represents average of 6 replicate pens per treatment

Bone mineral contents are the absolute weight of ash or individual minerals present in the bone collected. These can be related back to bone weight to determined % minerals in the bone

3.3.2 Digestibility and total tract retention

Table 3.4 show the ileal nutrient digestibility response of broilers to the dietary treatments. There were significant dEB × phytase interaction ($P<0.01$) for all the nutrients and energy except phytate P and hence the simple effects are presented. Decreasing the dietary P and Ca increased ($P<0.05$) ileal phytate-P disappearance. In the diets with 214 mEq/Kg dEB, phytase supplementation decreased ($P<0.01$) ileal DM, energy and N utilisation. In the diets with 234 mEq/Kg dEB, there were no phytase effects. However, in the diets with 266 mEq/Kg dEB, phytase supplementation increased ileal nutrient and energy utilisation. In the diets without phytase, increasing dEB from 214 to 266 decreased ($P<0.01$) ileal nutrient digestibility except for phytate-P whereas in the diets with phytase, increasing the dEB from 214 to 266 mEq/Kg increased ($P<0.01$) ileal nutrient digestibility with the exception of ileal digestible energy and phytate-P.

Table 3.5 shows the ileal mineral digestibility of the broilers to the experimental diets. Reducing the dietary P and Ca increased ($P<0.05$) digestibility of Ca, Mg, Zn, and P. In the diets with 214 mEq/Kg, phytase supplementation decreased ($P<0.01$) ileal mineral digestibility. In the diets with 234 mEq/Kg phytase supplementation increased ($P<0.05$) ileal digestibility of Fe, Zn and P while in the diets with 266 mEq/Kg, phytase supplementation increased ($P<0.01$) Mg, K and P. In the diets without phytase, increasing dEB from 214 to 266 mEq/Kg decreased ($P<0.01$) ileal mineral digestibility whereas in the diets with phytase supplementation, increasing dEB from 214 to 266 mEq/Kg increased ileal digestibility of Mg, Fe, Zn, and K but had no effect on Ca and P.

Table 3.4 - Coefficients of ileal nutrient digestibility in broilers fed dietary phytase supplementation at different dEB levels

Diets	IDE, kcal/Kg	Energy	DM	N	Phytate-P
Main effect means: dEB (mEq/Kg)					
214	3019[ab]	0.732[ab]	0.725	0.696	0.847[b]
234	3129[b]	0.753[b]	0.742	0.724	0.762[ab]
266	2903[a]	0.713[a]	0.704	0.667	0.740[a]
SEM	26.7	0.01	0.01	0.01	0.03
P-value for dEB effect	<.001	<.001	0.001	0.010	0.034
Main effect means: Phytase (FTU/Kg)					
0 (NC)	3035	0.739	0.728	0.703	0.749
1500	2999	0.726	0.719	0.688	0.817
SEM	21.8	0.01	0.01	0.01	0.02
Main effect of phytase	0.265	0.112	0.206	0.272	0.052
P-value for dEB × phytase	0.001	<.001	<.001	<.001	0.649
Simple effects means*					
1 Positive control (PC)	3119	0.732	0.709	0.698	0.529
2 Negative Control 1 (NC1)	3127	0.777	0.772	0.764	0.834
3 NC2	3117	0.746	0.733	0.718	0.712
4 NC3	2860	0.694	0.680	0.628	0.701
5 NC1 + Phytase	2912	0.686	0.677	0.628	0.859
6 NC2 + Phytase	3140	0.761	0.751	0.730	0.812
7 NC3 + Phytase	2946	0.732	0.728	0.705	0.778
Pooled SEM	35.2	0.009	0.008	0.016	0.057
P-values for contrasts					
PC vs NC (1 vs 3)	0.961	0.248	0.054	0.371	0.030
2 vs 5 (phytase)	<.001	<.001	<.001	<.001	0.750
3 vs 6 (phytase)	0.638	0.245	0.127	0.596	0.223
4 vs 7 (phytase)	0.092	0.004	<.001	0.002	0.346
2 vs 4 (dEB extremes)	<.001	<.001	<.001	<.001	0.110
5 vs 7 (dEB extremes)	0.488	<.001	<.001	0.002	0.321

*Note – data represents average of 6 replicate pens per treatment
IDE – ileal digestible energy; DM – dry matter; PC – positive control; NC – negative control; NC1, 2 and 3 had dEB levels of 214, 234 and 266 mEq/Kg, respectively

Table 3.5 - Coefficients of ileal mineral digestibility in broilers fed dietary phytase supplementation at different dEB levels

Diets	Ca	Mg	Fe	Zn	K	P
Main effect means: dEB (mEq/Kg)						
214	0.518[ab]	0.422[ab]	0.262	0.153[a]	0.744	0.546[b]
234	0.604[b]	0.477[b]	0.355	0.276	0.760	0.565[b]
266	0.476[a]	0.381[a]	0.261	0.088[a]	0.725	0.442[a]
SEM	0.01	0.01	0.02	0.01	0.01	0.01
P-value for dEB effect	<.001	<.001	<.001	<.001	0.099	<.001
Main effect means: Phytase (FTU/Kg)						
0 (NC)	0.600	0.454	0.338	0.184	0.748	0.503
1500	0.466	0.400	0.247	0.161	0.738	0.533
SEM	0.01	0.01	0.01	0.01	0.01	0.01
Phytase effect	<.001	<.001	<.001	0.110	0.473	0.005
dEB × phytase interaction	<.001	<.001	<.001	<.001	<.001	<.001
Simple effects means*						
1 Positive control (PC)	0.349	0.327	0.242	0.050	0.732	0.410
2 Negative Control 1 (NC1)	0.619	0.543	0.410	0.273	0.798	0.581
3 NC2	0.627	0.464	0.304	0.210	0.754	0.522
4 NC3	0.554	0.354	0.301	0.071	0.691	0.404
5 NC1 + Phytase	0.418	0.301	0.115	0.034	0.690	0.511
6 NC2 + Phytase	0.581	0.491	0.405	0.342	0.766	0.608
7 NC3 + Phytase	0.399	0.409	0.222	0.106	0.759	0.480
Pooled SEM	0.013	0.017	0.028	0.020	0.015	0.014
P-values for contrasts						
PC vs NC (1 vs 3)	<.001	<.001	0.128	<.001	0.293	<.001
2 vs 5 (phytase)	<.001	<.001	<.001	<.001	<.001	0.001
3 vs 6 (phytase)	0.016	0.269	0.016	<.001	0.564	<.001
4 vs 7 (phytase)	<.001	0.028	0.053	0.222	0.003	<.001
2 vs 4 (dEB extremes)	0.001	<.001	0.01	<.001	<.001	<.001
5 vs 7 (dEB extremes)	0.300	<.001	0.011	0.015	0.002	0.126

*Note – data represents average of 6 replicate pens per treatment
PC – positive control; NC – negative control; NC1, 2 and 3 had dEB levels of 214, 234 and 266 mEq/Kg, respectively

Table 3.6 shows the effect of the dietary treatments on total tract nutrient and energy utilisation. dEB × phytase interaction was significant ($P<0.01$) for all the nutrients and energy and tended to be significant for phytate-P. Decreasing the dietary Ca and P increased ($P<0.05$) energy, DM, N and phytate P retention. Supplementing phytase to diets with dEB 214 mEq/Kg decreased ($P<0.05$) total tract utilisation of energy, DM and N. In the diets with dEB 234 and 266 mEq/Kg, phytase supplementation increased total tract utilisation of DM and phytate-P but increased total tract utilisation of energy only in the diets with 234 mEq/Kg and total tract utilisation of N only in diets with 266 mEq/Kg.

Table 3.7 shows the effects of the dietary treatments on total tract nutrient utilisation of broilers. There were dEB × phytase interactions ($P<0.01$) for all the minerals except Na. Decreasing the dietary Ca and P increased ($P<0.05$) total tract mineral retention, except for Fe. In the diets with 214 mEq/Kg dEB, phytase supplementation decreased ($P<0.05$) total tract mineral retention. However, in the diets with 234 mEq/Kg, phytase supplementation increased ($P<0.05$) total tract retention of Ca, Mg, Fe, K, and P; decreased ($P<0.05$) Cu retention but had no effect on Na retention. On the other hand, phytase supplementation to diets with 266 mEq/Kg increased ($P<0.01$) total tract retention of Ca, Mg, K, and P; reduced ($P < 0.05$) retention of Na and Fe, but had no effect on Cu retention. In the diets without phytase, increasing dEB from 214 to 266 mEq/Kg decreased ($P<0.05$) total tract retention of all the minerals. On the other hand, in the diets with phytase, increasing dEB from 214 to 266 mEq/Kg increased ($P<0.01$) retention of Mg, Cu, Fe, K, and P but reduced ($P<0.05$) retention of Ca and Na.

