The comparative efficacy of novel phytase enzymes in young broiler diets through the use of different sampling and statistical methods

by

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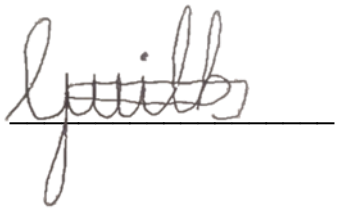
Declaration

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



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In loving memory of Dave Wilks. This one is for you.

Abstract

Currently, traditional South African broiler diets comprise primarily of plant-based ingredients of which phytate (IP_6) is the primary storage form of phosphorus. Phytate is a 6-carbon chained ring that has a strong affinity for binding minerals such as phosphorus (P), resulting in these minerals being poorly digested and utilised by broilers and therefore is largely considered as an unavailable source of P. To overcome phytate's poor digestibility, the supplementation of inorganic phosphorus sources (iP) such as monodicalcium phosphate (MDCP) are commonly used to meet the broilers phosphorus requirements. However, this method is not without its drawbacks as current research suggests that global P reserves are estimated to be depleted in the next 50-100 year. Furthermore, the over supplementation of iP has been shown to contribute towards environmental pollution as the over supplementation of inorganic phosphate sources can lead to the loss of flora and fauna biodiversity through the eutrophication of water bodies such as lakes, rivers, and dams. Therefore, it is of interest to broiler producers and researchers alike to find sustainable methods of reducing the amount of MDCP being supplied to the bird while still meeting the bird's requirement for phosphorus (P). One such method is the supplementation of exogenous phytases, which cleave phosphate groups bound to phytate making the P found in plant-based ingredients more available to the bird and thus reducing the amount of iP required in the diet. Currently there is a plethora of phytase products on the market all stating to be better than the rest. Methods used to determine the efficacy of phytase products include making use of trials whereby different phytase products are dosed at the same concentrations and performance and bone mineralisation are compared. Up until now there exists no standard methodology for analysing and interpreting data obtained from bone ash studies with various sampling criterion such as tibial bone, metatarsal bone and even the foot of the bird being used. Further complicating the matter is the various statistical equations that can be used to plot bone mineralisation against phytase dose such as non-linear regression or piecewise regression. Although there may not be a single method that will give you the correct answer it is of interest to understand how these various sampling criterion and statistical equations can influence the final result and our final conclusion when determining the efficacy of phytases from various sources. Therefore, this study aimed to investigate the efficacy of three different phytase products using two different sampling methods as well as two different statistical methods to analyse bone mineralisation. The current study made use of three phytase products supplemented at five dose levels (500, 1000, 1800, 2500 and 3500 FTU/kg) in a 5x3 factorial design, in which they were compared against three positive controls each containing incremental levels of MDCP and limestone as well as against a negative

control using two different sampling methods (metatarsal ash and tibia ash). The results from this study suggest large differences in the *in-vivo* efficacy of newly developed phytases based on differences in the gradient and asymptote of the phytase dose response in bone ash, body weight, and feed conversion ratio. When comparing the two different sampling criteria (tibia bones and metatarsal bones) differences in the degree of pression were observed. With differences in the reported asymptote and gradient between phytase being observed between the sampling criteria. Lastly, based on R^2 values, the statistical method used to analyse bone ash had an influence on the reported bone ash results. The results obtained from this study show that there is a need for scientists and poultry organisations to create a standard protocol for determining phytase efficacy in young broilers as the sampling method as well as the statistical model used can influence the efficacy values of phytase products.

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List of Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
AID	Apparent ileal digestibility
AOAC	Association of Applied Chemists
aP	Available phosphorus
ATTD	Apparent total tract digestible
BWG	Body weight gain
BW	Body weight
Ca	Calcium
CO_2	Carbon dioxide
CP	Crude protein
CT	Calcitonin
DM	Dry matter
dP	Digestible phosphorus
EE	Ether extract
FCR	Feed conversion ratio
FTU	Phytase units
g	Grams
GIT	Gastrointestinal tract
iP	Inorganic phosphorus
IP6	Phytate
kg	Kilograms
MDCP	Mono dicalcium phosphate
N	Nitrogen
nPP	Non phytate phosphorus
NRC	National Research Council
P	Phosphorus
pH	Potential hydrogen
PTH	Parathyroid hormone
PP	Phytate bound phosphorus
rP	Retainable phosphorus

SBM	Soybean meal
STTD	Standardised total tract digestibility
tP	Total phosphorus

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CHAPTER 1: Introduction

1.1 Introduction

Phosphorus (P) is an essential micromineral that plays a major role in bone development, growth, and productivity (Adeola & Cowieson, 2011). At present P is the third most expensive component in a broiler diet only behind energy and protein (Boling *et al.*, 2000; Li *et al.*, 2015). Due to the important role P plays in maintaining skeletal integrity and growth performance, nutritionists tend to formulate with wide safety margins, which inadvertently leads to an oversupply of P (Waldroup, 1999; Selle & Ravindran, 2007). This over supplementation can have economic as well as environmental consequences. There is growing concern regarding the over supplementation of P in broilers, with certain countries imposing strict legislation to govern the amount of phosphorus from broiler excreta that can be applied as fertilizer (Environmental Protection Agency, 2003). Therefore, optimising phosphorus nutrition in poultry production is important not only for environmental reasons but also from a profitability, and animal welfare point of view.

The main sources of P in a broiler diet can be grouped into three categories mainly: plant feedstuffs, animal-based feedstuffs, and lastly, inorganic phosphorus sources (Payne, 2005). The main source of P in a broiler diet comes from inorganic sources such as monodicalcium phosphate (MDCP), or monocalcium phosphate (MCP). However, the supplementation of inorganic phosphates is not a sustainable solution, with current global P reserves estimated to be depleted in the next 50-100 years (Cordel *et al.*, 2009; Neset & Cordell, 2012; Shastak *et al.*, 2012b). The main reason for the supplementation of inorganic phosphate sources is due to how P is stored in plants. Phytate is the anionic form of phytic acid and is the primary storage form of P in all plants, with approximately 60-80% of P in plants being stored in the form of phytate (Angel, 2011; Walters *et al.*, 2019). Due to the low digestibility of P found in broiler diets because of phytate, the supplementation of inorganic P (iP) has become essential to ensuring that the bird's P requirements are met for optimum performance (Li *et al.*, 2016). Thus, any method that could unlock the phytate-bound phosphorus found in cereal grains would not only reduce the amount of iP being supplemented but would have the added benefit of reducing feed costs (Boling *et al.*, 2000).

One such method is by supplementing poultry diets with exogenous enzymes such as phytase. The most common microbial phytase used in broiler diets are derived either from fungi (*Aspergillus niger* and *Peniophora lycii*) or bacteria (*Escherichia coli*) (Selle & Ravindran, 2007). Phytases are

typically expressed according to phytase unit (FTU), with one FTU being the amount of phytase required to liberate 1 mmol of inorganic phosphate per minute from 0.0051 mol L^{-1} sodium phytate at a pH 5.5 and a temperature of 37^{o}C (AOAC, 2000). Current industry practice is to include anywhere between 500-1000 FTU/kg feed of phytase depending on the diet. Researchers agree that increasing the level of phytase can have a positive effect on the rate of PP hydrolysis by rapidly dephosphorylating the phytate molecule and thus reduce the anti-nutritional effects associated with phytate. Super dosing refers to the inclusion of phytase above and beyond normal inclusion rates, usually greater than 1000 FTU/kg. It has been suggested that at current industry levels, there exists the opportunity to further increase the amount of phytase being dosed to further improve the availability of phytate bound P in a maize-soyabean-meal diet (Shirley & Edwards, 2003; Pieniazek *et al.* 2017). However, work done by Menezes-Blackburn *et al.* (2015), has shown that not all phytases exhibit the same level of efficacy. Furthermore, the efficacy of different phytases has been shown to be somewhat inconsistent between studies (Selle & Ravindran, 2007). The reason for this inconsistency can be down to a wide range of factors that have been shown to influence the efficacy of phytase *in vivo* (Dersjant-Li *et al.*, 2015; Wealleans *et al.*, 2016). Another factor contributing to variation in reported efficacy values is down to the lack of standard methodology used to determine phytase efficacy (Rodehutscord, 2009). A commonly used response criterion to determine phytase efficacy is the use of tibia bone ash percent (Li *et al.*, 2015; Adeola & Walk, 2013). The reason for this being due to bone being highly sensitive to changes in dietary P availability (Nelson and Walker, 1964; Shastak *et al.*, 2012a). However, due to tibia sampling being labour intensive other sampling methods have also been developed such as the use of metatarsal ash (Yoshida & Hoshii, 1983). Various authors such as Yan *et al.* (2005) and Shastak (2012), have shown a strong correlation between tibia and metatarsal ash.

With all the above information in mind, this study was formulated to help clarify some of the important factors that can contribute the variation seen in reported phytase efficacy values by shedding light on key factors such as sampling and statistical methodology used to report phytase efficacy values.

1.2 Research aims

This study aimed to compare the effect of different statistical methods and sampling criterion on the efficacy of three phytase enzymes in young broiler diets on performance parameters (body weight (BW), body weight gain (BWG) and FCR) as well as bone mineralisation. To achieve this research, aim the following objectives were set.

1. To determine the Ca and P contribution of three different sources of phytase, relative to MDCP.
2. To evaluate if the asymptote of phytase response at which tibia ash is maximised (FTU/kg feed) is affected by the source of phytase.
3. To evaluate the effect of two different statistical methods on final bone mineralisation results.
4. To evaluate the correlation between the metatarsal and tibial sampling techniques and their effects on the reported ash percentage value.

1.3 Hypothesis

H0: Phytase inclusion level will have no significant dose-dependent effect on final body weight, body weight gain and feed conversion ratio.

H1: Phytase inclusion level will have a significant dose-dependent effect on final body weight, body weight gain and feed conversion ratio.

H0: Phytase inclusion level will have no significant dose-dependent effect on tibia ash and metatarsal ash.

H1: Phytase inclusion level will have a significant dose-dependent effect on tibia ash and metatarsal ash.

H0: The optimal dose of phytase at which the asymptote of the response is achieved will be the same for the different phytase sources.

H1: The optimal dose of phytase at which the asymptote of the response is achieved will be different for the different phytase sources.

H0: There is a strong correlation between metatarsal and tibia ash.

H1: There is no correlation between metatarsal and tibia ash.

CHAPTER 2 Literature review

2.1 Introduction

According to Dierick *et al.* (2019), over 90 million metric tonnes of broiler meat is produced every year, with that value forecast to rise by 24% in the next decade. In South Africa alone approximately 983 million broilers were produced for slaughter in 2018 (SAPA, 2018). Due to the intensive nature and high concentration of poultry operations, there is considerable concern surrounding the impact these intensive production systems have on the environment with regards to phosphorus (P) and nitrogen (N) excretion. This concern is not unfounded. Although broilers are highly efficient in converting feed to food products, they still excrete as much as 45% of the total phosphorus that they consume (Plumstead, 2007). The increased demand for poultry meat and other poultry-related products has left the industry in a predicament as what to do with all the excreta being produced. Traditional methods of disposing of large amounts of excreta and litter include the application of broiler excreta and litter as fertilizer for crops (Plumstead, 2007). However, research is starting to show this is not a sustainable solution, as poor management and the continuous application of broiler excreta and litter have led to high levels of P being found in the soil, which has had a substantial effect on the amount of P contained in surface runoff (Pautler and Sims, 2000). This is thought to be further compounded as increased demand for poultry products far outweighs the land's capacity to accommodate the increase in excreta production (Vadas *et al.*, 2004; Foy *et al.*, 2014). Increased P concentrations found in surface runoff have been shown to result in the acceleration of eutrophication, which can lead to the reduction in biological biodiversity of flora and fauna found in surrounding water bodies (Sharpley and Moyer, 2000). This potential loss of biodiversity has resulted in increased pressure from the public, causing broiler producers and various stakeholders to act accordingly. A consequence of the increased pressure from the public has led to governments in certain countries imposing strict legislation to govern the amount of phosphorus from broiler excreta that can be applied as fertilizer (Environmental Protection Agency, 2003).

Optimising phosphorus nutrition in poultry production is becoming of increasing importance not only for environmental reasons but also from a profitability, and animal welfare point of view. Phosphorus is the third most expensive component in a broiler diet only behind energy and protein (Boling *et al.,* 2000 and Li *et al.,* 2015). Furthermore, P is an essential micromineral that plays a major role in bone development, growth, and productivity (Adeola & Cowieson, 2011). To reduce the amount of P being exposed to the environment and maintain adequate skeletal development, one

must first look at the composition of the excreta itself, and only then can possible methods be established to reduce the quantity of minerals being exposed to the environment. The question that should be asked is: “Why is the amount of P found in the excreta higher compared to other nutrients commonly found in poultry excreta?”. One reason is that the P found in excreta consists mainly of undigested portions of phytate-bound P and none-phytate phosphorus (nPP) from plant sources, undigested portions of P from animal by-products and mineral supplements, and lastly, surplus amounts of bioavailable inorganic P more than what the animal requires (Waldroup,1999). Broiler diets comprise primarily of plant-based ingredients of which phytate (IP_6) is the primary storage form of phosphorus. Phytate and phytate-bound phosphorus are present in all poultry diets with a traditional maize-soyabean-meal diet containing 8-9 g of phytate per kg of feed (Selle & Ravindran, 2007). According to Ravindran *et al.* (1995), phytin accounts for approximately 50-80% of the P found in cereal grain feedstuffs, which is inherently poorly digested and utilised by broilers and therefore is largely considered as an unavailable source of P (Plumstead *et al.,* 2008; Dersjant-Li & Kwakernaak, 2019). Phytate is poorly used by monogastric animals, such as broilers, with Nelson *et al.* (1968) reporting P availability feedstuff values of plant origin in the range of 30 to 40%. Both Ravindran *et al.* (1995) and Cowieson *et al.* (2011) reported phytate P-hydrolysis by poultry to be highly variable in the absence of supplemented exogenous phytase with both authors reporting ranges of 0-50% and 20-30%, respectively. These low levels of digestible phytate-P are a consequence of the broiler's inability to produce sufficient levels of endogenous phytase in their gastrointestinal mucosa (Maenz and Classen, 1998; Dersjant-Li *et al.,* 2015; Humer *et al.,* 2014). Work done by Olukosi *et al.* (2007) showed that young chicks have an issue retaining Ca and iP when fed a maize-soy diet, due to their lower production of endogenous phytase. However, according to Morgan *et al.* (2015), this amount of endogenous phytase increases as the bird ages. Apart from reduced P utilisation, work done by Cowieson *et al.* (2004) showed that phytate can increase the amount of total endogenous amino acids being excreted, showing that phytate readily binds to proteins making them less susceptible to digestion. Due to the low digestibility of P found in broiler diets because of phytate, the supplementation of inorganic P (iP) has become essential to ensuring that the bird's P requirements are met for optimum performance (Li *et al.,* 2016). The continuous supplementation of iP should not be viewed as a long-term sustainable solution as most, if not all, the iP used in broiler diets comes from phosphate rock which is a non-renewable source. Gunther (2005) estimates that 148 million tons of rock phosphate are used every year to supplement animal diets. At this rate, current global P reserves are estimated to be depleted in the next 50-100 years (Cordel *et al.,* 2009; Neset & Cordell, 2012; Shastak *et al.,* 2012b). This makes it imperative that the level of iP supplemented is monitored

carefully as a reduction in current supplemented iP levels could result in significant economic savings as well as having a positive effect on the environment (Scholey *et al.*, 2018)

Any method that could unlock the phytate-bound phosphorus found in cereal grains would not only reduce the amount of iP being supplemented but would have the added benefit of reducing feed costs (Boling *et al.*, 2000). One such method which is currently in use to improve the utilisation of phytate bound phosphorus is the supplementation of exogenous phytase enzymes. Dietary supplementation of exogenous phytase has shown to improve growth performance through the reduction of nutrient variability of feedstuff, and anti-nutritive effects of phytate, as well as improving the digestibility of P and Ca, all while reducing the amount of iP excretion being exposed to the environment (Oluyinka *et al.*, 2007; Manangi & Coon, 2008; Slominski, 2011; Sousa *et al.*, 2014; Dersjant-Li *et al.*, 2015). Research has also shown that phytase not only improves the digestibility and utilisation of phytate-bound phosphorus but also improves the digestion and utilisation of amino acids and other minerals through so-called "extra-phosphoric" effects (Dersjant-Li & Kwakernaak,. 2019). Although the benefits of phytase on bone mineralisation and performance in broilers is well documented. Current literature has only looked at the effects of phytase supplementation at current industry levels in the final diet. Therefore, the scope exists to formulate diets containing phytase inclusion levels above current industry standards to see if there are any additional effects. Formulating for a diet that contains a high inclusion rate of phytase could potentially not only reduce the amount of inorganic phosphorus being supplemented and subsequently reduce feed costs but may also reduce the antinutritive effects associated with phytate and thereby improve broiler performance through other "extra-phosphoric" effects.

2.2 Phosphorus terminology

Before one can fully understand and summarise the P requirements of a broiler, the terminology used must first be understood. Over the years, there have been several attempts by various authors to describe and measure the P requirements of a broiler, which has led to confusion and variation in reported results. As a result, this has led to various groups such as the Working Group No 2 (Nutrition) of the European Federation of Branches of WPSA being formed, with the aim of providing a set of standardised definitions, which can more accurately describing the available P in broilers.

Total phosphorus (tP) is generally referred to as P and encompasses all forms of phosphorus, irrespective of the binding form (Applegate & Angel, 2008; Angel, 2011).

Non-phytate phosphorus (nPP) composition and availability varies between feedstuffs and can be chemically determined by subtracting the analysed phytate bound P from the analysed tP (WPSA, 2013; Appelgate & Angel, 2014). An alternative explanation is that nPP refers to the P found in the feed that is not bound to phytic acid (Angel, 2011; Rodehutscord & WPSA, 2013). The term nPP has often been used predominantly in poultry nutrition as an expression of the phosphorus requirement of the bird and has been used interchangeably with the term "available phosphorus", which is incorrect (NRC, 1994; Plumstead, 2007; Angel, 2011).