Table 3.6 - Coefficients of total tract nutrient retention in broilers fed dietary phytase supplementation at different dEB levels

Diets	AME,kcal/kg	Energy	DM	N	Phytate-P
Main effect means: dEB (mEq/Kg)					
214	2993[a]	0.733[ab]	0.710[ab]	0.638[ab]	0.707
234	3174	0.764[b]	0.738[b]	0.684[b]	0.622
266	2888[a]	0.709[a]	0.681[a]	0.614[a]	0.573
SEM	17.18	0.004	0.004	0.01	0.03
P-value for dEB effect	<.001	<.001	<.001	<.001	0.036
Main effect means: Phytase (FTU/Kg)					
0 (NC)	3055	0.744	0.717	0.662	0.555
1500	2982	0.727	0.701	0.628	0.713
SEM	14.03	0.003	0.003	0.01	0.03
Main effect of phytase	<.001	0.002	0.002	0.001	<.001
dEB × phytase	<.001	<.001	<.001	<.001	0.071
Simple effects means*					
1 Positive control (PC)	3090	0.725	0.685	0.632	0.272
2 Negative Control 1	3137	0.779	0.761	0.717	0.693
3 NC2	3131	0.749	0.722	0.674	0.527
4 NC3	2898	0.703	0.670	0.595	0.444
5 NC1 + Phytase	2849	0.688	0.659	0.558	0.721
6 NC2 + Phytase	3218	0.780	0.754	0.694	0.716
7 NC3 + Phytase	2877	0.715	0.691	0.633	0.703
Pooled SEM	24.28	0.005	0.006	0.011	0.052
P-values for contrasts					
PC vs NC (1 vs 3)	0.250	0.006	<.001	0.011	0.002
2 vs 5 (phytase)	<.001	<.001	<.001	<.001	0.704
3 vs 6 (phytase)	0.016	0.001	<.001	0.220	0.015
4 vs 7 (phytase)	0.558	0.158	0.011	0.020	0.001
2 vs 4 (dEB extremes)	<.001	<.001	<.001	<.001	0.002
5 vs 7 (dEB extremes)	0.415	0.003	<.001	<.001	0.810

*Note – data represents average of 6 replicate pens per treatment
ME – metabolisable energy at excreta level; DM – excreta dry matter; N - Nitrogen; PC – positive control; NC – negative control; NC1, 2 and 3 had dEB levels of 214, 234 and 266 mEq/Kg, respectively

Table 3.7 – Coefficients of total tract mineral retention in broilers fed dietary phytase supplementation at different dEB levels

Diets	Ca	Na	Mg	Cu	Fe	K	P
Main effect means: dEB (mEq/Kg)							
214	0.507[b]	0.653[b]	0.187[ab]	0.099[a]	0.209	0.242[ab]	0.594[ab]
234	0.544[b]	0.716[b]	0.295[b]	0.292	0.288	0.303[b]	0.645[b]
266	0.402[a]	0.515[a]	0.095[a]	0.096[a]	0.227	0.172[a]	0.543[a]
SEM	0.01	0.02	0.01	0.01	0.01	0.01	0.01
P-value for dEB effect	<.001	<.001	<.001	<.001	<.001	<.001	<.001
Main effect means: Phytase (FTU/Kg)							
0 (NC)	0.478	0.662	0.214	0.205	0.278	0.251	0.587
1500	0.491	0.594	0.171	0.120	0.204	0.227	0.601
SEM	0.005	0.02	0.01	0.01	0.01	0.01	0.01
Main effect of phytase	0.069	0.019	0.016	<.001	<.001	0.233	0.075
dEB × phytase interaction	<.001	0.421	<.001	<.001	<.001	<.001	<.001
Simple effects means*							
1 Positive control (PC)	0.382	0.527	0.078	0.186	0.254	0.178	0.425
2 Negative Control 1 (NC1)	0.544	0.679	0.355	0.192	0.352	0.396	0.654
3 NC2	0.501	0.734	0.246	0.319	0.215	0.248	0.600
4 NC3	0.389	0.574	0.041	0.103	0.268	0.109	0.505
5 NC1 + Phytase	0.469	0.628	0.018	0.007	0.065	0.088	0.533
6 NC2 + Phytase	0.588	0.699	0.345	0.264	0.360	0.357	0.689
7 NC3 + Phytase	0.415	0.456	0.149	0.089	0.185	0.234	0.583
Pooled SEM	0.008	0.031	0.020	0.014	0.017	0.024	0.010
P-values for contrasts							
PC vs NC (1 vs 3)	<.001	<.001	<.001	<.001	0.112	0.044	<.001
2 vs 5 (phytase)	<.001	0.253	<.001	<.001	<.001	<.001	<.001
3 vs 6 (phytase)	<.001	0.433	0.001	0.012	<.001	0.003	<.001
4 vs 7 (phytase)	0.028	0.011	<.001	0.479	0.002	<.001	<.001
2 vs 4 (dEB extremes)	<.001	0.023	<.001	<.001	0.002	<.001	<.001
5 vs 7 (dEB extremes)	<.001	<.001	<.001	<.001	<.001	<.001	0.002

*Note – data represents average of 6 replicate pens per treatment

PC – positive control; NC – negative control; NC1, 2 and 3 had dEB levels of 214, 234 and 266 mEq/Kg, respectively

3.4 Discussion

This study focused on the effects of three levels of dEB on phytase efficacy with animal growth performance and nutrient utilisation as response criteria**.** The hypothesis was that by lowering or increasing the levels of dietary electrolyte balance (dEB) from the recommended level of 250 mEq/Kg (Mongin & Sauveur, 1977) this would reduce the efficacy of phytase in improving growth performance and nutrient utilisation.

The experimental diets were formulated to have 3 levels of dEB and 2 levels of phytase. A positive control (PC) diet that was adequate in all nutrients, and was designed to meet the dEB recommendation level of 250 mEq/Kg, was added for comparison. The NC diets were formulated to have lower P, Ca and Na than PC to ensure optimise the effect of supplemented phytase which is expected to release the amount of P, Ca and Na that were reduced from PC diet. The chemical analysis of the diets showed that the diets mineral content was in general higher than planned which caused differences between the expected and actual levels of dEB. These differences may be due to errors associated with sampling, analysis or combination of both. As done in similar studies where differentials were observed between expected and analysed values (Ravindran *et al.*, 2008), references will be made to the expected levels of dEB in the discussion.

3.4.1 Growth performance, bone mineralisation and blood pH

Overall, results indicated that the different levels of dEB did not have any significant effect on growth performance or tibia bone mineralisation which suggests that the birds were able to adjust their electrolyte balance or were tolerant to the range of dEB levels used in the current study. This adjustment might have occurred because the birds had access to water *ad libitum* during the whole experimental period and this was enough for them to balance or counterbalance the possible deleterious effects of the existing electrolyte range. As Borges (2001) suggested, an increase in water intake caused by increasing dEB levels is required to overcome the osmotic imbalance caused by higher Na^+ and K^+ levels but, as observed by Ravindran *et al.* (2008), the intake of drinking water electrolytes had no impact on dEB levels. A lack of response was also observed by Borges *et al.* (2003b) who observed no effect of increasing dEB levels from 120 to 240 mEq/Kg for a starter phase (0 to 21 days old) on broilers performance. At the two levels of dEB, although no effect was seen for weight gain, feed intake and FCR, water intake was significantly different which might also corroborate the suggestion that the electrolytes present in the water are taking

an important part in the bird's adjustment to the electrolyte imbalance or that, there might be a range of optimal dEB levels (Borges *et al.*, 2003b) rather than a single point as suggested by Mongin & Sauveur (1977). Nonetheless, water intake was not measured in the current experiment.

Koreleski *et al.* (2011) observed that a drop from 298 to 274 mEq/Kg of dEB improved growth responses in a starter phase (1 to 15 days old) and Murakami *et al.* (2001) reported that dEB between 245 and 315 mEq/Kg gave maximum growth performance in broilers 1 to 21 days old. Arantes *et al.* (2013) who used dEB levels between 200 and 320 mEq/Kg also reported no effect on broilers performance during the periods of 7 to 21 days of age. Different results were observed by Ravindran *et al.* (2008) who observed significant effects of dEB (150, 225 and 300 mEq/Kg) on broiler weight gain and feed efficiency and a negative impact on weight gain and feed conversion ratio at 375 mEq/Kg of dEB. Considerable differences in the results observed from these studies can mean that not only dEB, but also other ions, can contribute to changes in the effects of dEB in broilers performance (Mushtaq *et al.* (2013).

The effects of phytase on body weight gain observed in this study are consistent with previous studies (Cowieson & Adeola, 2005; Ravindran *et al.*, 2008; Shahsavari *et al.*, 2012). In this study phytase and dEB had no influence on feed intake. The same observations on the effect of dEB levels on feed intake were made by Ravindran *et al.* (2008) where increasing levels of dEB had no effect on feed intake. However, different results were observed for phytase supplementation, which had a positive effect on feed intake for all dEB levels, which is not consistent with the findings in the present study. This effect could be explained by the differences in Ca:P ratio observed in the two studies. Ravindran *et al.* (2008) maintained a Ca:P ratio at 1.3:1 while in the present study the Ca:P ratio increased from 1.3:1 in NC1 to 1.8:1 in NC3 which had been observed could compromise feed intake in broilers (Qian *et al.*, 1997). Also, the fact that dietary Ca levels are slightly higher in the present study than in Ravindran *et al.* (2008)'s study, which appears to influence feed intake in broilers (Ferket & Gernat, 2006) and could be another reason for the differences in feed intake responses. The strong effect of C1 on broilers chicks feed intake has been observed by Hurwitz *et al.* (1973) and Murakami *et al.* (1997b) and the higher levels of Cl present in the current study compared with Ravindran *et al.* (2008) study could also be a reason for the differences observed in the feed intake results. Apart from this, the diets in Ravindran *et al.* (2008) study were pelleted which has been

shown to increase density and intake of the ration, and also improves growth and feed efficiency (Engberg *et al.*, 2002; Brickett *et al.*, 2007).

Gain:feed was affected by phytase supplementation and these results are similar to Ravindran et al. (2008) and Shahsavari et al. (2012) findings, but there was a tendency observed by Ravindran *et al.* (2008) or an actual dEB × phytase interaction for gain:feed observed by Shahsavari *et al.* (2012) that is not consistent with the findings in the present study. Also, phytase addition increased gain:feed in the present study while Ravindran et al. (2008) observed a decrease in this response for phytase supplemented diets but, in both the present and Ravindran et al. (2008) study, the responses in gain:feed were greater at the lowest dEB level which suggests that phytase is more likely to improve gain:feed at lower dEB levels.

Hussein (2006) observed that phytase supplemented starter and finisher diets that had a decrease in available P by 40% did not support optimum growth rate of broilers and also resulted in a decrease of tibia and toe ash content compared to control diets but when birds were fed a normal starter diet without phytase and then a low P diet supplemented with phytase there was an improvement on bird performance and bone mineralisation. The level of available P reduction used in this study had a negative impact in weight gain, gain:feed, final body weight, bone ash and bone mineralisation compared to PC diet but when the diets were supplemented with phytase there was a significant improvement in growth performance and bone mineralisation of the birds which suggests that phytase can support bone mineralisation of low P diets and also works as a growth promoter probably due to the improvement of minerals (mainly Ca and P) availability (Shaw, 2010).

Maiorka *et al.* (2004) observed that dietary Na levels and different cation/anion balances (Na+K-Cl) had a quadratic effect on feed intake and weight gain of birds at 1 to 7 days of age. These results are contrary to the observations made in the current study where none of these parameters were affected by dEB levels. Murakami *et al.* (1997a) recommended the need of 0.20 to 0.25% of Na for maximum broiler growth performance during the first 21 days and later on Murakami *et al.* (2001) suggested 0.28 and 0.25% for broilers 1 to 21 days old. Sklan & Noy (2000) demonstrated that Na plays an important role in feed intake for post hatched birds and Maiorka *et al.* (2004) observed that maximum responses were achieved at Na dietary levels of 0.40% respectively for feed intake and weight gain. Nevertheless, the Na values used by Maiorka *et al.* (2004) were higher than the values recommended by the NRC (1994) for birds 1 to 7 days old and also much higher than the

ones used in the present study (0.16% to 0.24%) which might explain the differences on birds performance observed in the present study and Maiorka *et al.* (2004) study.

Ahmad & Sarwar (2006) suggested that growth performance can be affected if there is a change in blood pH. It is suggested that the blood pH parameters in broilers should be in the range of 7.35 to 7.45 for maintenance of protein structure and function (Carlson, 1997; DiBartola, 2011) which is a necessary condition for normal progression of metabolic events. A deviation from these parameters can cause metabolic disorders, microbiological diseases and loss of productivity (Haskins, 1977; Carlson, 1997; DiBartola, 2011). In the present study blood pH increased with increase in dEB levels at the extremity levels of 214 and 266 mEq/Kg for dEB however it was also observed that the difference in blood pH did not compromise growth performance. These findings are not in agreement with Hurwitz *et al.* (1973) who reported that the growth rate of broilers was higher at blood pH of 7.28 and it decreased when blood pH was greater than 7.3 or lower than 7.2 which suggests that the responses observed might not be totally related to pH changes but also due to other electrolyte or metabolic effects (Ahmad & Sarwar, 2006). Generally the blood pH observed in the current study were greater than the levels reported by Hurwitz *et al.* (1973) and thus it may be that the effect of narrow changes in pH is less inhibitory to growth performance at the range of pH observed in the current study.

Tibia weight, ash and mineral content were not affected by changes in dEB levels in this experiment. Oliveira *et al.* (2010) used dEB levels in the range of the ones used in this experiment and observed that there was no effect on broilers tibia weight, diameter height or length. Also Arantes *et al.* (2013) observed no effects on tibia density, ash and mineral content of broilers fed diets with dEB levels of 200, 240, 280 and 320 mEq/Kg. Bones function as a buffer for the electrolyte balance of body fluids and Ca and other cations can be released from the bones to the blood to correct pH in case of acidosis. The majority of the Ca existing in the body is present in the bone which suggests that bone is the source of higher excretion of this mineral by the kidneys (Bushinsky, 2001). Calcium loss should result in reduced bone mineralisation and could affect bone density Arantes *et al.* (2013) which was not observed in this study suggesting that the dEB levels used in this experiment did not affect bone mineralisation.

As mentioned before, the reduction of P and Ca had a negative effect on tibia bone mineralisation responses but phytase supplemented diets improved tibia bone mineralisation responses relative to diets without phytase. Phytase supplemented diets also

had better bone mineralisation responses than PC diets with the best phytase response to bone mineralisation being observed at the dEB of 234 mEq/Kg. These results are in accordance with what was observed by Selle *et al.* (2009a) who, in a review of the literature, noted that for poultry diets that are supplemented with phytase, the activity of the phytase is facilitated when dietary Ca levels are kept to a minimum as long as there is no compromise of the animal skeletal integrity or growth. Powell *et al.* (2011) observed similar results to the ones in the present study for diets without phytase supplementation where an increase in dietary Ca decreased growth performance and bone mineralisation but, on the other hand, for phytase supplemented diets, Powell *et al.* (2011) reported that growth performance and bone mineralisation responses were greater for the diets with higher level of dietary Ca, which is not in accordance with the results observed in the present study. Similarly to Powell *et al.* (2011)'s study, Driver *et al.*, (2005) had also observed earlier that phytase efficacy was better for growth performance and bone responses when dietary Ca was higher and then also concluded that phytase effects are different at each concentration of P and Ca and therefore no single efficacy value can be attributed to phytase.

3.4.2 Digestibility and total tract retention

There was dEB × phytase interaction effect on digestibility and total tract N utilisation. Suplementation of phytase improved total tract utilisation and digestibility of N for the lower and higher levels of dEB but there was no improvement observed in the diets with 234 mEq/Kg of dEB. Ravindran *et al.* (2008) also observed improvements for diets suplemented with phytase but in this case the interaction of dEB × phytase was not observed for N digestibility and total tract retention. There was no phytase response to higher dEB levels (375 mEq/Kg) in Ravindran *et al.* (2008)'s study but the lack of reponse observed in the present study for dEB at 234 mEq/Kg was not observed for similar levels (between 225 and 300 mEq/Kg) by Ravindran *et al.* (2008). This could be explained by the fact that perhaps at these levels other factors like phytate-P levels might be afecting N retention (Manangi *et al.*, 2009). Camden (2001) reported that the addition of phytase to diets with low Ca and available P improved apparent N retention in broilers, but more recent studies have reported no positive effects of phytase addition on N retention (Silva *et al.*, 2008; Donato *et al.*, 2013). The discrepancy of some of these results and the results obtained in the present study sugest that phytase capacity to improve ileal digestibility and retention of N (Ravindran *et al.*, 2000) can be inconsistent and that this inconsistency might be a reflection of changes in dEB levels.