Digestible P (dP) is the portion of total dietary P that is not recovered in the excreta (Li *et al.*, 2016). In poultry the faeces and urine are void together and often referred to as excreta. Therefore, the determination of dP requires colostomised birds to exclude the urine. This procedure can be avoided though as the P content of urine is negligible when P intake is below requirement (Rodehutscord & WPSA, 2013).

Retainable P (rP) refers to the portion of dietary total P that is retained in the body of the animal and can be determined quantitatively by measuring the difference between P intake and P found in excreta (faeces plus urine) (WPSA, 2013).

Available P (aP) is the part of dietary tP that can be utilised to cover the P requirement of the broiler when marginal levels of P are supplied. The term aP describes the potential of a diet or a raw material to meet the P requirement of the animal (i.e., feed P minus P in the distal ileum) (Angel, 2011; WPSA, 2013). This definition is somewhat different from the definition set out by the NRC (1994), which describes "available P" as being all phosphorus not bound to phytate i.e., nPP; this is one of the challenges facing nutritionists when it comes to determining broiler P requirements. A key difference between aP and nPP is that the term aP takes into account absorbed iP as well as organic P, which includes PP (phytate bound phosphorus), whereas the nPP doesn't take into account any PP, which may potentially be available to the bird, however, it does take into account any potentially unavailable iP (Angel, 2006).

2.3 Main sources of phosphorus

The main contributors of phosphorus in poultry diets can be divided into three main sources: plant feedstuffs, animal-based feedstuffs, and lastly, inorganic phosphorus sources (Payne, 2005). In a typical broiler diet, plant and animal-based feedstuffs can contribute as much as 52% and 13% of the P requirement, whereas inorganic phosphate can contribute as much as 35% (IFP, 2004).

2.3.1 Plant feedstuffs

Plant-based ingredients make up a large portion of poultry diets and include feedstuff such as cereal grains, oilseeds, and their respective by-products (Li *et al.*, 2016). Most of the P present in plant feedstuffs is in the form of phytate P (PP). The bioavailability of PP for broilers ranges from 0 to 80% and depends on various factors such as type of feedstuff, age of the bird, and metabolic absorption (Van der Klis & Versteegh, 1999). The bioavailability of tP and PP also varies according to the feed ingredient as seen in Table 2.1. The extent to which PP is utilised by the bird is dependent to a large degree on factors that influence the hydrolysis of PP in the gastrointestinal tract such as phytase activity (Li *et al.*, 2016).

Table 2.1 Summary of total phosphorus and phytate phosphorus found in common feed ingredients (Adapted from Viveros *et al.*, 2000)

	n	Total P (%)	Phytate P (%)	% Phytate P of total P
Cereals				
Maize	7	0.23 ± 0.01	0.18 ± 0.01	78 ± 0.01
Oats	9	0.29 ± 0.02	0.17 ± 0.03	59 ± 0.07
Rye	2	0.34 ± 0.03	0.20 ± 0.01	59 ± 0.02
Wheat	30	0.29 ± 0.03	0.23 ± 0.03	79 ± 0.07
Barley	21	0.31 ± 0.03	0.19 ± 0.02	61 ± 0.04
Legume seeds				
Soybean	4	0.73 ± 0.01	0.33 ± 0.01	45 ± 0.01
Oilseeds				
Canola oilcake meal	5	1.05 ± 0.01	0.76 ± 0.01	72 ± 0.01
Cereal by-products				
Wheat bran	6	1.16 ± 0.01	0.88 ± 0.01	76 ± 0.01
Rye bran	10	0.96 ± 0.01	0.73 ± 0.01	76 ± 0.01
Oat bran	3	0.83 ± 0.01	0.68 ± 0.01	82 ± 0.05

2.3.2 Animal feedstuffs

The main animal protein sources used in broiler diets include meat meal, meat and bone meal, fish meal, poultry by-product meal, blood meal, and feather meal. These feedstuffs can contain high levels of protein and higher levels of tP and rP, when compared to their plant-based feedstuff counterparts, as seen in Table 2.2. Their inclusion rate is limited in poultry diets mainly due to their relatively high cost as well as wide variation in reported nutrient values, which exist between batches due to the quality of raw material available and rendering process used (Li *et al.*, 2016).

Table 2.2 Summary of total phosphorus and phytate phosphorus found in common feed ingredients (Adapted from Van der Klis & Versteegh, 1999)

Animal Feedstuffs	Total P (g/kg)	Retainable P (% of TP)
Bone meal	76	59
Fish meal	22	74
Meat meal	29	65
Meat and bone meal	60	66

2.3.3 Inorganic phosphorus sources

A typical maize-soyabean-based broiler diet can contain anywhere between 2.5–4 g/kg of phytate phosphorous (Selle & Ravindran, 2007). Due to the poor digestibility of phytate-phosphorus found in plant feedstuffs, and the key role P plays in ensuring maximum growth performance, nutritionists aim to meet the birds' P requirements through the supplementation of inorganic P sources (iP) (Walters *et al.,* 2019). These iP sources are commonly derived from natural rock phosphates. These naturally occurring phosphates need to be chemically modified before they can be included in broiler feed as they are not readily utilised by broilers when they are in their natural form (Payne, 2005). The iP sources most used commercially include monocalcium phosphate (MCP), monodicalcium phosphate (MDCP), dicalcium phosphate (DCP), defluorinated phosphate (DFP), and monosodium phosphate, all of which differ in the total amount of P, as well as available P supplied, as can be seen in Table 2.3. Although the supplementation of iP in broiler diets is a common practice globally, it does have its drawbacks. One such drawback associated with the supplementation of iP is the cost, with P being the third most expensive component in a broiler diet only behind energy and protein (Boling *et al.,* 2000; Van der Klis & Fledderus, 2007). Secondly, the rock from which iP is extracted is non-renewable, with global reserves estimated to be depleted in the next 50-100 years (Cordel *et al.*, 2009). Lastly, iP sources differ in their mineral content and mineral availability, which can have a negative effect on feed formulation and subsequent bird performance if not accounted for. This variability in iP sources can be attributed to various factors such as chemical structure, particle size, pH, crystallinity, the production process, source of ingredients used, and lastly, the level of impurities present in the rock (Lamp *et al.,* 2020).

Table 2.3 Examples of inorganic feed phosphates and their respective total phosphorus and retainable phosphorus compositions (Adapted from Van der Klis & Versteegh, 1999)

Inorganic P source	Total P (g/kg)	Available P (g/kg)
Calcium sodium phosphate	180	106.2
Dicalcium phosphate (anhydrous)	197	108.4
Dicalcium phosphate (hydrous)	181	139.4
Monocalcium phosphate	226	189.8
Mono-dicalcium phosphate (hydrous)	213	168.3
Monosodium phosphate	224	206.1

2.4 Phosphorus and calcium requirements

2.4.1 The metabolic role of phosphorus and calcium

To formulate diets that contain the minimum requirement of Ca and P needed for optimum performance and bone mineralisation, nutritionists need to know what the bird's true requirements for these nutrients are, at different growth phases. To get a better understanding of the bird's true requirements the metabolic role of Ca and P needs to be understood. Phosphorus is an essential micromineral that plays a major role in bone development, growth, and productivity (Adeola & Cowieson, 2011). Due to advancements in genetics, management, and feed formulation, the broiler of today is different from the broiler from two decades ago. An example of this rapid progress is evident in the fact that in South Africa it now takes only 33-days to achieve a slaughter weight of 1,9 kg. Such rapid growth can only be sustained by adequate nutritional supply and in the case of proper bone development, Ca and P are essential and need to be supplied in sufficient quantities (Proszkowiec-weglarz & Angel, 2013).

It is impossible to discuss the metabolic role P plays in a broiler without making mention of the role of Ca. Plasma Ca and P concentrations are regulated within a narrow physiological range through feedback mechanisms consisting of the parathyroid hormone (PTH), active vitamin D3 [1,25-dihydroxyvitamin D3; 1,25(OH)2D3], calcitonin (CT), and their respective receptors, which are found in the small intestine, bone, and kidneys (Veum, 2010). The role Ca and P plays in bone health and mineralisation is beyond question, with 99% of the total body Ca and 80-85% of the total body P being stored in the skeleton, with the remaining 20% of P being contained in nucleotides, such as ATP, nucleic acids, phospholipids, and a multitude of other phosphorylated compounds needed for metabolism (Hurwitz *et al.*, 1987; Soares, 1995; Driver, 2004). The remaining Ca is found in the extracellular fluid, plasma, and within cells, and plays a pivotal role in metabolism, blood clotting

enzyme activation, and other essential metabolic functions (Veum, 2010). Phosphorus, in turn, plays an important role in maintaining osmotic and acid-base balance, amino acid metabolism, and protein synthesis (Li *et al.,* 2016). Due to their metabolic importance, deficiency symptoms of these minerals manifest rapidly if animal requirements are not met, resulting in nutritionists formulating with considerably wide safety margins, which often leads to an oversupply of Ca and P. This is further compounded by the fact that Ca is relatively cheap and readily available in "ready to feed" forms such as limestone (Bedford & Roussea, 2017). Although oversupplying Ca has no adverse effects on broiler performance directly, excess Ca has been shown to bind with phytic acid to form an insoluble complex, which has been shown to adversely affects phytate hydrolysis in the small intestine, as well as increase precipitation of insoluble Ca-P complexes in the digesta, which reduces P utilisation (Driver *et al.*, 2005; Toor *et al.*, 2005).

2.4.2 Phosphorus and calcium recommendations

Due to the growing pressure from environmental groups and governments to reduce the amount of P found in the excreta, care must be taken to ensure that P deficiencies do not arise (Fleming, 2008). Calcium and P deficiencies lead to increased skeletal abnormalities, such as rickets and tibial dyschondroplasia, which can lead to an increased number of mortalities and loss of income (Proszkowiec-Weglarz & Angel, 2013). An imbalance in these minerals can potentially result in an excessive amount of P excreted, leading to eutrophication and environmental pollution (Sharpley, 1999). Poultry diets used to be formulated according to the ratio of tCa to tP (Ca:P). As more research about the utilisation of P becomes available there has been a shift towards formulating according to tCa and aP to better reflect the utilisation of P by the bird, leading to commercial broiler companies changing their Ca and P recommendations over the years (Angel, 2011). For example, the recommendations put forward by Aviagen for the Ross 308 broiler show a reduction in Ca and aP of approximately 6.3% and 4.4%, respectively, from 2009 to 2014. Cobb-Vantress have reduced their Ca and aP requirements by 14.1% and 12.7%, respectively, from 2006 to 2012. Research has shown that these levels have still not been fully optimised (Necmettin *et al.*, 2020).

Due to the important role P plays in maintaining skeletal integrity and growth performance, nutritionists tend to formulate with wide safety margins, which inadvertently leads to an oversupply of P (Waldroup, 1999; Selle & Ravindran, 2007). Thus, according to Shastak (2012), it is important to try and formulate for an adequate supply of P that is not in excess of the bird's requirements. One reason as to why nutritionists are struggling to formulate to meet the bird's true requirements for P is

due to the numerous variables involved that can influence the bird's requirement, such as the amount of Ca and P required to sustain a broilers' physiological requirements, which varies with age, Ca source, and phytate content of the diet (Walk *et al.,* 2012). The difference in strain requirements is highlighted by just looking at the aP requirements set out by the genetic companies. For example, according to the Aviagen Ross broiler nutrition specifications (2019), they recommend 0.48% aP for 0-10 days, 0.435% aP for 11-24 days, and 0.405% aP for greater than 25 days to market. Cobb-vantress (2018), recommends a slightly lower amount with 0.45% aP for 0-8 days, 0.42% aP for 9-18 days, 0.38% aP for 19-28 days and 0.38% aP for 29 days till market. This is further complicated by the widespread supplementation of poultry diets with phytase, which has further reduced the need for the supplementation of diets with iP (Li *et al.*, 2017).

In the most recent NRC (1994), requirements for nPP range from 0.45 g/kg for broilers from 0-3 weeks during the starter period, with the requirement dropping to 0.35 g/kg for 3-6 weeks and 0.30 g/kg for 6-8 weeks during the finishing period. These values must be used with caution and should be considered outdated as most of the studies published from 1952 to 1983 made use of non-commercial strains of broilers, and without supplementing phytase in the diet (NRC, 1994). According to Leske & Coon (2002), the P requirements for broilers provided by the NRC (1994) are expressed in terms of tP and nPP, which does not consider the phytate P of the feed ingredients, which may be partially available to the bird. Recent research suggests that the NRC (1994) recommendations for nPP for broiler chickens exceed requirements and that the minimum P requirements of broilers are much lower (Li *et al.*, 2016). This is further illustrated by Li *et al.* (2017), who showed that aP levels of 3.5 g/kg from day 1-14 and 3.0 g/kg from day 15-45 were enough to meet the broilers requirements without the supplementation of phytase. Li *et al.* (2017) went on to show that birds given diets with supplemented levels of phytase (500 FTU/kg) showed improved growth performance at aP levels of 2.5 g/kg from day 1-14 and 2.0 g/kg of aP during day 15-21, when compared to birds fed diets without phytase. Summers (1997) suggested that dietary P levels can be reduced by up to 20% for most classes of poultry without any detrimental effects on performance. One reason for the wide range in reported Ca and P requirements according to Walk *et al.* (2012), is because these requirements vary with age, Ca source, and the phytate level in the diet being used. Work done by Angel *et al.* (2000a) demonstrated that by making use of Ross 308 broilers and an industry-accepted four-phase feeding program, the nPP requirements for maximum tibia ash and body weight gain (BWG) were 0.32% and 0.28%, respectively, from days 18-32 and that the nPP requirements decreased from 0.24% to 0.19% during the finisher phase (32-42 days). Ling *et al.* (2000) reported that the nPP requirements of modern broiler strains were lower than the NRC (1994)

recommendations and that values ranged from 0.32 – 0.26% in the grower phase, 0.26 – 0.18% in the finisher phase, and 0.19 – 0.14% during the withdrawal phase. Research done at the University of Maryland determined that the nPP requirements for the starter (1-18), grower (18-32), finisher (32-43), and withdrawal phase (42-49 days of age) to be 4.5, 3.1, 2.3 and 1.8 g/kg, respectively (Angel *et al.,* 2005). Angel *et al.* (2005) suggested the nPP recommendations of broilers supplemented with 600 FTU/kg to be 3.9, 2.5, 1.7, and 1.2 g/kg for the same age groups. Despite the abundance of research suggesting that nPP requirements are lower than current recommendations, nutritionists still tend to supplement nPP levels over and above the birds' requirements to create a "safety margin" during dietary formulations to accommodate the wide variation in aP content found in different feedstuffs (Knowlton *et al.,* 2004; Plumstead, 2007). Further reasons, as stated by Angel (2011), include the inconsistency in detail provided as well as differences in methodology and response criteria used between studies. Table 2.4 highlights how variation in sampling criteria and the age of bird can lead to differences in reported aP values. What is clear from looking at Table 2.4 is that there is no standardised method used and that the criteria used to determine aP values play a role in explaining the variation observed between different trials. When one considers all the different variables mentioned above, it is clear why it is not easy to accurately predict P requirements for the various phases of the bird's life cycle. This is further complicated by the widespread supplementation of poultry diets with phytase which has further reduced the need for inorganic P as a source of P (Li *et al.,* 2017).

Table 2.4 Summary of selected published available phosphorus requirements (g/kg) for broilers and various criteria. (Adapted from Li *et al.*, 2017)

Reference	Days-of-age		Criteria
	1-21	21-42	
Moran and Todd (1994)	4.5	4.0	Growth, bone ash and processing losses
Van Der Klis & Versteegh (1999)	3.7(d 0-10); 3.0 (d 10-30)	2.4 (d 30-40)	Carcass P analysis
Angel *et al.* (2000[ab])	3.2-2.8 (d 18-32)	2.4-1.9 (d 32-42)	Growth and tibia ash
Waldroup *et al.* (2000)	3.2-3.4		Weight gain
	1.7-1.8 or 2.2-2.7		Feed conversion ratio
Yan *et al.* (2001)		1.86	Weight gain
		1.63	Feed conversion ratio
		3.3	Tibia ash
Leske & Coon (2002)	3.9	3.0	Bone breaking strength
Persia & Saylor (2006)	3.2-3.5		Weight gain
	3.3-3.9		Feed conversion ratio
	3.5-3.9		Tibia ash
Yan & Waldroup (2006)	2.8-2.9		Weight gain

One possible way of minimising the variation surrounding broiler P requirements and, more accurately, determining the availability of P, is to formulate a standard protocol for the determination of available P based on current scientific knowledge. The Working Group 2, Nutrition, of the 94 European federations of branches of the World's Poultry Science Association has developed such a protocol, which is a commonly accepted system whereby they recommend making use of precaecal mineral digestibility to determine aP (WPSA, 2013). By implementing such a protocol across the broiler industry, much of the confusion, as stated by Angel (2011), surrounding P availability will be eliminated and it will allow for the comparison of data from various laboratories. This will have the added benefit of allowing nutritionists to formulate comprehensive and up-to-date feeding tables which will be able to better meet broiler requirements and subsequently reduce the environmental load associated with intensive broiler production (Rodehutscord, 2013).