An influence of dietary Ca and P levels on phytate-P retention and digestibility are observed in this study. Selle & Ravindran (2007) mentioned in their review that low dietary Ca and P levels are more beneficial to phytase activity. Other published work aslo reported that cations, including Ca, negatively influenced phytate-P hydrolysis (Gifford & lydesdale, 1990; Tamim & Angel, 2003). Lei *et al* (1994) observed that phytate-P hydrolysis was improved by phytase at a low dietary Ca level for weanling pigs fed a corn-soybean meal based diet. The results in the present study suggest that high dietary Ca concentrations can result in insoluble Ca-phytate complexes being formed, leaving phytate-P and Ca unavailable for absorption (Wise, 1983).

In the present study there was no effect of phytase on phytate-P digestibility at the ileum level, and increasing dEB levels for diets with and without phytase had no effect on phytate-P digestibility. There was no dEB × phytase interaction at ileum level but there was an influence of dEB on phytate-P digestibility and a tendency for phytase effect on phytate-P digestibility. Furthermore, total tract utilisation of phytate-P was only observed in diets supplemented with phytase at higher dEB levels with the greatest utilisation observed for the diets with the highest dEB level of 266 mEq/Kg and there was a tendency for dEB × phytase interaction for phytate-P utilisation. These results on phytate-P digestibility and retention suggest that phytase still had the ability to dephosphorylate phytate but the extent of phytase effect might be compromised by dEB levels.

The effects of dEB and phytase on total tract retention of Ca and P were not consistent with Ravindran *et al.* (2008)'s findings. In the later study it was observed that Ca and P retention were influenced by dEB, phytase and dEB × phytase interaction, whereas in the present study Ca and P retention were influenced only by dEB levels and by dEB × phytase interaction but not phytase. On the other hand, Ca retention was improved by phytase suplementation at lower dEB levels which is suported by the findings by Ravindran *et al.* (2008). Also, in the present study, phytase improved P and Ca retention and ileal P and Ca digestibility at all dEB levels apart from the lowest dEB level where there was a decrease. These results suggest that phytase was effective in releasing the P and Ca from phytate but that phytase efficacy was more effective at lower dEB levels, which is in accordance with observations made by Ravindran *et al.* (2008), who observed that phytase is more effective at lower dEB levels.

Total tract retention of Na was influenced by dEB levels with an increase in retention as dEB levels increased from 214 to 234 mEq/Kg but then, Na retention decreased with a

further increase of dEB from 234 and 266 mEq/Kg. Ravindran *et al.* (2008) observed a decrease in Na retention for levels higher than 225 mEq/Kg as well as an increase in ileal Na availability with an increase in dEB levels. It was suggested in Ravindran *et al.* (2008)'s study that there is a clear change in the patterns of absorption and secretion of Na by poultry in responses to dEB. In both, Ravindran *et al.* (2008)'s and the present study, there was no dEB × phytase interaction for total tract Na retention, but in the present study there was an influence of phytase in Na retention that was not observed in Ravindran *et al.* (2008)'s study. These differences in responses might have been influenced by the use of $NaHCO_3$ as source of Na which has been shown to be similar to the use of NaCl (Murakami *et al.*, 1997a) but which buffering properties of the bicarbonate may be influencing the relationship between phytase and Na in the intestinal tract (Goodgame *et al.*, 2011). The results from the present study and Ravindran *et al.* (2008)'s study suggest that Na absorption and retention patterns are dependent on poultry responses to dEB levels and also from phytase inclusion, but that phytase and dEB are, independently, influencing Na digestibility patterns.

3.5 Conclusions

It can be concluded from this experiment that dEB ranging from 214 to 266 mEq/Kg had no negative effect on growth performance and tibia mineralisation and had a general positive effect on mineral retention and nutrient digestibility for phytase supplemented diets.

The level of 234 mEq/Kg dEB for phytase supplemented diets seem to be more adequate for broilers requirements and performance, since higher dEB levels can sometimes compromise phytase efficacy in improving the availability and retention of important minerals like Ca, P and Na.

The effects of phytase and dEB on nutrient utilisation and growth performance indicates that treatment effects seen in nutrient utilisation do not always reflect effects observed in growth performance.

4. Effects of phytase alone or in combination with xylanase on broiler performance, bone ash, caecal volatile fatty acids, and pH at different parts of the GIT

4.1 Introduction

Certain types of cereal grains are more likely to induce greater digesta viscosity, which will reduce the nutrient diffusion and absorption in the gut and compromise birds performance (Bedford, 1996). This is especially the case for diets containing high levels of NSP's which are not degraded by the animal and increase digesta viscosity (Hayat *et al.*, 2005). Moreover, the effect of digesta viscosity does not only limit the nutrient digestion and absorption. It can also increase fermentation in the small intestine of broilers due to a decrease in oxygen tension (Choct & Hughes, 1996) which will provide a relative stable environment for fermentative microflora to become established (Wagner & Thomas, 1978). Some studies suggest that volatile fatty acids (VFA), mainly acetate, propionate and butyrate, play an important role in the development of the microflora of the caeca in broiler chicks during growth (Nisbet *et al.*, 1996; van der Wielen *et al.*, 2000) and (Lee & Gemmell (1972) reported that increasing concentrations of butyrate in the intestine of mice was related with decreasing numbers of *Enterobacteriaceae.*

The anti-nutritive effects that are associated with the presence of NSP in feedstuff of plant origin can be alleviated with the supplementation of poultry diets with exogenous NSP degrading enzymes - such as xylanases (Woyengo & Nyachoti, 2011). Xylanases can improve digestibility of nutrients in broilers by lowering the viscosity of intestinal contents which will lead to greater apparent metabolisable energy (AME) of wheat-based diets (Gehring et al., 2013). Apart from being used as an effective mitigator of the viscosity of intestinal contents they also have other benefits to the animal since, by breaking down hemicellulose (component of plant cell walls), they can release any nutrients that might be encapsulated in these cells. This characteristic of these enzymes is especially important in corn based diets where there is no significant amount of soluble NSP and these diets are not associated with adverse digesta viscosity (Gehring *et al.*, 2013).

Phytic acid is an organic complex that occurs in cereal grains and oil seeds, and which primary physiological role is to store nutrients, mainly P (phytate-P). Apart from storing P,

this molecule also has the ability to chelate cations and form insoluble mineral-phytate complexes (Ravindran *et al.*, 1999b). Phytases are normally added to poultry feeds to help the animals break down the phytic acid and release the P and any other nutrients (such as starch) bound to the phytic acid molecule. The animal is then able to absorb the P and any other "trapped" minerals such as Ca, Cu, and Zn (Ravindran *et al.*, 1999b).

The portion of phytate-P being released by phytase has been shown to be dependent on the amount of phytase added to the feed (Ravindran *et al.*, 1999b; Olukosi *et al.*, 2007b) but there seems to be a considerable amount of phytate-P that remains undigested even when high amounts of phytase are used. This can be explained by the fact that phytate might be "locked" away in intact cells (Karimi, 2013). This encapsulating effect of cell walls can be reduced or even eliminated with the supplementation of NSP degrading enzymes, which may increase the efficacy of phytase by eliminating the phytate chelating effects of NSP (Kim *et al.*, 2005). Non-starch polysaccharides have the capacity to bind multivalent cations which are associated with phytate in both feedstuffs and in digesta (Slominski, 2011). Since the presence of NSP and phytate can reduce the efficiency of nutrient utilisation, supplementation of poultry diets with phytase and xylanase may alleviate the anti-nutritional effects that are associated with phytic acid and NSP, respectively (Woyengo & Nyachoti, 2011). The synergistic effect of phytase and xylanase on broiler performance might also be due to increased retention time in the gizzard allowing more complete digestion as pointed out by Singh *et al.* (2013). This is supported by results of Zeller et al. (2013) who reported a tendency towards an improved inositol phosphate reduction in wheat based diets supplemented with phytase and xylanase compared to phytase added diets only.

The objective of the present study is to determine the influence of xylanase on phytase activity and the effects of phytase alone or in combination with xylanase on broiler performance, bone ash, VFA and pH in different parts of the gut. The influence of dEB on performance as well as the ability of phytase to reverse its possible negative effects are already addressed in the first study, hence in the current study, there was only marginal differences in dEB and hence the main focus was on the use of phytase alone or in combination with xylanase.

4.1.1 Objective

To investigate the effects of phytase, alone or in combination with xylanase, on broiler growth performance, bone ash, caeca VFA and digesta pH along the GIT.

4.1.2 Hypothesis

The addition of xylanase will improve the efficacy of phytase in promoting growth performance, bone ash and VFA production. The impact of marginal dietary differences in dEB on growth performance will be reduced by high phytase dosing.

4.2 Materials and Methods

4.2.1 Experiment Design

A total of 336 day-old male Ross 308 broilers were used in this study. On arrival, all birds were allocated to 7 treatments in a randomised complete block design (diets were randomly allocated within blocks) with a 3x2 + 1 factorial arrangement, using initial body weight as the blocking criterion to ensure that the treatments had the same average body weight at day 0. A positive control diet (PC) was formulated to meet the Ross 308 guidelines for energy and nutrient requirements. In the factorial arrangement, the factors included 3 levels of enzyme (no enzyme, phytase alone or combination of phytase and xylanase) and 2 types of negative control (NC1 and NC2). Each treatment had 6 replicate metabolism pens and 8 birds per replicate pen. All the birds and feed were weighed on day 0 and 21 for measurement of growth performance responses. On day 21 all the birds were euthanised and the left tibia bones from two randomly selected birds per pen were used for determination of tibia bone ash and the pH of the gizzard, jejunum and caeca (left and right) were taken. Bird's caeca contents were collected into centrifuge tubes for subsequent analysis for volatile fatty acids.

4.2.2 Diets

Phytase alone or combined with xylanase was incorporated into corn-soybean meal based control diets. The diets were a positive control diet (PC) formulated to meet the Ross 308 guidelines for energy and nutrient requirements and the negative control (NC1) diet formulated to have 0.16% less Ca, 0.15% less available P and 0.3% less Na than PC (this reduction was expected to be counterbalanced by a phytase matrix inclusion of 500 FTU/Kg), NC2 had 0.22% less Ca, 0.19% less available P and 0.5% less Na than PC (this reduction was expected to be counterbalanced by a phytase matrix inclusion of 1000 FTU/Kg). In addition some AA were reduced in diet NC1 and NC2 to follow the matrix recommendations given for the phytase. Diets 4 and 5 were NC1 and NC2, respectively supplemented with phytase at the rate of 1500 FTU/kg; and diets 6 and 7 were NC1 and NC2, respectively supplemented with phytase at the rate indicated above, plus xylanase added at the rate of 16000 BXU/kg. The diet formulas for the control diets are presented in Table 4.1.

4.2.3 Husbandry

On the day of arrival (day 0), the birds were housed in metabolism pens where they remained until the end of the experiment, day 21. Water and experimental diets were provided *ad libitum* during the experimental period. The birds were housed in an environmentally controlled room. The temperature was maintained at 31°C on day 1 and then gradually reduced to 22°C by day 21. The relative humidity was kept below 70% during the 21-day trial.

Table 4.1 - Ingredient composition (g/kg) of the experimental diets

Description of diets	*Positive Control*	*Negative Control 1*	*Negative Control 2*
Ingredients	1	2	3
Corn	453	493	501
Wheat	50	50	50
Soybean meal	348	332	328
Methionine	3.2	2.9	2.7
Lysine	3.0	2.9	2.9
Threonine	1.2	0.9	0.8
Soybean oil	55.0	40.0	39.0
DCP(A)	21.5	13.1	10.5
Limestone (B)	9.5	11.0	11.3
Titanium dioxide premix (C)	25.0	25.0	25.0
Corn gluten meal	20.0	20.0	20.0
Phytase premix (D)	0.0	0.0	0.0
Xylanase premix (E)	0.0	0.0	0.0
Vitamin-mineral premix (F)	5.0	5.0	5.0
NaHCO3	2.5	1.7	1.0
NH4Cl	0.0	0.0	0.0
Salt NaCl	2.9	2.9	2.9
Total	1000	1000	1000
Calculated nutrients and energy			
Protein, g/kg	235	231	230
ME, kcal/kg	3050	3020	3031
Ca, g/kg	10.0	8.4	7.9
P, g/kg	6.9	5.4	5.0
Available P, g/kg	5.0	3.5	3.1
Na	2.0	1.7	1.5
K	8.5	8.3	8.3
Cl	2.0	2.0	2.0
Mg	1.4	1.4	1.4
S	1.5	1.4	1.4
Lysine	12.9	12.5	12.4
Threonine	8.5	8.1	7.9
Total sulphur amino acids	9.6	9.2	9.0
dEB, mEq/Kg	246.9	232.2	222.5

Positive Control - adequate in all nutrients, with recommended dEB of 250mEq/Kg; Negative Control 1 – had 0.16% less Ca, 0.15% less available P and 0.3% less Na than Positive Control; Negative Control 2 – had 0.22% less Ca, 0.19% less available P and 0.5% less Na than Positive Control;

A - 16% Ca, 21% P; B - 38% Ca; C - Prepared as 2.7Kg titanium dioxide (TiO_2) added to 10.8Kg corn gluten meal;

D - Phytase premix to be made to desired enzyme activity unit (1500FTU/Kg);

E - Xylanase premix to be made to desired enzyme activity unit (16000FTU/Kg);

F - Supplies the following per kg DIET: Vit. A, 5484 IU; Vit. D3, 2643 ICU; Vit E,11 IU; Menadione sodium bisulfite,4.38 mg; Riboflavin, 5.49 mg; d-pantothenic acid, 11 mg; Niacin, 44.1 mg; Choline chloride, 771 mg; Vit B12, 13.2 ug; Biotin, 55.2 ug; Thiamine mononitrate, 2.2 mg; Folic acid, 990 ug; Pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug. Also contains per g of premix: Vit. A, 1828 IU; Vit. D3, 881 ICU; Vit E,3.67 IU; Menadione sodium bisulfite,1.46 mg; Riboflavin, 1.83 mg; d-pantothenic acid, 3.67 mg; Niacin, 14.69 mg; Choline chloride, 257 mg; Vit B12, 4.4 ug; Biotin, 18.4 ug; Thiamine mononitrate, 735 ug; Folic acid, 330 ug; Pyridoxine hydrochloride, 1.1 mg; I, 370 ug; Mn, 22.02 mg; Cu, 1.48 mg; Fe, 14.69 mg; Zn, 14.69 mg; Se, 100 ug.

4.2.4 Sample collection

Samples of each of the 7 diets and of the enzyme premix were collected and stored in a refrigerator until chemically analysed. Birds were weighed per pen on day 0 and individual body weights in each pen were collected on day 21. Feed weight data were recorded on day 0 and day 21.

All the birds were euthanised on day 21 in two stages: in the first stage, 6 randomly selected birds were euthanised by cervical dislocation and the left tibia bones from two randomly selected birds (among the six euthanised) in each pen were collected, placed in a polythene bag, and stored in the freezer prior to bone ash determination.

Bones were processed as follows: all attached muscle, tendon and cartilage were removed and the bones were frozen. Consequently, each of the bones were cut into top 25% (epiphysis), middle 50% (diaphysis) and lower 25% (epiphysis). This was done to separate the faster (epiphysis) and slower (diaphysis) growing parts of the bone and to determine the bone ash in each of the bone regions.

The remaining two birds per pen were used for determination of digesta pH and VFA production. After euthanasia by overdose of sodium pentobarbital, the digestive tract was exteriorized, and the digesta pH was measured in situ at the gizzard, jejunum and caecum (the pH was measured for both the right and left caeca). The caeca was then removed and the contents (both caeca) were squeezed into centrifuge tubes and then stored frozen prior to analysis of VFA.

4.2.5 Chemical analysis

4.2.5.1 Dry matter

Dry matter content was determined by drying the samples in an oven (Uniterm, Russel-Lindsey Enginering Ltd., Birmingham, England, UK) at 80°C for 24 hours (Method 934.01; AOAC, 2006).

4.2.5.2 Bone ashing

Bone ash content was determined by placing the dried bone samples in a muffle furnace (Carbolite Furnace, Bamford, Sheffield, England, UK) for 24 hours at 500-600^{o}C (Method 934.01; AOAC, 2006). Samples were then weighed and analysed for ash content.

4.2.5.3 pH

Digesta pH of the gizzard, jejunum and caeca (both left and right caeca) were measured using a sterile glass pH electrode (HI 99163, HANNA Instruments, Romania).

4.2.5.4 Volatile fatty acids

The caeca pools of volatile fatty acids (VFAs) were measured following the procedure outlined by Choct *et al.* (1996).