2.5 Factors influencing phosphorus digestibility

Various factors can influence PP utilisation. These factors can be grouped into two categories: dietary-related, and bird-related. Some of the major dietary-related factors that influencing PP hydrolysis in the gastrointestinal tract include the level of Ca, nPP, tP, and vitamin D, as well as feed processing and feed or ingredient particle size (Angel *et al.*, 2002). It is a common misconception that broilers lack the necessary enzymes to break down and utilise phytate-bound phosphorus. Van

der Klis & Versteegh (1996) estimated that in 3-week-old broilers PP hydrolysis ranges from 2% to 80% in an assortment of feedstuffs, thus highlighting the fact that poor phytate digestion is not a result of a lack of endogenous enzymes but rather a result of the substrate being poorly soluble in the small intestine. Cowieson *et al.* (2011) stated that, in the absence of exogenous phytase, phytate-phosphorus digestibility is in the region of 20-30%, and this response is strongly linked with dietary calcium concentrations, as an increase in dietary Ca concentration results in a decrease in PP digestibility. These values are lower than the values obtained in studies done by Leytem *et al.* (2008), Zeller *et al.* (2015), and Sommerfeld *et al.* (2018), all of which showed the percentage of phytate hydrolysis to be in the range of 56-89% in the digestive tract of broilers fed diets low in P and Ca with no supplemented exogenous phytase. Tamin *et al.* (2004) showed that in the absence of Ca or exogenous phytase enzyme, 69.2% of dietary phytate P was hydrolysed by the terminal ileum.

2.5.1 Phytate

When discussing dietary-related factors that influence the digestibility of P and Ca, three culprits are worth mentioning and they are the compounds phytate, phytin, and phytic acid (Selle & Ravindran, 2007). These three terms are often used interchangeably when discussing P and Ca absorption, however, upon closer inspection each compound is slightly different and deserve to be discussed in their merit. Another term that is often used is phytate-bound-phosphorus (*PP*). Phytate-bound-phosphorus refers to all the P contained in the raw feed material as phytic acid (WPSA, 2013). The majority of the tP present in broiler diets is present as PP, with a standard maize-soyabean-meal diet containing anywhere from 2.5 to 4.0 g kg^{-1} PP (Ravindran *et al.*, 1995; Selle & Ravindran 2007). Raboy *et al.* (2000) and Oltmans *et al.* (2005) estimate that 65-80% of the tP in maize and ~70% in soy occurs in the bound form phytate. Phytate (myo-inositol-1,2,3,4,5,6-hexakisdihydrogenphosphate, $C_6H_{18}P_6O_{24}$) is a naturally occurring polyanionic molecule and is synthesised from myo-inositol via a series of phosphorylation steps, resulting in an inositol ring with six phosphate ester bonds (IUPAC-IUB, 1989; Humer *et al.*, 2014) (Figure 2.1).

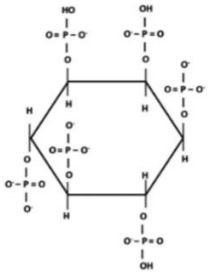


Figure 2.1 Structure of phytic acid at neutral pH, based on the Anderson Model (Adapted from Humer *et al.,* 2014)

Phytate is the anionic form of phytic acid and is the primary storage form of P in all plants, with approximately 60-80% of P in plants being stored in the form of phytate (Angel, 2011; Walters *et al.*, 2019). Phytate is poorly used by monogastric animals such as broilers with Nelson *et al.* (1968) reporting P availability feedstuff values of plant origin in the range of 30 to 40%. Lastly, phytin is a term that is used to describe when phytic acid binds/chelates to form insoluble mineral complexes with potassium, magnesium, calcium, and other divalent minerals (Selle & Ravindran, 2007). Phytic acid is considered an anti-nutritive factor in broiler diets because it exhibits a negative charge under pH conditions encountered in the stomach and small intestine, making it a strong chelating agent (Reddy *et al.*, 1982). As a result, phytic acid can bind/chelate with multivalent cations such as calcium (Ca^{2+}), zinc (Zn^{2+}), iron (Fe^{2+}), magnesium (Mg^{2+}), manganese (Mn^{2+}), cobalt (Co^{2+}), and copper (Cu^{2+}) and trivalent minerals to form enzyme resistant phytate-metal complexes in the digestive tract (Tamim & Angel, 2003; Tamim *et al.*, 2004; Selle & Ravindran 2007; Dersjant-Li *et al.*, 2015). In poultry, phytate hydrolysis mainly takes place in the stomach (i.e., crop, proventriculus, and ventriculus) (Humer *et al.*, 2014), whereas the formation of phytic acid mineral complexes takes place mainly at higher pH levels found in the small intestine (Dersjant-Li *et al.*, 2015). Apart from the effect phytate has on Ca and P utilisation, phytate has also been shown to have detrimental effects on amino acid (AA) digestibility and subsequent protein and AA absorption (Ravindran *et al.*, 2000; Choct *et al.*, 2010; Walk *et al.*, 2012).

2.5.2 Phytase

Phytases (myoinositol hexakisphosphate phosphohydrolase) are a group of enzymes used to catalyse the cleavage of phosphate groups in a stepwise manner from phytate (Zeller *et al.*, 2015). As stated by Angel *et al.* (2002), there are four possible sources of phytase: 1) phytase from the feed ingredients, 2) supplementation of exogenous microbial or fungal phytases, 3) endogenous phytases secreted by the bird in its intestinal mucosa, and 4) phytase produced by the microflora in the lower gastrointestinal tract of the bird. The ability of phytase to increase total P digestibility in broilers has been well documented and reviewed (Shirley & Edwards, 2003; Angel *et al.*, 2005; Angel *et al.*, 2006; Selle & Ravindran 2007; Gautier *et al.*, 2017). The first commercially available phytase feed enzyme that was derived from *Aspergillus niger* was introduced in the year 1991 (Selle & Ravindran, 2007). However, phytase activity was first detected in rice bran almost a century ago (Suzuki *et al.*, 1907). The negative effects of phytate on both Ca and P availability, as well as the improved P utilisation by chicks fed maize-soyabean-meal diets containing microbial phytase, is reflected in

research done by Nelson (1967) and Nelson *et al.* (1968). Nowadays, microbial phytase enzymes are the most widely supplemented exogenous enzyme in feed for monogastric animals, making up approximately 60% of the global feed enzyme market (Adeola & Cowieson, 2011). Much like the rapid genetic progress seen in broilers over the years, there has been considerable development in phytase efficacy resulting in a diverse range of phytase products. Phytase belongs to a class of phosphatases, with their main objective being to break down the molecule phytate via dephosphorylation and in doing so increase the availability of phytate bound P (Haefner *et al.,* 2005; Adeola & Cowieson, 2011; Woyengo & Nyachoti, 2011; Desrjant-Li *et al.,* 2019). The International Union of Pure and Applied Chemistry/International Union of Biochemistry groups phytases into three different types: 3-phytases (EC 3.1.3.8), 4-/6-phytases (EC 3.1.3.26), and 5-phytases (EC 3.1.3.72). This classification is based on the initial site of where dephosphorylation begins on the inositol ring (Zeller *et al.,* 2015). Phytases can further be grouped according to their optimum working pH: acidic phytases have a pH optimum of 3.0-5.5 where alkaline phytases have a pH optimum of 7.0-8.0 (Yin *et al.,* 2007; Vijayaraghavan *et al.,* 2013).

The most common microbial phytase used in broiler diets come from either fungus (*Aspergillus niger* and *Peniophora lycii*) or bacteria (*Escherichia coli*) (Selle & Ravindran, 2007). Phytases are typically expressed according to phytase unit (FTU), with one FTU being the amount of phytase required to liberate 1 mmol of inorganic phosphate per minute from 0.0051 mol L^{-1} sodium phytate at a pH 5.5 and a temperature of 37^{o}C (AOAC, 2000). However, not all phytases exhibit the same level of efficacy. Research done by Menezes-Blackburn *et al.* (2015) showed that bacterial phytases are often resistant to pepsin digestion and have a lower pH spectrum of activity compared to fungal phytases, giving them the capacity to degrade phytate in the gizzard. These authors continue to illustrate via a series of *in vitro* assays that not only did different types of phytase differ in their pH optima but also varied in their efficacy in degrading IP6 in the different sections of the gut. These difference in enzymatic properties across various phytase products are illustrated in Table 2.5. According to Bedford *et al.* (2015), the only difference between phytases is the fact that some require higher levels of supplementation to achieve the same amount of nutrient release. Even though this statement might be correct, the factors that influence the efficacy of various phytase sources can still be examined. Dersjant-Li & Kwakernaak (2019) investigated this point under *in vivo* conditions whereby they compared a *Buttiauxella* sp. phytase expressed in *Trichoderma reesei* against an *E. coli* phytase expressed in *Pichia pastoris* at four various dose levels and found that although both phytases increased both BWG, P digestibility and bone mineralisation (tibia ash) in 21-day old broilers in a

curvilinear manner with increasing phytase doses; the *Buttauxella* phytase exhibited greater dose-equivalent effects when compared to those of the *E. coli* phytase.

Table 2.5 A breakdown of the enzymatic properties of various phytase products available on the market (Adapted from Menezes-Blackburn *et al.,* 2015)

Trademark	Donor organism	pH range (80% of the optimal activity)
Quantum®	*Escherichia coli*	4.0-5.0
Quantum Blue®	*Escherichia coli*	3.5-5.0
Phyzyme XP®	*Escherichia coli*	3.0-5.0
AxtraPHY®	*Buttiauxella* sp.	3.0
Ronozyme Hiphos®	*Citrobacter braakii*	3.4-4.5
Ronozyme NP®	*Peniophora lycii*	4.5-5.5
Natuphos®	*Aspergillus niger*	4.5-5.5

2.5.3 Mode of Action

Enzymes only work if they have substrate to act upon. In the case of phytase that substrate is the molecule phytate. Not all phytase products are alike in their efficacy to reduce nutrients from the diet as proven *in vitro* by Menezes-Blackburn *et al.* (2015). Studying enzyme's mode of action helps to explain these differences between the different enzymes. Phytase (myo-inositol hexakisphosphate phosphohydrolase) works by catalysing the stepwise removal of inorganic phosphate and *myo*-inositol from phytic acid (Wyss *et al.,* 1999; Yu *et al.,* 2012) (Figure 2.2). The removal of the phosphate group starts with a fully phosphorylated phytic acid (IP6), followed by penta- (IP-5), tetra-(IP-4), tri-(IP-3), di- and mono-esters of inositol in descending order of preference, liberating the once undigestible phosphate digestible (Adeola & Cowieson, 2011; Dersjant-Li *et al.,* 2014).

Dietary supplementation of exogenous phytase has been shown to reduce the anti-nutritive effects of phytate, increase the accuracy of feed formulation as well as reducing the amount of dietary nPP being supplemented, with the crop being the primary site of action for exogenous phytase (Manangi & Coon, 2008; Humer *et al.,* 2014; Sousa *et al.,* 2014). Under *in vivo* conditions, complete hydrolysis of IP_6 is not always the case and often results in a mixture of IP esters, most notably IP_4 and IP_3, even if phytase was supplemented (Leske & Coon, 1999; Shirley & Edwards, 2003). According to Powell *et al.* (2011), less than 35% of the phytate present in broiler diets is hydrolysed by phytase enzymes once ileal disappearance has been accounted for. Although several reviewed

papers (Selle & Ravindran, 2007; Dersjant-Li *et al.*, 2015) have been published illustrating the efficacy of phytase in releasing phytate-bound P, there are still several reasons as to why incomplete hydrolysis occurs. Yueming *et al.* (2014) best summarises these reasons into three broad categories namely, phytase related, dietary related, and animal-related, with the main factors affecting the efficacy of phytase being pH, diet composition (non-phytate phosphorus levels and dietary calcium levels), and phytase dosing level just to name a few (Kebreab *et al.*, 2012; Karimi *et al.*, 2013).

To ensure maximum phytase performance benefits in the animal, phytate needs to be hydrolysed as rapidly as possible and as completely as possible in the upper part of the digestive tract. The reason for rapid and complete hydrolysis is because lower phytate esters (IP-4,3,2) exhibit reduced capacity to chelate with Ca and other minerals when compared with IP-6 and IP-5 (Luttrell, 1993).

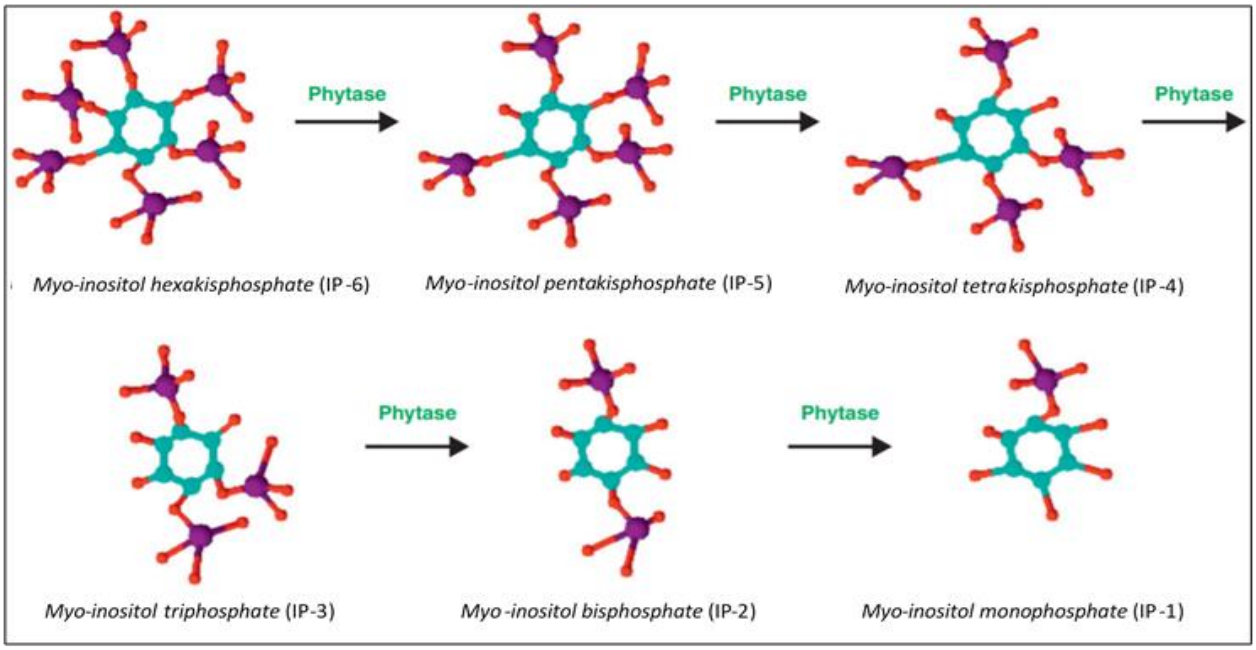


Figure 2.2 The enzymatic degradation of phytate (myo-inositol hexakisphosphate (IP-6)) to lower inositol phosphates (IP-5, IP-4, IP-3, IP-2, and IP-1) by the enzyme phytase. Different types of phytases start to cleave phosphate off at different positions (Adapted from Kebreab *et al.*, 2012)

2.6 Factors that influence phytase efficacy

According to Dersjant-Li *et al.* (2015) and Wealleans *et al.* (2016), a number of factors can play a role in influencing the efficacy of phytase products *in vivo*. These factors can be broadly grouped into three categories namely, phytase-related, animal-related, and dietary related factors. Potential phytase- and dietary related factors that have been shown to influence phytase efficacy include the concentration of phytate in the diet, the level of added phytase, the intrinsic properties of the phytase

enzyme used, source of phytase, feed particle size, and P sources used (Ravindran *et al.*, 2006; Amerah & Ravindran, 2009; Selle *et al.*, 2009; Wealleans *et al.*, 2016).

2.6.1 Effect of optimal pH range

The pH optimum of phytase activity determines the site of maximum phytase activity within the gastrointestinal tract of the animal, with most microbial phytase activity occurring in the crop, gizzard, and proventriculus (Plumstead, 2007; Simon & Igbassan, 2002). The hydrolyses of phytate bound minerals is only possible if the phytate mineral complex is soluble (Tamim *et al.*, 2004). Although commercial phytase activity is standardised at a pH of 5.5 the activity of various phytases at lower pH levels differs significantly (Menezes-Blackburn *et al.*, 2015). This has to do with the characteristics of the specific phytase as each product has its own unique set of characteristics, such as different pH optima (Dersjant-Li & Kwakernaak, 2019). The conventional view is that the formation of insoluble phytate-mineral complexes and its relative solubility in the gastrointestinal tract (GIT) is a function of the pH and molar ratio of minerals to phytate present, with the varying pH levels found in the gastrointestinal tract having a significant effect on IP6 degradation (Wise, 1983; Selle *et al.*, 2009). Ravindran (2013) reported average pH values for the crop, gizzard, and proventriculus in the range of 5.5 and 2.5-3.5, respectively. Work done by Selle *et al.*, (2000) showed that changes in pH alter the charge distribution of the phytate molecule. These authors stated that maximum insolubility occurs at a pH of between 4 and 7, which is in the pH range of the small intestine (pH of 5.5-6.6), and that most phytate-mineral complexes are soluble at lower pH levels that are associated with the crop (pH 5.5), gizzard and proventriculus (pH 2.5-3.5), as reported by Ravindran (2013).

Oberleas (1973) determined that the order of stability of metal-phytate complexes was found to be in the order of most stable (i.e., most insoluble) to least stable Cu > Zn > Co > Mn > Fe > Ca. This order is slightly different from the order proposed by Maenez (1999), where mineral potency as inhibitors of PP hydrolysis at a neutral pH of 7 is Zn^{2+} > Fe^{2+} > Fe^{3+}> Ca^{2+} > Mg^{2+}. Regardless of the rankings, Ca^{2+} has one of the lowest affinities for phytate, yet it has the greatest impact on PP availability.

2.6.2 Effect of calcium level

Traditional maize-soyabean-meal broiler diets can provide high amounts of protein and energy. Cereal grains and protein meals are low in various minerals, such as Ca, with cereals containing Ca concentrations in the range of 0.02 and 0.06%, and protein meals containing 0.25 to 0.70%. Broiler diets are, therefore, supplemented with various Ca sources such as limestone and oyster shells to meet the Ca requirements of the bird (Anwar & Ravindran, 2016). Due to its metabolic importance and low-cost, Ca is often oversupplied, with the primary source being from limestone. A typical commercial broiler starter-diet can contain 9-10 g/kg Ca (Walk, 2016; Dersjant-Li *et al.,* 2018). Although Ca is considered an essential nutrient and plays an important role in optimum broiler performance, up to a third of dietary Ca is inaccessible to the bird as it is bound to phytate in the digesta (Selle *et al.,* 2009). Furthermore, the level of dietary Ca affects the efficacy of phytase in broilers (Tamim *et al.,* 2004).

The apparent absorption of Ca and P takes place primarily in the small intestine, more specifically the duodenum and the jejunum (Veum, 2010). Not only can the dietary level of Ca influence the efficacy of supplemented exogenous phytase, but the Ca:P ratio in the diet has been reported to influence phytase activity (Angel *et al.,* 2002; Tamim *et al.,* 2004; Amerah *et al.,* 2014). Understanding the optimum dietary Ca:P ratio is of the utmost importance in ensuring optimum growth and bone mineralisation (Li *et al.,* 2016). Firstly, an increase in either P and/or Ca concentrations may harm the apparent digestion of these nutrients. Secondly, the correct ratio of Ca and P ensures that there is neither a deficiency nor over-supplementation of these minerals (Dersjant-Li *et al.,* 2018). Decreasing dietary Ca may improve P utilisation, whereas an increase in Ca relative to P can result in reduced P digestibility and impaired broiler performance due to the chelation of excess Ca with phytate to form insoluble mineral complexes (Létourneau–Montminy *et al.,* 2008; Plumstead *et al.,* 2008).

Originally the ratio of Ca:iP was defined as total Ca (tCa) and total P (tP) in the diet, however, over the years there has been a shift to Ca:aP, which better reflects the influence phytase has on releasing bound Pi found in plant sources (Majeed, 2008). The latest NRC requirements (1994) recommend a Ca: nPP of 2.22:1 in 1 to 21-day old birds and 2.57:1 in 22 to 42-day old birds. In the current broiler industries, many broiler companies such as Cobb-Vantress, Inc (2018) and Aviagen (2019) recommend a 2:1 Ca:P ratio for optimum broiler performance.

These recommendations have been challenged by Driver *et al.* (2005), which showed that the optimum Ca:aP for BWG, feed to gain ratio, and tibia ash percentage in broilers from age 1-16 were

1.1:1, 1.4:1, and 1.6:1, respectively. This contrasts with research done by Leske & Coon (2002), which showed the ideal ratio to be 2:1 and that maximum retention of dietary retainable P was achieved at a dietary inclusion level of 4.8 g/kg Ca and 2.4 g/kg retainable P. Lei & Stahl (2000) stated that the level of dietary P can harm the efficacy of phytase and subsequent P liberation from phytate and that phytase is more efficacious in diets containing low levels of supplemented iP. Two explanations have been put forward to explain how high levels of dietary P can harm phytase efficacy. The first explanation is that iP, which is the end-product of phytate hydrolysis, inhibits the catalytic activity of phytase. Secondly, the increased liberation of P, because of phytase, may lead to an imbalance of Ca and P in the gastrointestinal tract (Selle & Ravindran, 2007). Plumstead *et al.* (2008) reported that a ratio of Ca:nPP that achieved the highest P retention and lowest P excretion was 2.53:1, 2.40:1 and 2.34:1 for diets with 0.28%, 0.24% and 0.10% PP. Plumstead *et al.* (2008) also suggested that increased dietary Ca levels reduced the extent of phytate P hydrolysis and P digestibility, and that the optimum Ca:nPP ratio should be reduced when diets contain lower levels of phytate. A study by Dersjant-Li *et al.* (2018) suggested that the presence of phytase at a lower dietary Ca level maintained a better Ca:P balance and lead to improved P digestibility. This is in accordance with Appelgate *et al.* (2003), who investigated the effect of dietary supplemented Ca on intestinal phytase present in broilers fed two different levels of dietary Ca (0.90% vs 0.40% dietary Ca). What these authors found was that the 0.90% Ca diet reduced intestinal phytase activity by 9% and PP hydrolysis by 11.9% when compared to the 0.40% Ca diet.

An increase in Ca:P ratio is further supported by research done by Lei *et al.* (1994), Tamim *et al.* (2004), and Walk *et al.* (2012), which showed that high levels of dietary Ca reduced phytase efficacy, broiler performance, and increased the gastrointestinal pH. Amerah *et al.* (2014) demonstrated that broilers fed non-phytase supplemented diets containing constant levels of tP and nPP exhibited ileal phytate degradation in the range of 39.8 to 51.4% depending on the dietary Ca concentrations. Not only can the dietary level of Ca influence the efficacy of supplemented exogenous phytase in broiler diets and subsequent dP improvements, but the Ca:P ratio in the diet has been reported to influence phytase activity (Angel *et al.,* 2002; Tamim *et al.,* 2004; Amerah *et al.,* 2014). Tamim *et al.* (2004) illustrated that in the absence of phytase that the addition of 0.5% Ca from limestone reduced ileal P digestibility by 38.5% compared to a control diet without added limestone. Plumstead *et al.* (2008) demonstrated that increasing dietary Ca levels from 4.7 to 11.6 g/kg decreased ileal phytate-P digestibility by 71% in broilers. Research published by Li *et al.* (2016) reported a decrease in ileal IP-6 disappearance at a high dietary Ca level (10 g/kg) vs. a lower dietary Ca level (7 g/kg). Work done by Powell *et al.* (2011) in which three levels of diet Ca were used (0.67, 1.00,

and 1.33%) showed that increasing Ca supplementation without supplemented phytase decreased growth performance and bone characteristics. The supplementation of 500 FTU phytase increased average daily gain (ADG), average daily feed intake (ADFI), and bone response variables measured (bone weight, ash weight, and tibia ash percentage) at the higher levels of dietary Ca. The authors concluded that the higher dietary Ca levels had no negative effects on the efficacy of the phytase supplemented compared to the lower levels of dietary Ca. The reason for this is due to the relationship between Ca and P at the lower supplemented levels of dietary Ca, there was simply not enough Ca to enable the absorption and utilisation of the extra phosphorus available owing to the supplementation of phytase. This highlights the importance of ensuring the correct ratio of Ca to P in the diet.

Research done by Desjant-Li *et al.* (2018) demonstrated that there are significant performance and production benefits associated with reducing Ca levels in broiler diets in the presence of phytase. They were able to show during the finisher phases (day-22 to 42) that a high reduction level in dietary Ca significantly reduced FCR when compared to a low reduction level. This agrees with Selle *et al.* (2009), who reported that a lower dietary Ca level may reduce the incidence of insoluble Ca-phytate complexes from forming. Nelson *et al.* (1968) showed that in a typical maize-soybean-meal diet, 1% of the phytate in the diet can chelate 0.36% Ca, rendering the Ca unavailable to the bird. Thus, complete phytate hydrolysis through the supplementation of phytase may reduce the amount of supplemental dietary Ca found in broiler diets without harming broiler performance (Walk *et al.*, 2012). A better understanding of the optimum dietary levels of Ca is important due to its high inclusion rate in poultry diets and the negative impact it has on exogenous phytase efficacy (Angel *et al.*, 2002).

Besides the Ca concentration in the diet, it is well documented that the source of Ca, as well as the rate at which the Ca source is solubilised, may affect phytase efficacy and subsequent Ca and P digestibility. Work done by Walk *et al.* (2012) and Kim *et al.* (2019) has shown that the rate of solubility of the limestone used is strongly corelated to the particle size of limestone with a finer particle size resulting in a faster solubilising limestone. Kim *et al.* (2019) compared the *in vivo* digestibility of Ca and P in a maize-soybean-meal based diet using two limestones of the same source with differing particles sizes and subsequent rates of solubility in the presence and absence of phytase. The research showed that the more rapid solubilising limestone had a significant ($P<0.05$) detrimental effect on P digestibility compared to the slower solubilising limestone in the presence of phytase (1000 FTU/kg). The researchers go on to explain the potential mechanism by which a more rapidly solubilising limestone can have a detrimental effect on phytase efficacy and subsequent P digestibility. They state that the faster a limestone dissolves, the more Ca ions become available to

bind to phytate to form insoluble mineral-complexes. Consequently, these insoluble mineral-complexes form before the enzyme phytase gets a chance to cleave the phosphate groups from the phytate molecule. This leads to a reduction in phytase efficacy resulting in reduction in the amount of P available for digestion.

2.7 Methods used to determine phosphorus availability

There are various phytase products available on the market, with each product promoting different matrix values for nutrient and energy values. The overall aim is the same for all researchers and nutritionists alike, and that is to define the relationship between the enzyme dose and the amount of nutrients "spared". Not only is this useful for determining optimum inclusion rates, but also for determining the return on investment (Bedford *et al.,* 2015). Caution must be used, however, when comparing matrix values between various phytase products, as commercial phytases can differ in their efficacy within the gastrointestinal tract of the animal (Dersjant-Li *et al.,* 2019). This can be a result of the range of methods used to determine the matrix values, as well as other factors that can influence the efficacy of the enzyme, such as on-farm management practices, and environmental and dietary factors (Dersjant-Li *et al*. 2019; Bedford & Cowieson, 2020). A P matrix is designed to assign a contribution (improvement) value for a given unit of phytase added. This contribution can take the form of either aP based on an inorganic source usually MCP, or it can take the form of dP based on ileal absorbed P (Dersjant-Li *et al.,* 2019). The importance of accurate matrix values cannot be stressed enough, with overestimations leading to detrimental effects on not only performance but also having the potential to cause animal welfare issues, while conservative matrix values could lead to the benefits associated with phytase supplementation not being fully realised (Bedford *et al.,* 2015). In poultry production, the precise knowledge about the birds' P retention and its variation is of great importance for two reasons: firstly, precise knowledge about P retention aids in better establishing the bird's true P requirement, and secondly because the P availability of raw materials used in poultry feed is often evaluated based on P retention (Rodehutscord, 2009).

Various methods can be used to determine phosphorus availability. The methods used to evaluate the biological value of P can be grouped into three categories, each with their own strengths and weaknesses, namely,

1) Qualitative measurements of P availability, which include blood, bone, and growth assays, which require a standard curve to be generated.

2) Quantitative measurements of P availability which include balance trials to determine P digestibility, which do not require a standard curve.

3) *In vitro* or indirect tests which can be used to predict P availability such as Solubility tests or near-infrared reflectance spectroscopy (Payne, 2005; Shastak & Rodehutscord, 2013; Sanni, 2017).

2.7.1 Qualitative measurements

Indirect measurements are based on methods that determine the biological value of phosphorus sources. These measurements often require a smaller number of *in vivo* trials compared to direct measurements and usually involve feeding chicks varying amounts of test phosphates in a P deficient diet for 2-3 weeks (Coon *et al.*, 2007). Indirect methods are therefore quicker and less expensive than direct measurements. One limitation of indirect methods, according to Dersjant-Li *et al.* (2019), is that they may overestimate values, resulting in larger safety margins required. To obtain valid comparisons between phosphates based on qualitative measurements, the following criteria need to be met: firstly, a sensitive enough criterion of measure needs to be used; secondly, the levels of P being supplemented must not be more than the animals' requirements; and lastly, a suitable standard must be used for comparison with the phosphates being tested (Nelson & Peeler, 1961; Shastak & Rodehutscord, 2013). Numerous criteria have been used to estimate P availability, however, upon review of phytate P utilisation in poultry, Nelson (1967) and Shastak & Rodehutscord (2013) both stated that bone ash is one of the most used and most sensitive criteria in evaluating dietary P availability. This review will henceforth focus on bone ash as a criterion for estimating P availability.

Bone ash and bone phosphorus

The most common method used by nutritionists to assess the amount of aP in feed ingredients is to make use of a slope-ratio procedure that measures and compares the response (tibia ash, metatarsal ash, or bodyweight gain) of a broiler fed a low-phosphorus, semi-synthetic diet that is supplemented with graded levels of P (known as the test diet) from a known source, such as monocalcium phosphate (MCP,) with that of a control diet (Soares, 1995). Over the years various poultry bones have been evaluated to determine their ash content. Such bones analysed include the femur (Hemme *et al.*, 2005), tibia (Onyango *et al.*, 2003; Coon *et al.*, 2007), feet (Garcia & Dale, 2006; Shastak *et al.*, 2012a), and metatarsal (Yoshida & Hoshii, 1983; Karimi *et al.*, 2013). Tibia ash concentration is often used to estimate the degree of bone mineralisation in broilers with the zone of proliferation being shown to be especially sensitive to nutritional P deficiencies (Nelson & Walker,

1964; Ammerman, 1995; Shastak *et al.,* 2012a). The reason why bone is commonly used as a criterion for estimating P availability, is because approximately 80% of tP is found in the skeleton of growing birds (De Groote & Huyghebaert, 1997), mainly in the form of hydroxyapatite (Breves & Schroder, 1991). Gillis *et al.* (1954) was the first to use tibia ash concentration as a reference criterion to determine the availability of P in chicks. The method is based on relating the percentage of tibia ash obtained from chicks fed a known level of test iP against chicks fed a reference source (beta-tricalcium phosphate) to establish a relative biological value. One of the main disadvantages of making use of the tibia ash approach is the fact that the dissection and cleaning of the bone is very labour intensive (Shastak & Rodehutscord, 2013). In 1942, Baird & Macmillan proposed a less labour-intensive method, which made use of metatarsal ash as the response criterion. However, using metatarsal ash as a criterion has not been fully accepted as Shastak *et al.* (2012a) stated that there is no fixed methodology as to which metatarsal or joint metatarsal should be removed, leading to ambiguity when it comes to sampling and interpreting results. Furthermore, due to the relatively small size, and subsequent low weight of the metatarsals, any variation in sampling technique can lead to large standard deviation in reported results (Scholey & Burton, 2017). A disadvantage of using bone ash studies to calculate matrix values is that the matrix value determined in the study depends upon the quality of material used to establish the standard curve, therefore making it difficult for future research to replicate and verify the results unless the same material is available for use (Shastak & Rodehutscord, 2015). Bone ash responses can also be influenced by the duration the test diet is fed for (Shastak *et al.,* 2012). This was shown by work done by Ammerman *et al.* (1961), in which they reported that a 10-d bioassay was less sensitive than a 28-d bioassay for generating data for a tibia ash response curve.

2.7.2 Quantitative measurements

Direct measurements can be used to obtain dP improvement matrix values. To achieve a reliable dP value, many *in vivo* studies are required to generate enough representative data and may underestimate the matrix values. Data obtained from retention measurements and precaecal mineral digestibility have the advantage of allowing nutritionists to formulate diets that more accurately meet the P and Ca requirements of the bird (Lamp *et al.,* 2020). According to Sanni (2017), three main quantitative assays are commonly used to quantify phosphorus availability, which are phosphorus retention studies, mass balance and whole-body phosphorus analysis, and lastly, precaecal digestibility. According to Bedford & Cowieson (2020), care must be taken when only making use of digestibility trials as they do not account for intake and post-absorptive effects.

Retained phosphorus

The retention of P can be measured using either complete excreta collection or by making use of an ingestible marker (Shastak & Rodehutscord, 2013). Van der Klis & Versteegh (1996) developed a method by which the apparent digestion is calculated by measuring the P concentration in the terminal ileum by employing an indigestible marker and the calculation of "ileal digestibility". Leseke & Coon (2002) developed a 5-day bioassay that makes use of acid-insoluble ash as the indigestible marker for determining the retainable P. During this assay, birds are given a 3-day adaption period before excreta is collected over a 48-h period to determine retainable P values of feed-grade calcium phosphates. Based on their report, retainable P was determined as the difference between the amount of P ingested and the total voided from the GIT and urinary tract at a determined dietary P level with the following equation:

$$\text{Phosphorus retention } (\%) = \frac{(\text{TPI} - \text{TPE})}{\text{TPI}} \text{x } 100$$

Where:

- TPI = total phosphorus ingested
- TPE = total phosphorus excreted

One of the main disadvantages of these commonly used methods is the fact that they do not account for endogenous losses and metabolic excretions. Although the endogenous P found in poultry excreta is relatively low, it still can affect the results obtained from digestibility and metabolism studies (France *et al.,* 2010). Secondly, these digestibility and metabolism studies are labour intensive and an assay that could separate endogenous and dietary P fraction would be of value.

Precaecal digestibility

According to Rodehutschord (2009), the main advantage precaecal digestibility assays have compared to retained P assays, is that they are less labour intensive as they do not require metabolic cages, and reported values are not affected by post-ileal microbial activity. Precaecal digestibility assays involve feeding birds graded levels of P below requirements combined with an indigestible marker. Digesta is then collected post-mortem as described by Ravindran *et al.* (1999). The collected digesta is frozen, freeze-dried, and then ground and analysed. Precaecal digestibility (%) is calculated according to the following equation:

$$100 - 100 \text{ x } [(TiO_{2Diet} \text{ x } P_{Digesta})/(TiO_{2Digesta} \text{ x } P_{Diet})$$

Where:

- TiO_{2Diet} and $TiO_{2Digesta}$ = the analysed concentration of TiO_2 in the diet or excreta (g/kg).
- P_{Diet} and $P_{Digesta}$ = the analysed concentration of phosphorus in the diet or digesta (g/kg).

Two issues that need to be accounted for when making use of digestibility methods are feed intake and the length of time the test diet is supplied for. According to Bedford *et al.* (2015), many digestibility trials do not provide the test diet for a long enough period, meaning that the long-term effects of the diet may not be observed.

Balance and Whole-body phosphorus analysis

According to Payne (2005) and Shastak *et al.* (2012b), P retention can be determined by two different methods. The first method is known as a balance study, which involves feeding marginal dietary levels of P to minimise P excretion by the kidneys. P retention is then calculated as a percentage of total P ingested and the total P excreted over a given period. The second approach involves determining the P concentration in homogenised samples of the whole body and calculating the difference between the start and the end of the determined feed period (Sanni, 2017). Both methods are regarded as labour intensive and expensive, with both Haag (1939) and Rodehutscord (2009) stating that although whole-body analysis can be determined without the use of metabolic cages, it is complicated by trying to obtain representative samples.