4.2.6 Statistical analysis

All the data were analysed using the generalized linear model of GenStat (11th edition, VSN International, 2008). The model included block and diets to test for the effect of the diets on the response criteria. Factorial analysis was used on six diets to test for main effects (effect of enzyme inclusion and phytase matrix separately) and interactions. The reference diet (PC) was not included in this factorial arrangement. Because of the hierarchical order of factorial arrangement, only the main effects (i.e. of enzyme inclusion: no enzyme, XAP alone or XAP + phytase) or phytase matrix levels (500 or 1000 FTU/kg)) are discussed below when there were no interactions whereas simple effects (effects of individual treatments) are discussed when there were factor interactions. Tukey's test (Snedecor & Cochran, 1989) was used to separate significantly different means for the main effect of dEB whereas pre-planned pair-wise orthogonal contrasts were used to separate the means for the simple effects. Significance was set at $P \leq 0.05$.

4.3 Results

4.3.1 Growth performance

The data on the growth performance of broilers receiving the experimental diets are presented in Table 4.2. There were no significant matrix × enzyme interactions for any of the responses. Reducing the dietary levels of P and Ca in both NC1 and NC2 reduced ($P<0.05$) gain:feed compared with PC but had no significant effect on the other growth performance responses. Phytase supplementation had no effect on growth performance compared to diets with no enzyme supplementation but when xylanase was supplemented with phytase there was an increase ($P<0.01$) in gain:feed response and no significant effect on any of the other growth performance responses.

4.3.2 Tibia ash and weight

Table 4.3 shows the effect of the dietary treatments on the epiphyseal and diaphyseal tibia ash and weight. Matrix × enzyme interaction was significant ($P<0.05$) for epiphysis ash but not significant for epiphysis weight or any of the responses at the diaphysis. Epiphysis ash was lower ($P<0.01$) at NC1 compared to PC. Phytase supplementation increased ($P<0.01$) epiphysis ash for NC1 diets but had no effect on epiphysis ash for NC2 diets. Xylanase plus phytase supplementation increased ($P<0.01$) epiphysis ash in NC1 relative to the PC diet but had no effect on epiphysis ash for birds receiving NC2 diets. Phytase supplementation alone or combined with xylanase increased ($P<0.05$) epiphysis weight and diaphysis ash and diaphysis weight. Xylanase supplementation to diets did not show different responses than the ones obtained for phytase supplementation apart from diaphysis weight responses where an increase ($P<0.05$) in weight was observed.

Table 4.2 - Growth responses of broilers to dietary xylanase and phytase supplementation

Diets	Wt gain, g	FI, g	G:F g/Kg	Final Wt, g
Main effect means: Enzyme				
No enzyme	872	1379	633[a]	914
Phytase	916	1378	663[ab]	957
Phytase + Xylanase	928	1352	686[b]	969
SEM	28.1	38.1	9.11	28.0
P-values for main effect of enzyme	0.358	0.847	0.001	0.358
Main effect means: Phytase				
500 FTU/kg	928	1383	670	969
1000 FTU/kg	883	1357	651	924
SEM	22.9	31.1	7.44	22.9
P-values for matrix	0.179	0.556	0.09	0.179
P-values for matrix × enzyme interaction	0.640	0.546	0.988	0.639
Simple effects means*				
1. Positive control (PC)	928	1356	684	969
2. Negative control 1 (NC1) (no enzyme)	873	1358	643	914
3. NC2 (no enzyme)	872	1401	623	913
4. NC1 + Phytase (500 FTU/Kg)	947	1406	672	988
5. NC2 + Phytase (1000FTU/Kg)	885	1350	655	926
6. NC1 + Phytase (500FTU/Kg) +	963	1385	696	1004
7. NC2 + Phytase (1000FTU/Kg) +	892	1319	677	933
P-values for diet effect	0.495	0.905	0.003	0.494
Pooled SEM	38.6	52.5	12.1	38.6
P-values for contrasts				
PC vs NC1	0.327	0.977	0.022	0.327
PC vs NC2	0.314	0.550	0.001	0.315
2 vs. 4 (phytase)	0.189	0.520	0.106	0.189
3 vs 5 (phytase)	0.810	0.503	0.068	0.810
2 vs. 6 (phytase + xylanase)	0.110	0.721	0.005	0.110
3 vs 7 (phytase + xylanase)	0.714	0.280	0.004	0.715

*Note – data represents average of 6 replicate pens per treatment
WG – weight gain; FI – Feed intake; G:F - Gain:Feed; Final Wt – final body weight;
PC – positive control; NC – negative control

Table 4.3 – Effect of dietary xylanase and phytase supplementation on broilers tibia bone (epiphysis and diaphysis) responses

Diets	Epiphysis		Diaphysis	
	Ash, %	Wt, g	Ash, %	Wt, g
Main effect means: Enzyme				
No enzyme	42.23[a]	2.27[a]	52.09[a]	1.52[a]
Phytase	44.79[b]	2.54[b]	56.18[b]	1.59[a]
Phytase + Xylanase	44.74[b]	2.58[b]	56.17[b]	1.86
SEM	0.43	0.06	0.45	0.07
P-values for main effect of enzyme	<.001	0.002	<.001	0.005
Main effect means: Phytase				
500 FTU/Kg	43.58	2.48	55.02	1.68
1000 FTU/Kg	44.26	2.45	54.61	1.64
SEM	0.35	0.05	0.37	0.06
P-values for matrix	0.182	0.676	0.443	0.622
P-values for matrix × enzyme interaction	0.017	0.703	0.279	0.799
Simple effects means*				
1. Positive control	45.14	2.58	56.37	1.75
2. Negative control 1 (NC1) (no enzyme)	40.85	2.32	51.72	1.55
3. NC2 (no enzyme)	43.60	2.21	52.46	1.49
4. NC1 + Phytase (500 FTU/Kg)	44.75	2.55	56.52	1.57
5. NC2 + Phytase (1000FTU/Kg)	44.84	2.54	55.84	1.61
6. NC1 + Phytase (500FTU/Kg) + Xylanase	45.15	2.56	56.81	1.91
7. NC2 + Phytase (1000FTU/Kg) + Xylanase	44.32	2.59	55.53	1.81
P-values for diet effect	<.001	0.011	<.001	0.031
Pooled SEM	0.59	0.08	0.65	0.09
P-values for contrasts				
PC vs NC1	<.001	0.030	<.001	0.141
PC vs NC2	0.073	0.003	<.001	0.057
2 vs. 4 (phytase)	<.001	0.053	<.001	0.880
3 vs 5 (phytase)	0.147	0.008	<.001	0.385
2 vs. 6 (phytase + xylanase)	<.001	0.047	<.001	0.012
3 vs 7 (phytase + xylanase)	0.393	0.002	0.002	0.021

*Note – data represents average of 6 replicate pens per treatment
Wt – weight; PC – positive control; NC – negative control

4.3.3 Digesta pH along the digestive tract

The data on digesta pH in different sections of digestive tract of broilers receiving the experimental diets are presented in Table 4.4. There were no significant matrix × enzyme interactions for any of the pH measurements at any part of the gut. Phytase supplementation had no effect on pH measurements at any part of the gut but when phytase was supplemented with xylanase there was a decrease ($P<0.05$) in caeca pH (L and R caeca respectively).

4.3.4 Caeca VFA

Data on caeca volatile fatty acids production by broilers receiving the experimental diets are presented in Table 4.5. There were no significant matrix × enzyme interactions for any of the VFA. Ethanol production was lower ($P<0.05$) in NC1 compared to PC. There was a tendency ($P<0.10$) observed for the effect of enzyme supplementation where phytase supplementation tended ($P<0.10$) to increased ethanol production in NC1 diets but not NC2 diets. Combination of phytase and xylanase had no effect on VFA production in the caeca.