2.8 Impact of phosphorus on the environment

Although broilers are considered highly efficient at converting feed into food products they can still excrete as much as 45% of the P they consume, which is high in relation to other nutrients found in poultry excreta (Plumstead *et al.*, 2007). Both Salyor *et al.* (2001) and Haggard *et al.* (2003) demonstrated that a broiler chicken, weighing 2.8 kg at 42-d of age, can produce as much as 1.0-1.3 kg of excreta. If not properly managed, poultry excreta can be seen as a potential source of water contamination (Chapman, 1996). Animal manure, including poultry excreta and litter are typically land-applied as fertilizer for crops. Although this is seen as a cost-effective way of dealing with the large amounts of excreta and litter that is produced annually, it does have its drawbacks as illustrated by Figure 2.3. Chief amongst these drawbacks is the imbalance in nitrogen (N) and P in the excreta relative to the crop needs (Plumstead, 2007). The traditional method of applying poultry waste to fields can be compared to a sponge soaking up water. At first, the sponge can absorb and retain the water but as more water is applied, the sponge begins to reach its threshold, and instead of absorbing

water it begins to leak water. The same principle can be applied to the application of poultry waste to fields. If not managed properly the quantity of excreta being applied to the field can outweigh the field's capacity to absorb the nutrients. This can result in the accumulation of surface runoff high in water-soluble P, which can end up in water bodies leading to eutrophication. From a broiler production point of view, improving the utilisation of P could aid in reducing the amount of P being excreted and subsequent environmental contamination, as well as helping to preserve world P reserves without negatively affecting broiler production (Sanni, 2017).

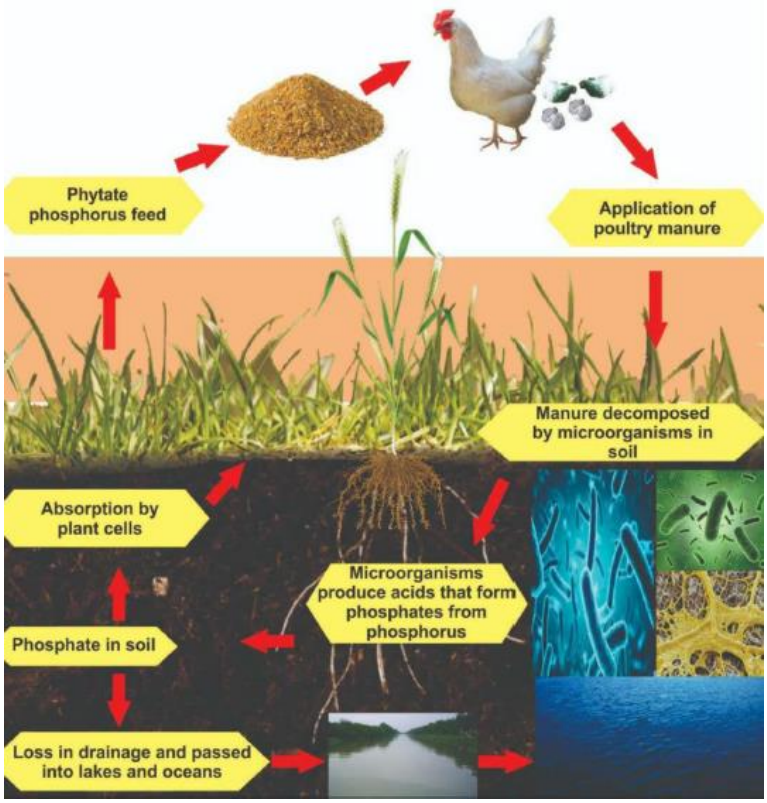


Figure 2.3. A diagram representing the application of poultry manure as fertiliser, and its pathway into the soil, plants, and water bodies (Abbasi *et al.*, 2019)

2.9 Key strategies to mitigate environmental phosphorus load

Potential mitigation strategies can be grouped according to three main categories: 1) intensification and structural changes of current livestock systems; 2) innovative technical and management interventions; and 3) moderation of demand for livestock products (Herrero *et al.*, 2016). Even though there are calls from certain groups to reduce the number of livestock products consumed,

it is the opinion of this author and Rojas-Downing *et al.* (2017) that the reduction of livestock products might be unfeasible, due to the increasing global demand for protein and products of animal origin. In keeping with the aim of this section and the overall literature review, only practices that can reduce the amount of P excreted without negatively affecting broiler production profitability will be looked at. Such strategies are already being implemented but have room for improvement. These P reduction strategies according to Wicker (1999) can be broken down into three areas: 1) Formulating and feeding to meet the animals' exact needs; 2) The use of highly digestible feedstuff that contains low levels of phytate; 3) Making use of improved management techniques and latest genetic lines. A successful solution needs to consider a combination of various solutions, such as feeding closer to nPP requirements by making use of multi-phase feeding, coupled with a better understanding of phytase matrix values, which would allow nutritionists to make use of lower dietary P feed formulation strategies. These strategies coupled with the effective use of enzymes could aid in reducing the current levels of iP supplement, as well as reduce total P in the excreta by at least 29% without compromising bone quality (Plumstead, 2007; Bello *et al.,* 2019).

2.9.1 Supplementation of feed additives

Exogenous phytase

One factor that has yet to be mentioned in this paper is the effect that the level of supplemented phytase has on the phytase response. Bougouin *et al.* (2014) and Wealleans *et al.* (2016), both agree that higher doses of phytases can influence the rate of PP hydrolysis by rapidly dephosphorylating the phytate molecule and thus ameliorate the anti-nutritional effects associated with phytate. All phytases appear to follow the same log-linear relationship between phytase dose and response (Rosen, 2001; Rosen, 2002). Current industry practice is to include anywhere between 500-1000 FTU/kg of phytase depending on the diet. According to Cowieson (2011), 500 FTU/kg will release between 0.05-0.15% digestible phosphorus. Super dosing refers to the inclusion of phytase above and beyond normal inclusion rates, usually greater than 1000 FTU/kg. Super dosing is thought to improve bird performance through improved phytate P utilisation by removing as much of the dietary IP6 and lower esters of phytate as possible (Pieniazek *et al.,* 2017). Work by Walk (2016) demonstrated that a standard dose of phytase is only sufficient to partially hydrolyse phytate, however, when the phytase dose was increased to three times industry levels, complete hydrolysis of the lower phytate esters was observed. According to Zyla *et al.* (2004), the complete hydrolysis of phytate is essential in producing inositol which has been shown to improve broiler performance. Shirley & Edwards (2003) showed

that birds fed maize-soybean-meal diets that were supplemented with log-doses of phytase up to 12,000 FTU/kg exhibited increased performance. Research by various authors looking at the effects of dosing phytase above current industry standards has shown to improve Ca and P digestibility as well as improve broiler performance (Cowieson *et al.,* 2011; Walk *et al.,* 2014). Walk *et al.* (2012) suggests that supplementing exogenous phytase above industry standards may reduce the amount of Ca being supplemented in broiler diets while maintaining broiler performance, bone ash, and improving amino acid digestibility.

Vitamin D_3

Apart from supplementing broiler diets with phytase, another feed additive that is used in conjunction to reduce the amount of P excreted is the supplementation of vitamin D and its metabolites 25-hydroxycholecalciferol ($25[OH]_2D_3$) and 25-hydroxy-cholecalciferol ($1,25[OH]_2D_3$) (McGrath *et al.,* 2010). Not only does vitamin D_3 stimulate transport mechanisms in the intestine but it also has the added benefit of enhancing the activity of supplemental phytase (Baker *et al.,*1998; Carlos & Edwards, 1998). McGrath *et al.* (2010), stated that the mode of action by which vitamin D_3 metabolites improves phytase efficacy is two-fold. Firstly, vitamin D_3 metabolites can increase Ca utilisation in broilers, and reduce the incidence of Ca-phytate complexes in the digestive tract, thereby improving phytase efficacy. Secondly, vitamin D_3 increases the absorption of inorganic P into the blood, thereby reducing the inhibitory effect that high levels of inorganic P have on phytase efficacy in the intestine. This was demonstrated by Ravindran *et al.* (1995), in which the supplement of vitamin D_3 into the diet was able to enhance phytate P digestibility in broilers. Work done by Mitchell & Edwards (1996) illustrated that the supplementation of both phytase and 1,25-dihydroxycholecalciferol has an additive effect on increasing phytate P retention along with reducing the amount of iP being supplemented in the diet.

2.9.2 Precision feeding

Pomar *et al.* (2019) defines precision feeding as the practice of feeding individual animals or groups of animals while considering changes in nutrient requirements that occur over time as well as variation amongst animals to meet production objectives. The primary objective for any broiler producer is to maximise growth on a least cost basis. To achieve this objective the level of nutrients needs to be provided at a level that will allow the most nutrient demanding birds in the flock to express their growth potential (Hauschild *et al.,* 2010). Broilers are typically raised and fed in large groups

and are given the same feed during a given period. Differences in nutrient requirements exist in large flocks meaning that some birds may be oversupplied nutrients, resulting in excessive nutrient excretion (Pomar *et al.*, 2003; Brossard *et al.*, 2009). According to the NRC (1994), the phosphorous requirements of a growing broiler expressed as a percentage of the diet decrease as the bird ages. One of the problems associated with making use of the NRC (1994) recommendations is the fact that the recommendation for nPP requirements is based on a conventional 3-phase feeding program. This program only makes use of three different diets over an 8-week growth period, resulting in the undersupply or oversupply of nutrients, which is not only costly but also wasteful (Plumstead, 2007). An increase in the number of feed phases would allow nutritionists to formulate diets that more closely meet the nutrient requirements of the bird throughout its various stages of growth. This form of formulating to meet the exact needs of the animal is known as precision feeding.

Precision feeding can result in a decrease in the amount of nPP and P fed to birds leading to a reduction in nutrient excretion as well as a reduction in feed costs (Ferket *et al.*, 2002). Wicker (1999) claimed a reduction of between 10-25% in P excretion when making use of precision feeding. It must be stated that there are economic and labour related factors that limit the number of phases that can be used. From a feed mixing perspective, increasing the number of feeding phases leads to more feeds to be mixed, resulting in increased costs. Therefore, a balance needs to be stuck between the reduction in the cost of feed due to less iP being added to the diet and the cost involved in mixing and transporting diets more frequently (Angel, 2011).

2.10 Conclusion

With climate change becoming an ever-increasing threat to the traditional way of farming, more efficient methods of producing food are needed now more than ever before. In the case of the poultry industry, the environmental concern and cost implications of supplementing broiler diets with a finite resource, such as inorganic phosphorus, have led to growing pressure to not only reduce the industries dependence on such a resource from an environmental standpoint, but also an economical and production efficiency standpoint. Methods which promote improved efficiency of dietary P utilisation in broiler diets are key to maximising the metabolic benefits, as well as economic profits, of this essential mineral. One such method that has proven successful in the poultry industry is the use of exogenous phytase. Microbial phytase is commonly and widely used in poultry diets to break down phytate, rand increasing the availability of phytate bound phosphorus. However, the efficacy of different phytases has been shown to be inconsistent between studies (Selle & Ravindran, 2007). The question remains if there are further benefits provided by supplying phytase at levels over and

above industry standard. Considering this information, the opportunity exists to explore various sampling and statistical methodology to determine the effect of dosing phytase above current industry standards.

CHAPTER 3: Material and methods

3. Introduction

All animal care procedures were approved by the Animal Ethics Committee (AEC) of the Faculty of Natural and Agricultural Science of the University of Pretoria (NAS240/2020). The main objective of the study was to determine if the asymptote of phytase response at which tibia ash is maximised (FTU/kg feed) is affected by the source of phytase. A secondary objective of the study was to determine the optimum regression model for describing the relationship between phytase dose and tibia ash in 14-day old broilers. The last objective was to determine the correlation between two different response criteria (tibia vs metatarsal) on reported ash values.

3.1 Facilities and animal husbandry

2000 sentient day-old broilers were used for this dose-response trial. The 14-day trial took place on the Hillcrest Experimental Farm, University of Pretoria (Hatfield, Pretoria). Twenty days before the placement of the birds the environmentally controlled broiler house was thoroughly cleaned and disinfected. After disinfecting, the house was left to stand for 14 days. Four days before the placement of the day-old birds, clean wood shavings were placed into each of the 30 pens and the house was preheated to 36ºC to allow for the house and litter to reach the correct brooding temperature. Each pen contained one nipple line, one tube feeder, and two fountain drinkers. One day prior to placement, brown chick paper and a feeder pan was laid in each pen and feed was sprinkled onto the chick paper and pan to encourage early feed intake. Fresh feed was sprinkled in the morning onto the feeder pan and brown chick paper for the first three days after placement. Throughout the 14-day trial, birds were supplied fresh feed and water *ad libitum.* A strict biosecurity protocol was followed for the duration of the trial to minimise the risk of disease transmission, which included the strict access of authorised personnel and the use of footbaths and designated work boots. Personnel working on the trial were expected not to have had any contact with live poultry for at least three days prior to working on the trial.

Upon arrival (day 0), 2000 A grade, male, day-old Ross 308 broilers sourced from National Chicks (Plot 33 Boschkop Road, Mooiplaats) were individually weighed and placed into one of 30 pens (8 m^2), with 66 birds per pen. The remaining 20 birds were individually randomly allocated to one of the 30 pens. For the duration of the trial the birds were exposed to the same lighting and temperature profiles as stipulated by the Ross 308 management handbook (Aviagen, 2019).

Temperatures were recorded twice daily (once in the morning and once in the evening) with an infrared thermometer (JPD-FR202). Pen temperatures were taken by measuring three random spots in each pen, whilst house floor temperatures were recorded by measuring the front, middle and back of the house. For the duration of the trial the birds were monitored for any signs of discomfort, stress, or illness. In the case of mortalities, the dead bird was weighed, and the weight and day of death was recorded to enable the calculation of a mortality corrected FCR (FCRm).

For the first three days the birds were fed a standard maize-soyabean-meal diet. For the remainder of this chapter this diet will be referred to as the adaptation phase or adaptation diet. This adaptation diet was formulated to contain adequate levels of all nutrients, including Ca and P, as recommended by the Ross 308 nutrition specifications (Aviagen, 2019). This diet was formulated and fed to provide the birds with an initial reserve of bone mineralisation to prevent any detrimental effects when birds were switched over onto the Ca and P deficient test diets. From day 4 to 14, birds were fed one of the 19 treatment diets. Throughout the trial, feed was provided *ad libitum* in the form of mash along with water.

3.2 Placement of test birds

On day 3, 10 birds from each pen (300 birds in total) were individually weighed and the mean flock weight, as well as standard deviation (SD) and percentage of coefficient of variation (CV%), was calculated. A total of 1920 birds, closest to the mean flock weight and that fitted within one standard deviation of the mean, were selected, and transferred to the preheated, environmentally controlled and artificially lit metabolic house located adjacent to the broiler house on the Experimental Farm at the University of Pretoria (Hatfield, Pretoria). Birds were handled with care during transport between the broiler house and the metabolic house to avoid stress as well as prevent injury. The metabolic house was prepared the same way as outlined for the broiler house.

A total of 1920, 4-day old Ross 308 male broilers were randomly assigned to one of 19 treatments (16 birds/cage, 6 replicates for treatments 2-14 and 12 replicates for the negative control (NC)). The metabolic house contained 120 metabolic cages (length x width x hight; 90 cm x 34 cm x 52 cm) stacked in groups of three to form a tier, with two tiers being combined to form a single battery unit; in total there were 20 battery units. Each metabolic cage was equipped with a height adjustable feed trough and nipple drinking system (4 nipples per line), as well as an excreta tray. Prior to placement each metabolic cage, as well as its corresponding feed trough, was labelled. To consider

variation within the metabolic house during the test period, trial treatments were randomly allocated within the metabolic house by making use of a completely randomised design.

After placement, the birds from each cage were weighed and the weights recorded. The weights obtained were analysed using JMP® (Version 15.0. SAS Institute Inc., Cary, NC, 1989-2019) to identify any outliers. This was done to ensure that no cage was significantly heavier or lighter than the average. If a cage was found to be an outlier, a bird was randomly taken from the cage and replaced with one of the spare birds and the cage was reweighed. Once all cages had been weighed and all outliers removed, the feed troughs were filled with the adaptation feed. Birds were only transferred onto the test diet on day 4 of the trial to provide the birds with a brief period to adapt to their surroundings.

On day 4, 1.4 kg of test feed was weighed and placed into the corresponding labelled feed troughs. For the duration of the trial period (day 4 to day 14) birds were checked three times daily and house temperatures recorded in the morning and afternoon at three different spots within the house using an infrared thermometer (JPD-FR202). The test diets were provided ad libitum in mash form for the duration of the trial period (day 4 till day 14) and water was freely available. The diets fed was all corn SBM – based diets of which all major constituents were analysed for dry matter (DM), crude protein (CP), crude fat (EE), crude Fibre (CF), ash, Ca and P before formulation.

3.3 Experimental design

For this study three different bacterial 6-phytase products were used. The first being a *Buttiauxella* sp. bacterium sourced 6-phytase (Product A), The second being a novel consensus bacterial 6-phytase variant expressed in *Trichoderma reesei* (product B). The last phytase being an *E. coli* phytase expressed in *Komatagaella phaffii* (Product C).

A mash corn-soy based NC diet (3.4 g/kg P and 7.0g/kg Ca) was supplemented with each phytase product based on analysed phytase units (FTU) at five dose levels (500, 1000, 1800, 2500 and 3500 FTU/kg) to form a dose response, which was compared against three positive control (PC) diets that contained incremental amounts of monodicalcium phosphate. PC1: (2.70 g/kg rP (retainable P), 4.88 g/kg P & 5.98 g/kg Ca), PC2: (3.00 g/kg rP, 5.24g/kg P & 6.82g/kg Ca) and PC3: (3.1 g/kg rP, 5.6 g/kg P & 7.2 g/kg Ca) as outlined in Table 3.1. Body weight (BW), body weight gain (BWG) and mortality corrected feed conversion (FCRm) were determined from 4-14 days. On day 14, eight broilers/cage were sampled, the right tibia and right middle metatarsal were removed, pooled by cage, and defatted tibia bone ash weight and metatarsal ash weight were determined.