Table 4.4 - Effect of dietary xylanase and phytase supplementation in broilers GIT pH

Diets	Gizzard	Jejunum	Caeca L	Caeca R
Main effect means: Enzyme				
No enzyme	1.9	5.9	6.7[b]	6.6[b]
Phytase	2.1	5.9	6.6[b]	6.5[b]
Phytase + Xylanase	2.0	6.0	6.3[a]	6.3[a]
SEM	0.11	0.06	0.05	0.05
P-values for main effect of enzyme	0.645	0.59	0.001	0.001
Main effect means: Phytase				
500 FTU/Kg	2.2	6.0	6.6	6.5
1000 FTU/Kg	1.8	5.9	6.5	6.5
SEM	0.09	0.05	0.04	0.04
P-values for matrix	0.007	0.552	0.108	0.149
P-values for matrix × enzyme interaction	0.870	0.427	0.856	0.775
Simple effects means*				
1. Positive control	1.8	6.2	6.7	6.8
2. Negative control 1 (NC1) (no enzyme)	2.1	5.9	6.7	6.7
3. NC2 (no enzyme)	1.7	6.0	6.6	6.6
4. NC1 + Phytase (500 FTU/Kg)	2.2	6.0	6.6	6.6
5. NC2 + Phytase (1000FTU/Kg)	2.0	6.0	6.5	6.5
6. NC1 + Phytase (500FTU/Kg) + Xylanase	2.2	6.1	6.4	6.4
7. NC2 + Phytase (1000FTU/Kg) + Xylanase	1.8	6.0	6.3	6.3
P-values for diet effect	0.108	0.491	0.005	<.001
Pooled SEM	0.157	0.102	0.080	0.067
P-values for contrasts				
PC vs NC1	0.145	0.069	0.942	0.195
PC vs NC2	0.659	0.125	0.483	0.096
2 vs. 4 (phytase)	0.767	0.610	0.382	0.329
3 vs 5 (phytase)	0.347	0.845	0.310	0.161
2 vs. 6 (phytase + xylanase)	0.594	0.199	0.019	0.015
3 vs 7 (phytase + xylanase)	0.596	0.895	0.003	0.001

*Note – data represents average of 6 replicate pens per treatment
Caeca L – left caeca; Caeca R – right caeca; PC – positive control; NC – negative control

Table 4.5 – Effect of dietary xylanase and phytase supplementation in broilers caeca VFA (mg/Kg) production

Diets	Ethanol	Acetic Acid	Propionic Acid	iso-Butyric Acid	n-Butyric Acid	iso-Valeric Acid	n-Valeric Acid
Main effect means: Enzyme							
No enzyme	74.2	3931	365	70.5	1213	69.3	107.6
Phytase	83.8	3646	333	62.3	1112	70.2	96
Phytase + Xylanase	46.9	3506	349	61.6	1007	67.4	96.8
SEM	10.54	652.6	50.7	10.12	208.6	10.45	15.81
P-values for main effect of enzyme	0.054	0.896	0.909	0.789	0.784	0.981	0.847
Main effect means: Phytase							
500 FTU/Kg	68.8	3476	336	60.8	1061	62.2	91.5
1000 FTU/Kg	67.7	3912	362	68.8	1160	75.8	108.8
SEM	8.61	532.9	41.4	8.26	170.3	8.53	12.91
P-values for matrix	0.928	0.568	0.67	0.503	0.684	0.268	0.353
P-values for matrix × enzyme interaction	0.130	0.496	0.432	0.924	0.397	0.778	0.746
Simple effects means*							
1. Positive control	113	3635	306	44.2	1268.0	52.3	83.8
2. Negative control 1 (NC1) (no enzyme)	63.2	3086	305	63.5	1001.0	56.8	91
3. NC2 (no enzyme)	85.2	4776	425	77.5	1426.0	81.8	124.2
4. NC1 + Phytase (500 FTU/Kg)	102.2	3850	368	61.0	1292.0	68.2	96.5
5. NC2 + Phytase (1000FTU/Kg)	65.3	3442	299	63.7	933.0	72.3	95.5
6. NC1 + Phytase (500FTU/Kg) + Xylanase	41.2	3493	336	58.0	891.0	61.5	87
7. NC2 + Phytase (1000FTU/Kg) + Xylanase	52.7	3519	361	65.2	1122.0	73.3	106.7
P-values for diet effect	0.029	0.875	0.821	0.780	0.783	0.781	0.843
Pooled SEM	15.9	846.0	66.1	13.6	278.3	14.2	20.7

*Note – data represents average of 6 replicate pens per treatment
Caeca L – left caeca; Caeca R – right caeca; PC – positive control; NC – negative control

4.4 Discussion

After addressing in the first study the influence of dEB in performance and the capacity of phytase to reverse dEB possible negative effects, the focus of this second study was to determine the effects of phytase alone or in combination with xylanase on broiler performance, tibia ash, caeca VFA and digesta pH at different parts of the gut. By reducing the impact of marginal dietary differences in dEB on growth performance by high phytase dosing we also intended to determine the influence of xylanase on phytase activity.

4.4.1 Growth performance

It is well documented that phytase is effective in releasing not just P from phytate, but also other nutrients such as minerals, which might limit growth under some circumstances (Ravindran *et al.*, 1999b; Selle *et al.*, 2009a). In the present study there was a negative effect of dietary reduction of P and Ca as shown in reduced gain:feed in the NC diets compared to PC diets, but there was no effect of phytase on any of the growth performance responses. Angel *et al.* (2002) noted that dietary levels of Ca and Ca:P ratios are important aspects to consider in dietary formulation due to their influence on phytase efficacy and that low Ca:P ratio should be used in broilers diets.

When xylanase was supplemented with phytase there was an increase in gain:feed but no effect on any of the other growth performance responses. In a study by Yang *et al.* (2008) weight gain and gain:feed ratio were improved from day 8 to 21 in broilers fed wheat based diets supplemented with xylanase. Similar results on FCR of broilers fed a wheat based diet were observed by Nian *et al.* (2011) but no effect on weight gain was observed in this study.

Luo *et al*. (2009) also observed no effect of wheat based diets with xylanase on broilers daily feed intake but an improvement in this response was observed for 1-21 day old and 1-42 day old broilers. The results on performance observed in the present study for phytase and xylanase supplementation can be explained by the fact that the breakdown of the gel-forming capacity of NSP by xylanases can improve nutrient digestibility and availability (Bedford & Classen, 1992). The use of cell wall degrading enzymes like xylanase could have exposed phytate to phytase and accelerate its hydrolysis (Bedford, 2000). In the current study combination of phytase and xylanase improved gain:feed above the control

diet and marginally improved gain:feed beyond the level observed for xylanase alone, helping to emphasise the beneficial effect of the use of a combination of the two enzymes.

4.4.2 Tibia mineralisation

Reduction in dietary P and Ca resulted in decreased bone ash, but supplementation of NC1 diets with phytase alone or in combination with xylanase reversed this effect. However, the same observations were not made for diets with higher reductions of Ca and P (NC2) which may indicate that the enzymes did not contain sufficient activity to reverse the negative effect of severe reduction of dietary Ca and P. Powell *et al.* (2011) observed that diets with lower level of Ca (0.33% less than recommended by the NRC (1994) limited growth and bone ash responses to phytase supplementation and, more recently, Singh *et al.* (2013) observed that broilers performance was sustained when they were fed diets with adequate P but reduced Ca (7.5 g Ca/kg during 0–3 weeks and 6 g/kg during 3–6 weeks of age), and that phytase supplementation improved mineral utilisation and therefore tibia bone mineralisation. In Hussein (2006)'s study there was a significant decrease in tibia and toe ash for birds fed low-P (40% less available P) starter and finisher diets with phytase supplementation when compared to PC, but an increase in these responses was observed in the later study for birds fed a normal starter diet without phytase a finisher low-P diet with phytase supplementation.

The diaphysis ash responses to enzyme supplementation (phytase alone or in combination with xylanase) observed in the present study suggests that the increase in available Ca and inorganic-P from phytate hydrolysis by phytase improved bone mineralisation (Pourreza & Classen, 2001) at both levels of mineral reduction (NC1 and NC2), which supports the fact that phytase can support bone mineralisation at low–P diets (Sebastian *et al.*, 1996). Kornegay *et al.* (1996) observed an influence of dietary level of non phytate-P to phytase response and a decrease of released P per unit of phytase as the amount of phytase increases per unit of diet. The lack of response observed for epiphysis ash in broilers fed NC2 diets compared to PC and to phytase supplementation observed in the present study suggests that a reduction of 1.5g/Kg available P and 1.6g/Kg available Ca can sustain broiler bone ash at the period of 0-21 days and that phytase supplementation can facilitate tibia mineralisation.

The addition of xylanase to phytase supplemented diets in the present study appeared to be marginally effective in improving bone mineralisation in broilers compared to diets

supplemented with only phytase. Pourreza & Classen (2001) observed an interaction between phytase and xylanase on tibia ash responses but there was no significant responses to tibia ash for xylanase supplemented corn-soybean-wheat bran diets. Contrary to these results, Conte *et al.* (2003) observed that phytase supplemented to rice bran diets with reduced available P (40% of the recommended) increased ash in tibia bones but xylanase had no effect on tibia bone mineralisation. The results obtained in the present study and the range of results from the other studies suggest that phytase addition to P deficient diets is effective in promoting bone mineralisation but the effectiveness of phytase differs according to the content of non phytate-P (Karimi *et al.*, 2013).