Table 3.1 Experimental design and treatment description

Treatment (T)	Diet	Phytase Product	Phytase dose (FTU/kg)
			Formulated values
T1	NC[a]		
T2	NC	Product A	500
T3	NC	Product A	1000
T4	NC	Product A	1800
T5	NC	Product A	2500
T6	NC	Product A	3500
T7	NC	Product B	500
T8	NC	Product B	1000
T9	NC	Product B	1800
T10	NC	Product B	2500
T11	NC	Product B	3500
T12	NC	Product C	500
T13	NC	Product C	1000
T14	NC	Product C	1800
T15	NC	Product C	2500
T16	NC	Product C	3500
T17	PC1[b] (NC + MDCP[e])		
T18	PC2[c] (NC + MDCP[e])		
T19	PC3[d] (NC + MDCP[e])		

[a]NC: Negative control
[bcd] served as the positive controls with incremental levels of MDCP
[b]PC1: (2.70 g/kg rP (retainable P), 4.88 g/kg P & 5.98 g/kg Ca)
[c]PC2: (3.00 g/kg rP, 5.24g/kg P & 6.82g/kg Ca)
[d]PC3: (3.1 g/kg rP, 5.6 g/kg P & 7.2 g/kg Ca)
[e]MDCP: Monodicalcium phosphate
[f]Phytase activity was determined by Chemuniqué Pty (Ltd), Lanseria, South Africa.

3.4 Diet formulation

3.4.1 Basal diet

All feed ingredients used in the trial were sourced from Simple Grow Agricultural Services (Centurion, South Africa). All feed analysis performed during the trial was done by Chem Nutri Analytical (Pty) Ltd (Olifantsfontein, South Africa). Each ingredient used to formulate the basal diet was analysed in accordance with the methods as outlined in Table 3.2. Each feed ingredient was analysed in duplicate for ash, CF, CP, DM and EE according to AOAC's official methods of analysis (2000). Furthermore, mineral analysis for Ca and P were analysed in duplicate using AOAC (2000), official method 985.01 (3.2.06) and AOAC (2016), official method 2011.14 (50.1.37), adapted for inductively coupled plasma optical emission spectrometry (ICP-OES), respectively. Phytase levels

were determined using the ISO 9001 (2008) procedure. Based on the individual proximate values obtained for each feed ingredient, a single batch (756 kg) of the basal diet was mixed according to the inclusion levels described in Table 3.3. The basal diet was mixed in five batches of 153 kg each in a 200kg ribbon blender at Chemuniqué (Pty) Ltd (Lanseria, South Africa). The basal diet was formulated to contain no phytase, and no monodicalcium phosphate (MDCP). The mixed basal diet would later go on to be used as the base for the adaptation diet and subsequent test diets used during the trial.

Table 3.2 Method of analysis for various feed analysis performed

Feed analysis	Method of analysis
DM and ash	Method 942.05 (2000)
Moisture	Method 943.01 (2000)
Crude Fiber (CF)	Method 962.09 (2000)
Crude Fat (EE)	Method 920.39 (2000)
Crude Protein (CP)	Method 988.05 (2000)
Calcium (Ca)	Method 985.01 (2000)
Phosphorus (P)	Method 2011.14 (2016)
Phytase	ISO 9001 (2008)

Table 3.3 Basal diet feedstuff inclusion levels

Ingredient	Inclusion level (%)
Maize	52.5
Soya oilcake eal	35.2
Sunflower oilcake meal (36%)	4.11
Gluten 60	4.11
HCL-Lysine (78%)	0.36
DL-Methionine (98%)	0.34
Threonine (98%)	0.16
Oil crude soya (Degummed)	1.29
Limestone	0.94
Salt (fine)	0.36
Sodium bicarbonate	0.15
Cycostat (Robenidine 6.6%)	0.05
Zinc bacitracin (15%)	0.07
Mould inhibitor	0.02
Premix[a]	0.31

[a] Supplied per kilogram of feed: Vitamin A, 12000 IU; Vitamin D3, 5000 IU; Vitamin E, 60.00 mg; Vitamin K3, 2.00 mg; Vitamin B1, 2.00 mg; Vitamin B2, 5.00 mg; Niacin (B3), 50.00 mg; B5, 12.00 mg; Pyridoxine, 3.00 mg; Folic acid, 2.00 mg; Vitamin B12, 0.01 mg; Biotin, 0.10 mg; Antioxidant, 125.00 mg; Manganese, 110.00 mg; Iron, 41.20 mg; Zinc, 100.00 mg; Copper, 10.00 mg; Cobalt, 0.50 mg; Iodine, 2.00 mg; Selenium 0.30 mg; Choline, 350 mg

3.4.2 Adaptation diet

An adaptation diet was mixed using a portion of the basal feed (Table 3.2) and supplementing it with inorganic P (MDCP) and Ca (limestone) to meet or exceed the nutrients requirements as stipulated by the Ross 308 breed recommendations (Aviagen, 2019). The reason for feeding the adaptation diet from 0-3 days was to ensure adequate bone mineralisation and prevent any welfare issues. The feed ingredient composition of the adaptation diet is shown in Table 3.4.

Table 3.4 Adaptation diet feedstuff inclusion levels

Feed ingredient	Inclusion level (%)
Maize	51.06
Soya oilcake meal	34.25
Sunflower oilcake meal (36%)	4.00
Gluten 60	4.00
HCL-Lysine (78%)	0.35
DL-Methionine (98%)	0.33
Threonine (98%)	0.16
Oil crude soya (Degummed)	1.25
Limestone	1.20
Monodicalcium phosphate	2.47
Salt (fine)	0.35
Sodium bicarbonate	0.15
Cycostat (Robenidine 6.6%)	0.05
Zinc bacitracin (15%)	0.07
Mould inhibitor (Bitek industries)	0.02
Premix[a]	0.30

[a] Supplied per kilogram of feed: Vitamin A, 12000 IU; Vitamin D3, 5000 IU; Vitamin E, 60.00 mg; Vitamin K3, 2.00 mg; Vitamin B1, 2.00 mg; Vitamin B2, 5.00 mg; Niacin (B3), 50.00 mg; B5, 12.00 mg; Pyridoxine, 3.00 mg; Folic acid, 2.00 mg; Vitamin B12, 0.01 mg; Biotin, 0.10 mg; Antioxidant, 125.00 mg; Manganese, 110.00 mg; Iron, 41.20 mg; Zinc, 100.00 mg; Copper, 10.00 mg; Cobalt, 0.50 mg; Iodine, 2.00 mg; Selenium 0.30 mg; Choline, 350 mg

3.4.3 Treatment diets

All treatment diets were formulated on a retainable phosphorus basis (0.11g/kg) according to the CVB (2016). The ingredient and nutrient composition of the NC and PC diets are presented in Table 3.5. The NC diet was tested as a stand-alone diet as well as forming the base for the phytase supplemented treatment diets.

The NC was mixed by taking a percentage of the basal diet (Table 3.3) and adding limestone, as described in Table 3.5. Degermed maize was used as a filler to maintain the same final volume in

each diet without influencing the overall nutrient value of each treatment. Each 40 kg treatment diet (Treatments 2-19) was mixed in a 25 kg mixer at Chemuniqué (Pty) Ltd. Due to the size of the mixer, and to prevent feed spillage, each treatment was done in two batches of 20 kg each. Each batch was mixed for three minutes to ensure uniformity of mixing. The three positive control (PC) diets were formulated from sub-batches of the NC with incremental levels of inorganic phosphorous (NC + 1.85, NC + 2.22 and NC + 2.58 g/kg P from MDCP). Treatments 2-16 were mixed by adding phytase to sub-batches of the NC diet according to analysed activity. After each batch was mixed the feed was poured into labelled 25 kg treatment bags and fed to the birds in mash form. A 1 kg sample was taken from each treatment diet and sent to Chem Nutri Analytical (Pty) Ltd to be analysed in duplicate for dry matter, ash, crude fat, crude fiber, crude protein, as well as Ca and P. A further sub-sample was taken of the negative control diet and three positive control diets and sent to Danisco Animal Nutrition (IFF) (Genencor International BV, The Netherlands) for phytate-P analysis. Phytate-P concentrations were determined using a modified version of the HPLC method described by Skoglund *et al.* (1998). The ingredient and nutrient composition of the negative control and positive control diets are shown in Table 3.6.

Table 3.5 Feed stuff ingredients and calculated nutritional composition of the control diets

Item	NC	NC + inorganic P from MDCP, g/kg		
		PC1	PC2	PC3
Ingredients (%), fixed part				
Maize	52.51	52.51	52.51	52.51
Soya Oilcake Meal	35.22	35.22	35.22	35.22
Sunflower Oilcake Meal (36%)	4.11	4.11	4.11	4.11
Corn Gluten 60	4.11	4.11	4.11	4.11
Lysine HCl (78%)	0.36	0.36	0.36	0.36
DL-Methionine (98%)	0.34	0.34	0.34	0.34
Threonine (98%)	0.16	0.16	0.16	0.16
Crude soya oil (Degummed)	1.29	1.29	1.29	1.29
Limestone	0.94	0.94	0.94	0.94
Salt (fine)	0.36	0.36	0.36	0.36
Sodium Bicarbonate	0.15	0.15	0.15	0.15
Cycostat (Robenidine 6.6%)	0.05	0.05	0.05	0.05
Zinc Bacitracin (15%)	0.07	0.07	0.07	0.07
Mould Inhibitor (Bitek industries)	0.02	0.02	0.02	0.02
Premix[a]	0.30	0.30	0.30	0.30
Ingredients (g/kg, as is) variable part				
MDCP[b]	0.00	9.5	11.60	13.50
Limestone	4.12	0.00	1.35	1.47
Degermed maize[c]	23.46	7.88	4.63	2.61

[a] Supplied per kilogram of feed: Vitamin A, 12000 IU; Vitamin D3, 5000 IU; Vitamin E, 60.00 mg; Vitamin K3, 2.00 mg; Vitamin B1, 2.00 mg; Vitamin B2, 5.00 mg; Niacin (B3), 50.00 mg; B5, 12.00 mg; Pyridoxine, 3.00 mg; Folic acid, 2.00 mg; Vitamin B12, 0.01 mg; Biotin, 0.10 mg; Antioxidant, 125.00 mg; Manganese, 110.00 mg; Iron, 41.20 mg; Zinc, 100.00 mg; Copper, 10.00 mg; Cobalt, 0.50 mg; Iodine, 2.00 mg; Selenium 0.30 mg; Choline, 350 mg

[b]MDCP: Monodicalcium phosphate

[c]Degermed maize was added as a filler ingredient to maintain a constant final weight across treatments

Table 3.6 Feed stuff ingredients, calculated and analysed nutrient composition of the control diets

Item	NC	NC + inorganic P from MDCP, g/kg		
		PC1	PC2	PC3
Calculated nutrients (%)				
Lysine	1.52	1.52	1.52	1.52
Threonine	1.06	1.06	1.06	1.06
Tryptophan	0.25	0.25	0.25	0.25
TSAA[1]	1.14	1.14	1.14	1.14
Dig Lysine	1.34	1.34	1.34	1.34
Dig Threonine	0.91	0.91	0.91	0.91
Dig Tryptophan	0.22	0.22	0.22	0.22
Dig TSAA	1.00	1.00	1.00	1.00
Retainable phosphorus[2]	0.11	0.27	0.30	0.33
Total phosphorus	0.30	0.49	0.52	0.56
Calcium	0.60	0.60	0.68	0.72
Phytate phosphorus	0.27	0.26	0.27	0.26
Analysed nutrients (%)[a]				
Dry matter	90.48	91.18	91.37	91.45
Ash	5.13	5.84	6.02	6.44
Crude fat	3.85	3.87	3.96	3.73
Crude fiber	3.55	3.61	3.93	3.83
Crude protein	24.26	23.68	23.64	23.52
Total phosphorus	0.50	0.57	0.60	0.63
Calcium	0.74	0.90	0.95	1.00
Phytate phosphorus[b]	0.27	0.26	0.27	0.26

[1]TSAA: Total sulfur containing amino acids
[2] Determined from CVB, 2018
[a]Analysed by Chem Nutri Analytical (Pty) Ltd.
[b] Determined by Danisco Animal Nutrition (IFF) Brabrand, Denmark.

3.5 Enzyme analysis

Phytase analysis was performed on all treatment diets in accordance with ISO 9001 (2008), Clause 7.2.1, 7.2.2, 7.2.3, 7.5.3, and 8.2.1 at Chemuniqué Pty (Ltd). Phytase activity (FTU/kg) was measured as stated in the AOAC (2000), in which phytase activity is defined as the amount of enzyme required to release 1 µmol of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37°C. The procedure outlined below involved the analysis of the test phytase in feed,

whereby the phytase in the test feed was incubated with sodium phytate (phytic acid dodecasodium). This resulted in the release of inorganic phosphorus, which reacted with a molybdate vanadate reagent, to create a yellow-coloured complex. This yellow colour was measured at a wavelength of 450 nm and the extent of colour formation was directly related to the enzyme activity. The activity was quantified by an absolute method using a phosphate standard calibration curve. To measure the activity of the test phytase in the feed, a blank sample, as well as a standard sample, were required. Each test feed was analysed in duplicate. The preparation of the test feed, blank sample, and standard sample are described in section 3.5.1 and section 3.5.2 respectively.

3.5.1 Test feed and blank sample preparation

To measure the phytase activity in the feed a one kg feed sample was randomly taken from each of the already mixed treatments and thoroughly mixed by hand. After mixing, a 200 g subsample was taken and ground until it could pass through a 0.75 mm screen. After passing through the screen the 200 g sub sample was again thoroughly mixed by hand and a 10 g sample was weighed into a labelled plastic cup. This procedure was done in duplicate for all 15 phytase treatments. A 100 mL of acetate buffer solution (F10X) and a magnet were placed into each labelled plastic cup. The sample cups were placed on a magnetic stirrer and allowed to mix for 10 minutes. After 10 minutes, the magnetic stirrer was switched off and the samples were left to settle. Once settled the samples were filtered by passing the solution through filter paper. A 250 µL sample of the filtrate was placed into corresponding labelled test tubes containing 750 µL of acetate buffer (F4X) in duplicate and vortexed. One set of the test tubes was used to analyse the test feed samples, while the other set was used to prepare the blank samples. After mixing, both sets of test tubes containing a solution of sodium phytate, were placed into a hot water bath, which had been preheated to 37ºC, and heated for five minutes. After five minutes, test feed samples were removed from the hot water bath and 2 mL of the Phytate substrate solution was pipetted into each test tube in five second intervals. The test feed samples were then placed back in the hot water bath for another hour at 37ºC to allow for equilibration to be achieved. Each test tube received 2mL of an already prepared stopper reagent in five second intervals following the same sequence as that followed when adding the phytate substrate solution. Another 2 mL of the stopper reagent was then added to each of the blank samples following the same sequence as the first time. Immediately after adding the stopper reagent, 2 mL of phytate substrate solution was added to the blank sample in five second intervals. Blank samples were then placed in a vortex mixer and centrifuged for 10 minutes at 3500 rpm. Test samples were then mixed in a vortex mixer and mixed at below 2000 rpm to ensure no liquid was spilled. The test feed samples were then

centrifuged at 35000 rpm for 10 minutes. Thereafter, 2 mL of both the test feed samples and the blank samples were pipetted into plastic cuvettes and placed into a spectrophotometer. The absorbance of both the test feed samples and the blank samples were measured 415 nm and compared against water.

3.5.2 Preparation of standard sample

To prepare the standard sample, a phosphate stock standard solution was diluted with acetate buffer to give the final phosphate concentration as outlined in Table 3.7. Two sets of each phosphate dilution were prepared, and the results were averaged. Next, 0.25 mL of phosphate standard solution and 0.75 mL of acetate buffer were pipetted into plastic tubes. Thereafter 2 mL of the stopper reagent was added followed by 2 mL of phytate substrate solution. Each tube was sealed and shaken vigorously before they were incubated at room temperate for 5-10 minutes. The tubes were then centrifuged for one minute at 3500 rpm before their absorbance was measured using the spectrophotometer at 415 nm.

3.6 Calcium and Phosphorus analysis of feed samples

All feed samples were analysed in duplicate for Ca and P content in accordance with the AOAC method 935.12 and method 965.17 (AOAC,1998), respectively, at Chem Nutri Analytical (Pty) Ltd. Prior to analysis, the feed samples were prepared as outlined in section 3.6.1.

3.6.1 Digestion of samples for calcium and phosphorus

A 0.5 g sample of feed was placed into a digestion-tube and the mass of the tube and sample was recorded before 25 mL of nitric acid (HNO_3) was added to the tube. The tube was heated on a pre-heated heating block set to 240^{o}C for 15 minutes. After 15 minutes the sample was removed from the heat block and allowed to cool for five minutes before 10 mL of perchloric acid ($HClO_4$) was added to the tube and heated for another 35 minutes. The tubes were then removed from the heating block and placed into a fume cupboard until all fumes had dissipated. After all fumes had dissipated, 30 mL of deionized water was added into the test tube. The solution in the test tube was transferred into a volumetric flask and thoroughly mixed and left to stand.

3.6.2 Calcium and phosphorus analysis

Calcium analysis was performed by making use of spectrophotometer analysis. Prior to analysis the spectrophotometer was calibrated by placing Ca solutions of different strengths into the spectrophotometer along with 1.0 mL of a certified Ca solution of known standard (1000 ppm). Once the spectrophotometer had been calibrated the Ca concentration of the digestion solution that was prepared as described in section 3.6.1, was analysed. The values obtained from the spectrophotometer were then used to calculate the Ca concentration of the original feed samples.

The P content of the feed was analysed by diluting 1.0 mL of the digestion sample described in section 3.6.1 with 7.0 mL of deionized water. For the spectrophotometer to analyse the sample solution a separate colour solution was prepared and then mixed with the sample solution and left for 30 min to allow the new mixture to develop. Once developed, the solution was analysed at 400 nm using the spectrophotometer and the results obtained from that analysis were used to determine the P content of the feed.

3.7 Limestone analysis

The limestone used for this trial was analysed to determine its rate of solubilisation, as well as mineral composition. The limestone was analysed for Ca and P in duplicate, according to AOAC (2000), official method 985.01 (3.2.06) and AOAC (2016), official method 2011.14 (50.1.37), which have been adapted for ICP-OES at Chem Nutri Analytical (Pty) Ltd. The mineral results of the two runs were averaged to obtain a Ca value of 39.74% and a P value of 0.02%. This was done to aid in calculating how much Ca and P the limestone would contribute to the test diets. All solubility analysis were performed at the Chemuniqué (Pty) Ltd. laboratory as described in section 3.7.1.

3.7.1 Limestone solubility assay

The dynamic solubility of the limestone used in this study was determined in accordance with the methods as set out by Kim *et al.* (2019), which is an adaptation of the method first described by Zhang & Coon (1997). This modified solubility method makes use of a pH 3 glycine buffered HCl solution designed to closely reflect the conditions in the gizzard of the bird. The test was performed in duplicate with solubility being calculated as the weight loss of the sample limestone at three different time points (5 minutes, 15 minutes, and 30 minutes) as described by Equation 1. The three

time points were selected as they are believed to represent the average retention time most closely in the gizzard and proventriculus of broilers (Kim *et al.*, 2019). The test limestone solubility was then plotted against the three time points and compared against a control limestone of known solubility. The results of the solubility assay can be seen in Figure 3.1. The control limestone used in Figure 3.1 to compare the test limestone is routinely used as a standard at Chemuniqué (Pty) Ltd when comparing analysed limestone samples as the Ca and P digestibility for this limestone have already been determined *in vivo* by Kim *et al.* (2019) and is believed to represent what the "ideal" limestone solubility profile should look like.

Equation 1: Limestone solubility

$$\text{Solubility (\%)} = \left(1 - \frac{\text{dried remaining limestone}}{\text{dry initial limestone}}\right) \text{x } 100$$

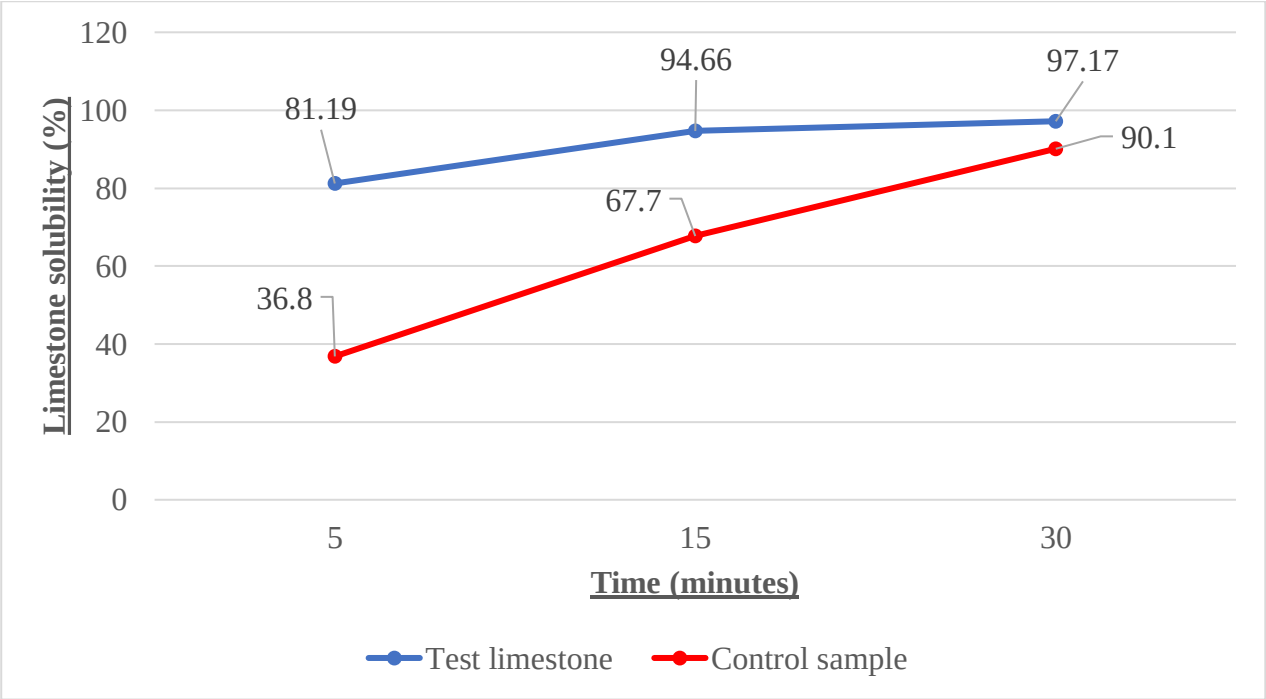


Figure 3.1 Rate of *in-vitro* solubility of fine limestone (< 1mm) used in study compared to a limestone control of known solubility.

3.8 Bone sampling

To determine tibial and metatarsal bone mineralisation, eight birds were randomly selected from each cage on day-14. Birds were humanely euthanised using a modified method as described by Zeller *et al.* (2015), via carbon dioxide asphyxiation and involved a two-step approach. The 1st step involved placing the eight birds into an air sealed dark box containing a thin layer of wood shavings.

The thin layer of wood shavings aided in minimising the amount of stress on the birds by preventing the birds from slipping on the smooth plastic box floor. A mixture of three gases in the proportion of 35% CO_2, 30% O_2 and 35% N_2 was administered for five minutes into the box to render the bird's unconscious. Thereafter, pure CO_2 was administered immediately for 2-3 minutes until the birds were deceased. The whole right leg was excised from each bird by separating the femur from the hip via dislocation, with the aid of a scalpel. The right whole leg samples were left for 48 hrs at room temperature to allow the flesh to rot. This was done to aid in the removal of the tibiotarsus (tibial) bones from the flesh, without causing any damage to the bones. The tibia bones were then separated at the tibio-tarsal junction and tibio-femoral junction. Once liberated, the cartilaginous caps were removed from the tibial head and the bones were cleaned of any excess flesh. Samples were pooled according to cage number with eight samples per replicate cage placed into prelabelled sampling cups. Samples were then placed into a freezer at -20^{o}C until defatting could occur.

Along with the removal of the tibias, the middle metatarsal of the right foot was removed at the metatarso-phalangeal joint, with the aid of a scalpel. Before the samples were frozen each metatarsal was inspected for any dried excreta. If extra excreta were found to be present on the metatarsal or under the claw it was removed with the aid of a pair of tweezers and a damp cloth. Once all metatarsals had been inspected, they were pooled according to cage number, with eight samples per replicate cage placed into clean sterilised sampling cups labelled as day-14. Once placed into their designated sample cups all samples were placed into a freezer at -20^{o}C along with the tibias until defatting could occur.

The reason for the removal of the tibia and metatarsal was to allow for a comparison between the two different sampling methods, as Yoshida & Hoshii (1983) first reported a high correlation between metatarsal and tibia ash. Both sets of samples were processed as mentioned in section 3.8.1 and analysed for dry-defatted ash percentage.

3.8.1 Bone defatting

All defatting of samples took place at Chemuniqué (Pty) Ltd. Laboratory. Before defatting, the samples were removed from the freezer and allowed to defrost at room temperature. Metatarsal samples were defatted with their skin still attached. Once defrosted the samples were then placed in a drying oven at 70^{o}C for 12 hours. After the allotted time had expired, the dried samples were left to cool before being placed into fat extraction thimbles, which had already been prelabelled according

to pen number. Cotton wool was used to plug the thimble opening to ensure the tibias remained in their designated thimbles. Thimbles were placed in sets of eight into organza bags. Eight organza bags containing eight thimbles per a bag. Samples were then refluxed for 48hrs in one of two Soxhlet apparatus using analytical grade petroleum ether sourced from Associated Chemical Enterprise (Theta Ext 6, South Africa). Following fat extraction, the samples were then placed into a fume hood for one hour to allow the ether to evaporate before being placed into their designated sampling cups.

3.8.2 Tibial and metatarsal ash

The defatted bone samples were then taken to Chem Nutri Analytical (Pty) Ltd to determine bone ash on a fat-free dry matter basis using a modified version of the AOAC (2000), method 942.05 (4.1.10). Before samples could be ashed the moisture content of the samples was first determined by placing the samples into dried crucibles, of known weight. The weight of the crucible with the sample was then weighed and subtracted from the initial empty crucible weight to get an initial sample weight. Crucibles containing the samples were oven-dried at a temperature of 70-100ºC for 12 hrs. Once dried, the crucibles containing the samples were transferred directly into a desiccator and allowed to cool completely. Once completely cooled, the crucibles containing the samples were individually weighed and the combined weight of the crucible and bone was recorded. Lastly, the crucibles containing the samples were placed into a muffle furnace to be ashed at 600ºC for a minimum of 12 hrs. After 12 hrs, samples were left to cool in the furnace until approximately a temperature of 100ºC was reached, after which the samples were taken and placed directly into a desiccator and allowed to cool completely. Each crucible containing either the tibia ash or metatarsal ash was individually weighed to determine dry ash weight. Percentage defatted bone ash was calculated based on the following calculations as described by Ravindran *et al.* (1999):

Equation 4: $\%\ \text{defatted bone ash} = \dfrac{\text{Dry bone ash weight}}{\text{Dry defatted bone weight}}\ \text{x}\ 100$

where the weight of dry bone ash

$$= (\text{Dry crucible} + \text{Dry bone ash weight}) - \text{Dry crucible weight}$$

where the weight of dry defatted bone

$$= (\text{Dry Crucible} + \text{Dry dry weight before ashing}) - \text{Dry crucible weight}$$

3.9 Performance

At placement (day 0) birds were individual weighed and their body weights were recorded. On day three birds were again individually weighed and transferred to metabolic cages (16 birds per cage). This was done to minimise the coefficient of variation across treatment pens. On day four and 14 the number of birds were weighed according to cage number to calculate average body weight gain (BWG), final body weight (BW) and feed conversion ratio (FCR) for the test period (day 4 to 14). Total feed intake was calculated by determining the difference between the total amount of feed added to the feed troughs at the beginning of the test period (day 4) from the total amount of feed that was left in the feed troughs at the end of day 14. Feed levels were monitored twice daily and if necessary extra feed was weighed and recorded before being placed into the required feeder. Along with the checking of feeders any spillage that may have occurred during the day was swept up and weighed. At day 14, all remaining feed was weighed back and recorded, and the spillage weight was subtracted to determine accurate feed intake for each treatment. Any mortalities that occurred during the test period were weighed and the weight and day of death was recorded and considered when calculating final FCRm as according to equation 5. In order to keep the data set balanced the three positive control treatments were not in included in the analysis of variance for all performance measurements.

Equation 5:

$$\text{FCRm} = \frac{\text{Total feed intake of pen}}{\text{Total weight gain of pen} + \text{Weight gain of all mortailities}}$$

3.10 Statistical analysis

Due to the odd number of treatments and space limitations in the metabolic house treatments were distributed in the house using a completely randomised design. Outliers were identified and removed from the dataset prior to statistical analysis using JMP® (Version 15.0. SAS Institute Inc., Cary, NC, 1989-2019) statistical package. Performance data (body weight, body weight gain mortality corrected feed conversion ration) and defatted tibia and metatarsal ash sampled from 14-day male broilers were used as the response criteria. Both performance data and bone ash percentage were plotted against analysed phytase values for each treatment. The results were then analysed using non-linear regression as illustrated in equation 6 and demonstrated by Dersjant-Li & Kwakernaak (2019). Bone ash percentage and analysed phytase values for each treatment were plotted using a

piecewise regression model as described by equation 7. A one-way ANOVA was used to determine the difference among the treatments. Non-linear regression was performed using JMP® (Version 15.0. SAS Institute Inc., Cary, NC, 1989-2019) statistical package, whereas piecewise regression was performed using the RStudio statistical package (R Core Team, 2021). To allow for comparisons between the nonlinear regression model and piecewise regression model the R^2 value was calculated using the RStudio package (R Core Team, 2021). The differences of means between treatments for both performance parameters and bone ash were determined using the Tukey's Honest Significant Difference (HSD) test. Differences were considered significant at a level of $P<0.05$.

To determine the correlation between defatted tibia ash and metatarsal ash. The final bone ash results for each criterion were analysed for outliers and removed using JMP® (Version 15.0. SAS Institute Inc., Cary, NC, 1989-2019) statistical package. In the case where a outlier was observed in only one criteria (i.e., defatted tibia ash percentage or metatarsal ash percentage), the corresponding values from the other criteria was also removed to keep a balanced data set. The reported correlation (r) value and R^2 value was determined by making use of multivarient analysis, whereby the pecentage defatted tibia ash was plotted against metatarsal ash percentage. To see if the different phytase products had any influence on the correlation between metatarsal ash and tibia ash, the same multi variant analysis was run, except this time for each individual product.

Equation 6: Non-linear regression

$$y = \mathrm{A} + \mathrm{B} * \mathrm{R}^{\mathrm{X}}$$

Where Y is your dependent variable (performance parameters or ash percentage per treatment)

A= asymptote value

B= maximum increment (a-b=response at x=0)

R= curve coefficient

x= independent variable (analysed phytase value)

Equation 7: Piecewise regression

$$y_i \begin{cases} \beta_0 + \beta_1 X_i + e_i & for\ Xi \leq \alpha \\ \beta_0 + B_1 X_i + B_2(X_i - \alpha) + e_i & for\ Xi > \alpha \end{cases}$$

Where y_i is the value for the i^{th} observation

X_i is the corresponding value for the independent variable (analysed FTU/kg)

α is the breakpoint

e_i are assumed to be indepent, additive errors with mean zero

$\beta_1$and $\beta_1 + \beta_2$ are the slopes of the lines

CHAPTER 4: Results

4.1 Diet analysis

The reason for some of the observed variation in analysed dose values vs targeted dose values amongst treatments as seen in Table 4.1 may be due to the over-reach of the activity in all three products. The analysed Ca and total P content of the treatment diets is shown in Table 4.1. The analysed Ca and P all test diets were higher than the expected formulated values. Similarly, on average, the analysed Ca and P content for phytase supplemented diets were higher than their formulated values. Due to the way the mixing process was carried out, the Ca and P values for each diet were relatively similar to one another. The Ca and P values for the Product A supplemented diets was 0.71% and 0.35%, for Product B it was 0.72% and 0.36% and lastly, for Product C it was 0.72% and 0.36%. The reason for these analysed values being higher than the formulated values may have been due to human error during the mixing process. As all raw material ingredients were analysed for Ca and P, and their respective contributions considered when formulating the final diets.

Table 4.1 Dietary analysis of treatment diets

	Formulated			Analysed		Product	Formulated	Analysed
Treatment no.	Ca (%)	oP (%)	Total P (%)	Ca (%)	Total P (%)		Phytase (FTU)	Phytase (FTU)
1 (NC)	0.60	0.11	0,31	0.74	0.35	-	0	146
2	0.60	0.11	0,31	0.72	0.35	Product A	500	636
3	0.60	0.11	0,31	0.71	0.35	Product A	1000	1291
4	0.60	0.11	0,31	0.73	036	Product A	1800	2224
5	0.60	0.11	0,31	0.73	0.36	Product A	2500	3067
6	0.60	0.11	0,31	0.68	0.34	Product A	3500	4516
7	0.60	0.11	0,31	0.72	0.36	Product B	500	458
8	0.60	0.11	0,31	0.71	0.35	Product B	1000	1022
9	0.60	0.11	0,31	0.70	0.35	Product B	1800	2166
10	0.60	0.11	0,31	0.73	0.37	Product B	2500	3142
11	0.60	0.11	0,31	0.73	0.36	Product B	3500	3621
12	0.60	0.11	0,31	0.71	0.35	Product C	500	646
13	0.60	0.11	0,31	0.72	0.35	Product C	1000	908
14	0.60	0.11	0,31	0.72	0.36	Product C	1800	1887
15	0.60	0.11	0,31	0.72	0.36	Product C	2500	2904
16	0.60	0.11	0,31	0.74	0.36	Product C	3500	3909
17 (PC 1)	0.60	0.27	0,49	0.90	0.57	-	0	200
18 (PC 2)	0.68	0.30	0,52	0.95	0.60	-	0	214
19 (PC 3)	0.72	0.33	0,56	1.00	0.63	-	0	198

4.2 Growth performance

In Table 4.2 the recorded performance parameters from day 4-14 are summarised. BW and BWG were significantly different (P<0.05) for the various phytase products compared to the negative control treatment. Furthermore, there were significant differences within the various phytase products as seen in Table 4.2. The results outlined in Table 4.2 show that as phytase dose increased so did BW and BWG. Product B at 3621 FTU/kg exhibited the greatest BW (405.14 g) and BWG (234.35 g/bird). Whereas product A at 636 FTU/kg and product C at 646 FTU/kg both elicited the lowest BW (327.11 vs 326.85 g) and BWG (161.43 vs 160.74 g/kg), respectively. However, when looking at the nonlinear regression parameter estimates outlined in Table 4.3. there was no significant difference (P<0.05) between any of the parameter estimates. The average mortality rate for the trial period was low (1.09 %) and was not significantly different among treatments. Lastly, there were no significant differences (P<0.05) in FCRm between treatments from day 4-14. However, as phytase dose increased, FCRm decreased numerically for each phytase product. Product A exhibited the best FCRm (1.28) at 4516 FTU/kg feed, whereas Product C exhibited the poorest FCRm (1.60) at 646 FTU/kg feed.