4.4.3 Digesta pH along the digestive tract

The reduction of Ca and P levels used in the present study did not interfere with the pH at any part of the GIT and the same was observed when these diets were supplemented with phytase. Supplementation of xylanase and phytase lowered caeca pH. Radcliffe *et al.* (1998) observed an increase in piglet's stomach pH when they were fed a reduce Ca and P corn-soybean meal diet supplemented with phytase. It was suggested by Radcliffe *et al.* (1998) that this increase in pH could have been caused by an increase in dietary Ca from limestone to maintain the Ca:P ratio as phytase supplementation increased. An in vitro digestion assay performed by Walk *et al.* (2012a) suggested that the hydrolysis of phytate by phytase could change protein and ion concentration and increase the pH. Walk *et al.* (2012b) observed that the pH at the gizzard and ileum decreased when broilers were fed diets with 0.64% Ca, compared to broilers fed with dietary Ca at levels higher than recommended by the industry, and that diets supplemented with phytase at levels also higher (5000FTU/Kg) than the recommended by industry (500 to 1000FTU/Kg) increased pH at the gizzard, duodenum, jejunum and ileum. The results observed in the present study do not support these findings, likely because the phytase levels used in Walk *et al.* (2012b) study were higher than the ones used in the present study and therefore, the levels of phytase used in the present study were not high enough to allow for the effect of the reduced Ca in the diets. On the other hand, the observations in this experiment could also mean that although phytate solubility is higher at lower gut pH (Campbell & Bedford, 1992) phytase presence at different parts of the GIT does not always indicate hydrolysis of phytate (Selle & Ravindran, 2007).

Although, in the present study, there was a reduction of caeca pH for both NC1 and NC2 diets when phytase was supplemented along with xylanase which suggests that xylanase

could have released the encapsulated phytic acid from the cells and therefore expose it to phytase hydrolysis and, as a consequence of this, have increased the available Ca from phytate and alter the pH patterns in the GIT. There is also the possibility that the oligosaccharides released after xylanase degradation of NSP are fermented and this will lead to a decrease in pH (Mathlouthi *et al.*, 2002).

4.4.4 Caeca VFA

The treatments had no effect on the VFA in the caeca but there was a tendency to increase ethanol production with phytase in the NC1 diets. Data on caeca VFA production by broilers receiving diets supplemented with phytase is limited (Zaefarian F. *et al.*, 2013) but there is evidence that phytate interacts with lipids in corn (Cosgrove, 1966) and that hydrolysis by phytase may reduce the formation of metallic soaps in the gut (Ravindran *et al.*, 2000).

Supplementation of xylanase with phytase had no effect on VFA production, contrary to results found by Tricarico & Dawson (2005) where the use of xylanase alone affected *in vitro* VFA production in ruminants. Similarly, Choct *et al.* (1999) observed that broilers fed a low metabolisable energy wheat diet supplemented with xylanase had a higher concentration of VFA at caeca level compared to PC diets. Diebold *et al.* (2004) on the other hand, observed that xylanase supplemented to wheat based diets for weanling pigs tended to lower the total VFA concentration in the faeces. The differences in these results could be explained by the nature of the diets and with the concentrations of NSPs whose negative effect is related to NSPs ability to increase digesta viscosity and, as a consequence, a change in gut microflora and nutrient utilisation (Choct *et al.*, 1999). Wang *et al.* (2005) observed that broilers fed a wheat based diet supplemented with xylanase and ß-glucanase had no effect on VFA at ileum level for birds at 41 days old, but there was an increase of VFA production at the caeca that was probably linked to the increase in microbial count. Choct *et al.* (2004) on the other hand did not observe any effect on VFA production at the ileum or caeca when feeding broilers a wheat based diet, with normal ME and supplemented with xylanase, which suggests that, for enzyme supplemented diets, the decreased viscosity might have led to a decrease in VFA at ileal and caeca level (Wang *et al.*, 2005).

4.5 Conclusions

It is concluded from this study that dietary Ca and P reductions caused a decrease in tibia mineralisation and gain:feed which were counterbalanced by phytase plus xylanase supplementation, but no effect on the rest of the growth performance responses, digesta pH or VFA production were observed.

5. General Discussion

In the first experiment it was shown that phytase supplementation has a beneficial effect in broiler diets in regards to performance and nutrient digestibility, although phytase efficacy seemed to be significantly affected by dEB and Ca levels in this study. Phytase improves nutrient digestibility in monogastric animals, but scepticism and concerns about production losses due to insufficient P make producers add extra Ca and P to diets to ensure that broilers Ca and P dietary requirements are met. In this study, the increase in dietary Ca levels in NC diets seemed to have influenced phytase activity and reduced feed intake. Moreover, the level of available P reduction used, compared to PC, compromised broilers weight gain, gain:feed, final body weight and bone mineralisation, which, as expected, were counterbalanced by the addition of phytase. The observations from the present study for effects of phytase-supplemented diets on broiler performance and bone mineralisation show that the practice of providing excess nutrients in phytase-supplemented diets can be deleterious to the animal, causing mineral imbalance and, as a consequence, increase the possible negative impact of animal production on the environment by increasing nutrient excretion.

The range of dEB levels used in this study (214 to 266 mEq/Kg) had no influence on broiler performance or bone mineralisation, which is contrary to the observations that others have made in that increases or decreases in dEB levels influence broiler growth performance. The difference in observations suggest that, although the monovalent minerals accounted for in the dEB calculation represent the acid-base balance of the diet, there are also other contributing elements for this balance that should be taken in account. Patience & Wolynetz (1990) showed that the balance of fixed inorganic anions (Cl, P, S) and fixed cations (Na, K, Mg, Ca) can be used as an indicator of the acid- or base-forming properties of a diet and are also contributors to growth performance. The balance of these elements is called the dietary undetermined anion (dUA) and has to be carefully controlled to avoid acidity or alkalinity effects which can negatively affect animal growth performance (Patience & Wolynetz, 1990; McDonald *et al.*, 2011). Although dEB is much simpler to use and in many cases adequately describes the acid-base potential of a diet, dUA is suggested by some authors as a more precise measure of acid-base balance of diet.

Although no dEB effect or dEB × phytase interaction was observed for bone mineralisation and growth performance responses in this study, and phytase was effective in promoting growth and tibia mineralisation at all dEB levels used, it seemed that responses to phytase

was higher for bone mineralisation at dEB level of 234 mEq/Kg. The FCR responses were also improved at lower dEB levels suggesting that, as long as the dEB levels used are not compromising bone mineralisation and growth performance, phytase activity is more significant at lower dEB levels.

It was also found that phytase and dEB responses observed for growth performance are not necessarily a reflection of the results obtained for nutrient utilisation. High levels of Ca have been shown to compromise phytase efficacy in the GIT and the findings in this study showed that phytase was more effective at lower dietary Ca levels. This, as mentioned previously, can minimize the deleterious effect for broilers of a dietary nutrient excess and bring, as a consequence, economic savings for producers. The decrease in dietary Ca can also have an effect on gut pH since there will be less acid secretion into the gut, making room for phytase to act in a less fluctuating gut pH, and therefore perform more optimally, and for a prolonged period of time, extending the time available for phytate-hydrolysis. There is also less chance for phytate-Ca complexes to form and jeopardize phytase efficacy.

Regarding the effectiveness of enzyme, it was observed that phytase, supplemented alone or in combination with xylanase, improved tibia mineralisation and gain:feed, which indicates a more optimal hydrolysis of phytate and utilisation of other dietary constituents. However, there were no effects on the rest of the responses analysed in this study. It has been suggested that non-phytate P can reduce phytase efficacy in promoting tibia bone mineralisation but the levels of reduction used in this study seemed sufficient to sustain broilers bone mineralisation during the 21 day trial period. The marginal efficiency in bone mineralisation improvement observed when xylanase was supplemented with phytase, compared with phytase alone, suggests that the level of NSPs present in the soybean meal were not detrimental to the broiler performance and bone mineralisation which might explain the lack of enzyme interaction for the majority of these responses.

Overall, these studies demonstrated that diet costs and P excretion can be reduced when phytase-supplemented diets for broilers are formulated with dEB values in the range of 213 to 266 mEq/Kg, and dietary levels of non phytate-P and Ca are kept to a minimum as long as neither of these values are compromising broilers performance or animal skeletal integrity. To minimise costs to producers there might also be the need to find a Na matrix for phytase supplemented diets, as well as a more detailed information on the digestibility of P along the GIT for various feed ingredients. Consequently, phytase supplemented diets

can be formulated more accurately in order to reduce the diet costs and P excretion. Lowering dietary nutrients will contribute to a reduction in dietary costs, and the inclusion of phytase and xylanse in broilers diets will lead to a better utilisation of phytate-P and other dietary constituents, resulting in broilers that are healthier and more productive.

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