Table 4.2 The effects of phytase treatment on feed intake, body weight and feed conversion ratio in broilers (4-14 days-of-age)

Treatment	Product	Analysed phytase dose (FTU/kg)	Feed intake	Body weight (g/bird)	Body weight gain (g/bird)	Feed conversion ratio (g/g)[1]
1	NC	146	300.46	277.36^{f}	85.18^{f}	1.93
2	Product A	636	339.92	327.11^{e}	161.43^{e}	1.55
3	Product A	1291	362.22	349.07^{bcde}	182.04^{bcde}	1.55
4	Product A	2224	372.50	376.60^{abc}	206.74^{abc}	1.41
5	Product A	3067	387.300	376.83^{abc}	207.20^{abc}	1.46
6	Product A	4516	311.96	382.41^{ab}	218.33^{ab}	1.28
7	Product B	458	305.08	333.15^{de}	163.54^{de}	1.52
8	Product B	1022	299.73	349.12^{bcde}	177.36^{cde}	1.40
9	Product B	2166	349.37	368.33^{abcd}	195.11^{abcd}	1.46
10	Product B	3142	359.70	376.67^{abc}	211.48^{abc}	1.39
11	Product B	3621	350.77	405.14^{a}	234.35^{a}	1.30
12	Product C	646	310.88	326.85^{e}	160.74^{e}	1.60
13	Product C	908	354.533	344.81^{bcde}	179.07^{cde}	1.49
14	Product C	1887	390.63	342.66^{cde}	174.19^{cde}	1.65
15	Product C	2904	370.40	374.63^{abc}	207.59^{abc}	1.48
16	Product C	3909	383.23	372.48^{abc}	201.04^{abcd}	1.48

[a-f] Data points bearing different superscript letters within columns are significantly different (P<0.05)
[1] Corrected for mortality.

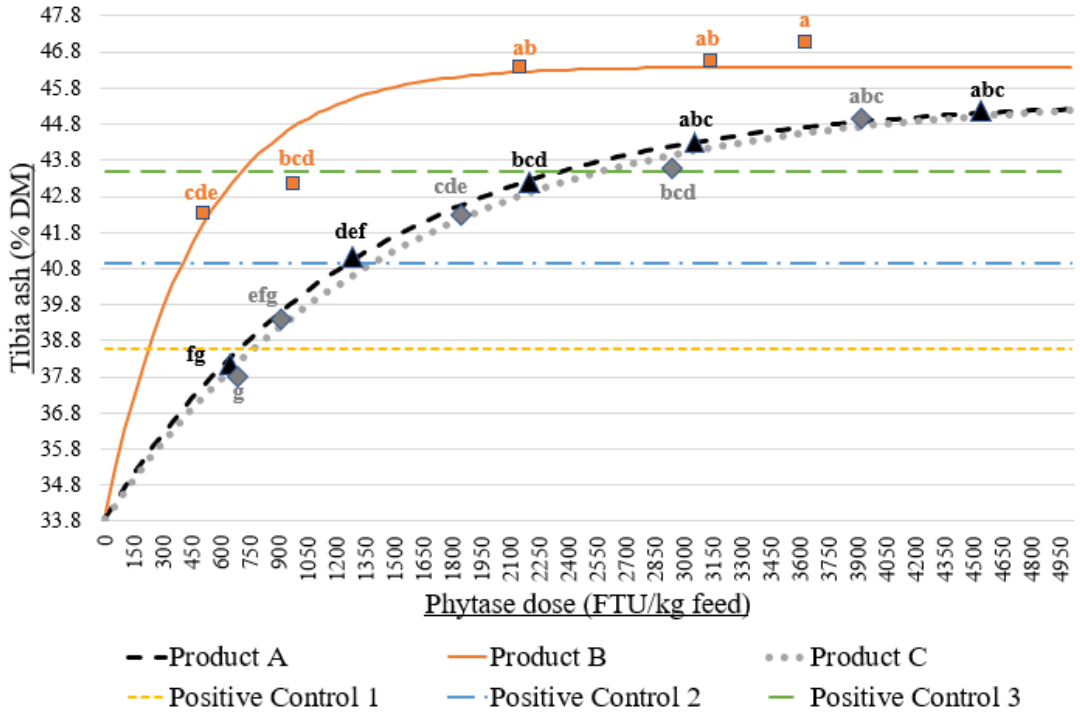
Table 4.3 Description of nonlinear parameters for body weight, body weight gain and FCRm in broilers (4-14 days-of-age)

Y	Parameters of nonlinear fitted curve				RMSE	R^2
	A^1	B^2	R^3	X		
Y= Body weight (g)						
Product A	384.51	-106.50	0.999	Analysed (FTU/kg)	16.25	0.87
Product B	390.89	-113.10	0.999	Analysed (FTU/kg)	23.41	0.78
Product C	372.72	-92.95	0.999	Analysed (FTU/kg)	16.48	0.84
Y= body weight gain (g)						
Product A	214.03	-128.97	0.999	Analysed (FTU/kg)	15.86	0.92
Product B	218.07	-132.13	0.999	Analysed (FTU/kg)	23.83	0.84
Product C	201.33	-115.98	0.993	Analysed (FTU/kg)	15.81	0.91
Y = FCRm						
Product A	1.378	0.547	0.999	Analysed (FTU/kg)	0.159	0.69
Product B	1.375	0.558	0.997	Analysed (FTU/kg)	0.175	0.66
Product C	1.523	0.411	0.997	Analysed (FTU/kg)	0.202	0.47

[1]A refers to the asymptotic value
[2]B refers to the gradient of the curve
[3]R refers to the curve coefficient

4.3 Bone ash

As expected, the addition of iP through the supplementation of MDCP in the three positive controls improved both tibia ash and metatarsal ash concentration at day 14, in a dose-dependent manner when compared to the P-deficient NC diet as seen in Figure 4.1 and Figure 4.2. Furthermore, the supplementation of the phytase enzyme improved tibia ash significantly ($P<0.05$) when compared to the NC diet. However, this same significant increase was not seen when comparing metatarsal ash concentrations against the NC diet, which contained no supplemented phytase. Increasing phytase dose for all three products resulted in a diminishing curvilinear increase in tibia and metatarsal ash concentration, respectively (Figure 4.1 and Figure 4.2).



[a-g] Data points bearing different superscript letters are significantly different (P<0.05)

Figure 4.1 Relationship between increasing phytase dose and tibia ash concentration of male broilers at 14 days of age

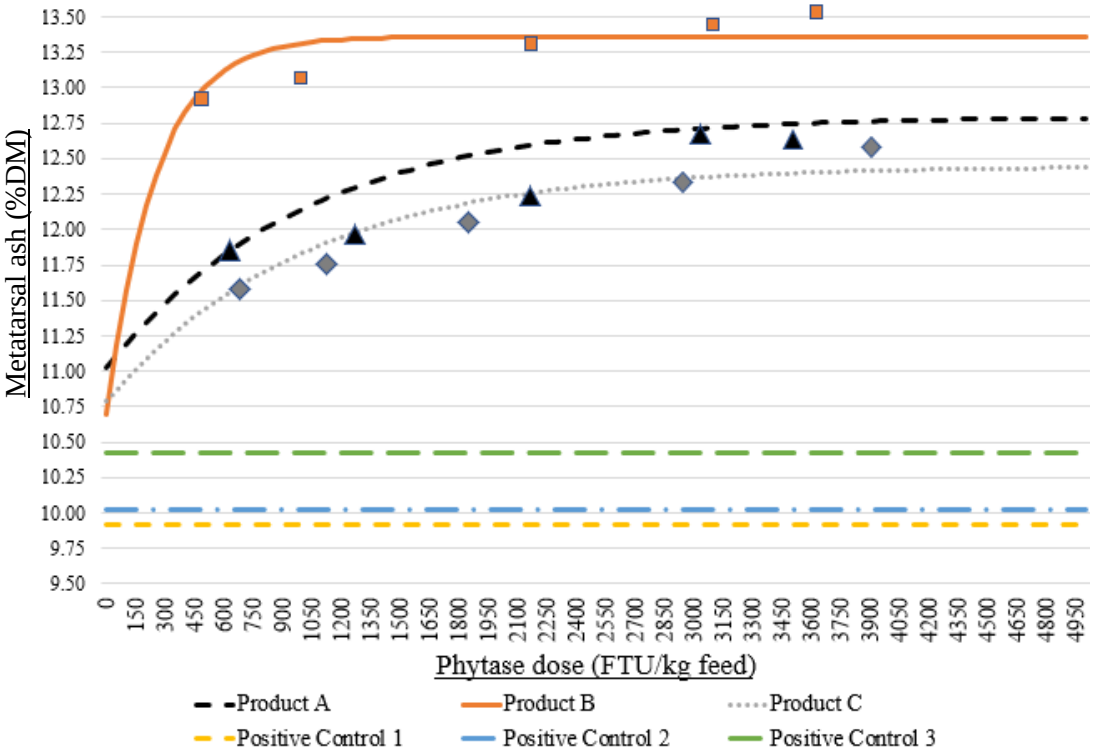


Figure 4.2 Relationship between increasing phytase dose and metatarsal ash concentration of male broilers at 14 days of age

4.3.1 Tibia ash

A dose response for each phytase product was noted as there were significant increases in tibia ash as phytase dose increased (P<0.05) until the asymptote was reached for each respective phytase product. The differences in tibia ash are reported in Table 4.4. Birds on the lowest dose of Product B (458 FTU/kg) exhibited significantly higher (P<0.05) tibia ash percentage values (42.60%) compared to the lowest dose of product A (636 FTU/kg; 38.30%) and product C (646 FTU/kg; 37.57%). There was a significant difference in tibia ash percentage between the third highest dose of product B (2166 FTU/kg; 46.15%) compared to its corresponding product C (1887 FTU/kg; 42.59%). This could potentially be explained by looking at Table 4.5 in which the gradient value (B) for each product was compared by making use of a comparison of means test at a $\alpha = 0.05$. The comparison showed that the gradient of the curve for tibia ash was significantly steeper for product B (-12.358) when compared with product A (-11.58) and product C (-11.71), respectively, at a α level of 0.05. However, there was no significant when comparing Product, A with Product C at $\alpha = 0.05$.

Table 4.4 Ranking of phytase products according to tibia ash (%)

Treatment	Product	Analysed phytase dose (FTU/kg)	Tibia ash (%), least square mean value
T11	Product B	3621	47.24^{a}
T10	Product B	3142	46.24^{ab}
T9	Product B	2166	46.15^{ab}
T6	Product A	4516	44.93^{abc}
T16	Product C	3909	44.84^{abc}
T5	Product A	3067	44.63^{abc}
T15	Product C	2904	43.58^{bcd}
T4	Product A	2224	43.19^{bcd}
T8	Product B	1022	43.14^{bcd}
T7	Product B	458	42.60^{cde}
T14	Product C	1887	42.59^{cde}
T3	Product A	1291	40.98^{def}
T13	Product C	908	39.50^{efg}
T2	Product A	636	38.30^{fg}
T12	Product C	646	37.57^{g}
T1	Negative Control	0	33.93^{h}

[abc] Data points baring different superscript letters within a column are significantly different at P<0.05

Looking at the ranking of the phytase products as outlined in Table 4.4, there were no significant differences in reported tibia ash percentage for the three highest phytase doses (4516, 3067, and 2224 FTU/kg) for Product A. Furthermore, there were no significant differences (P<0.05) between treatments 6, 5, 2 and 3. Birds fed Product B exhibited significant differences (P<0.05) in

ash percentage when comparing with treatments 7, 8 and 11. However, there was no significant difference between treatments 7 and 8. A significant difference ($P<0.05$) was reported between treatments 7 and 10 for ash percentage, whilst no difference was seen between treatments 9, 10 and 11. There were significant differences ($P<0.05$) in reported tibia ash percentage for Product C when comparing treatment 6 and 15 to treatments 12 and 13. However, there was no significance between treatments 14, 15 and 16 and treatments 12 and 13.

Table 4.5 Description of nonlinear parameters for tibia ash (%) and metatarsal ash (%) in 14-day old broilers

Y	Parameters of nonlinear fitted curve				RMSE	R^2
	A[1]	B[2]	R[3]	X		
Y=Tibia ash (%)						
Product A	45.51	-11.58[b]	0.999	Analysed (FTU/kg)	1.37	0.92
Product B	46.40	-12.36[a]	0.997	Analysed (FTU/kg)	1.67	0.95
Product C	45.59	-11.71[b]	0.999	Analysed (FTU/kg)	1.61	0.89
Y=Metatarsal ash (%)						
Product A	12.80	-1.78	0.999	Analysed (FTU/kg)	0.64	0.32
Product B	13.36	-2.67	0.996	Analysed (FTU/kg)	1.10	0.18
Product C	12.45	-1.66	0.999	Analysed (FTU/kg)	0.79	0.12

[1]A refers to the asymptotic value
[2]B refers to the gradient of the curve
[3]R refers to the curve coefficient
[abc] Data points baring different superscript letters within a column are significantly different at $\alpha=0.05$

Using the three positive controls as reference points, Table 4.6 shows that Product B was able to replace the three positive controls at a lower predicted phytase dose (FTU/kg) compared to the other two products. For the first positive control (PC1), Product B was able to replace 0.6 g Ca and 0.27 g iP at 223.07 FTU/kg compared to Product A and Product C which required 692.67 FTU/kg and 767.32 FTU/kg, respectively, to replace the same amount of Ca and P. With the second positive control (PC2), Product B was once again able to replace 0.68 g C and 0.27 g iP at a lower phytase dose (397.15 FTU/kg) compared to that of Product A (1250.18 FTU/kg) and Product C (1377.94 FTU/kg). Lastly, looking at the third and final positive control (PC3), Product B was able to replace 0.72 g Ca and 0.33 g iP at a lower predicted phytase dose (701.13 FTU/kg) compared to Product A (2340.42 FTU/kg) and Product C (2555.33 FTU/kg). This data infers that the higher the dose rate the greater the amount of Ca and P that can be replaced in the diet to a point. Furthermore, under the given trial conditions Product B was the most efficacious out of the three products as it was able to replace the same Ca and inorganic P in the diet at a lower dose compared to that of Product A and C.

Table 4.6 Amount of predicted phytase dose for each product to replace the equivalent amount of calcium and phosphorous found in each positive control diet using tibia ash as the response criterion

Positive control			Phytase Product	Predicted phytase dose (FTU/kg)
Treatment	Added inorganic Ca (g)	Added inorganic P (g)		
PC 1	0.6	0.27	Product A	692.67
			Product B	223.07
			Product C	767.32
PC 2	0.68	0.29	Product A	1250.18
			Product B	397.15
			Product C	1377.94
PC 3	0.72	0.33	Product A	2340.42
			Product B	701.13
			Product C	2555.33

4.3.2 Metatarsal ash results

No significant interaction in metatarsal ash percentage between the three different phytase products as well as across the various phytase dose levels was observed (Figure 4.2 and Table. 4.7). This is in direct contrast to results obtained for tibia ash percentage in which a significant ($P<0.05$) dose response was observed across the fived phytase doses for each phytase product. However, a high level of variation in reported metatarsal ash percentage values across treatments were noted, as indicated by the low R^2 values for all three products as evident in Table 4.5. Product B was able to express the highest asymptotic value of 13.36% compared to Product A (12.80%) and Product C (12.45%) as seen in Table 4.5, however, these differences between phytase dose were not significantly different between phytase products when making use of a comparison of means test ($\alpha=0.05$).

Table 4.7 Ranking of phytase products according to metatarsal ash (%)

Treatment	Product	Analysed phytase dose (FTU/kg)	Metatarsal ash (%), least square mean value
T11	Product B	3621	13.56
T10	Product B	3142	13.51
T9	Product B	2166	13.2
T8	Product B	1022	13.09
T7	Product B	458	12.98
T5	Product A	3067	12.73
T6	Product A	4516	12.69
T16	Product C	3909	12.55
T15	Product C	2904	12.31
T4	Product A	2224	12.24
T14	Product C	1887	12.05
T13	Product C	908	11.91
T3	Product A	1291	11.9
T2	Product A	636	11.83
T12	Product C	646	11.65
T1	Negative Control	0	10.66

4.4 Bone ash: Piecewise regression

As a part of the analysis of data a two-segment piecewise regression model was run for each phytase product. A piecewise regression model, or more commonly known as “broken-stick’’ model, is a statistical model that makes use of two or more straight lines which are joined at unknown point(s), called ‘‘breakpoint(s)” (Toms & Lesperance, 2003). Breakpoints are a result of when there are distinct differences in two or more linear relationships in the data, accompanied by a sharp change in direction i.e., gradient. The simplest form of piecewise regression and the form used in this study is made up of two straight lines in which a single breakpoint is used. This breakpoint value is interpreted as the point at which dosing beyond this point will lead to no further response in the dependent variable (i.e. bone ash %).

4.4.1 Tibia ash

The reported breaking point values along with their residual standard error and goodness of fit values (R^2) for both tibia and metatarsal ash can be seen in Table 4.8. By looking at Figure 4.3 and Table 4.8 together, it is evident that Product A achieved the greatest tibia ash break point value of 43.23%, although it was at a higher analysed dose level of 1723.29 FTU/kg compared to product B (42.79% at 468.79 FTU/kg) and Product C (41.89% at 1309.27 FTU/kg), respectively. The reason

for the lower reported analysed phytase level seen in product B is because product B exhibited the steepest gradient (0.02) allowing it to obtain its breakpoint of 42.79% as a lower phytase dose compared to the other 2 products. Lastly, the birds fed the positive control diets demonstrated the same incremental increase in tibia ash percentage as already described in section 4.2.1. The amount of predicted phytase dose required by each product to replace a certain amount of Ca and iP found in each of the positive controls are depicted in Table 4.9. Product B was able to replace 0.6 g Ca and 0.27 g iP at a lower dose level of 245.96 FTU/kg compared to Product A (861.96 FTU/kg) and Product C (765.11 FTU/kg). For the second positive control (PC2) Product B was able to replace 0.68 g Ca and 0.29 g iP at a predicted dose of 370.22 FTU/kg, compared to Product A, which needed 1297.41 FTU/kg and 1151.64 FTU/kg for Product C. Lastly, in the case of the third positive control (PC3), product B was able to replace 0.72 g Ca and 0.33 g iP at a predicted dose level of 950.22 FTU/kg, whereas Product A and C required 2177.55 FTU/kg and 2701.67 FTU/kg, respectively.

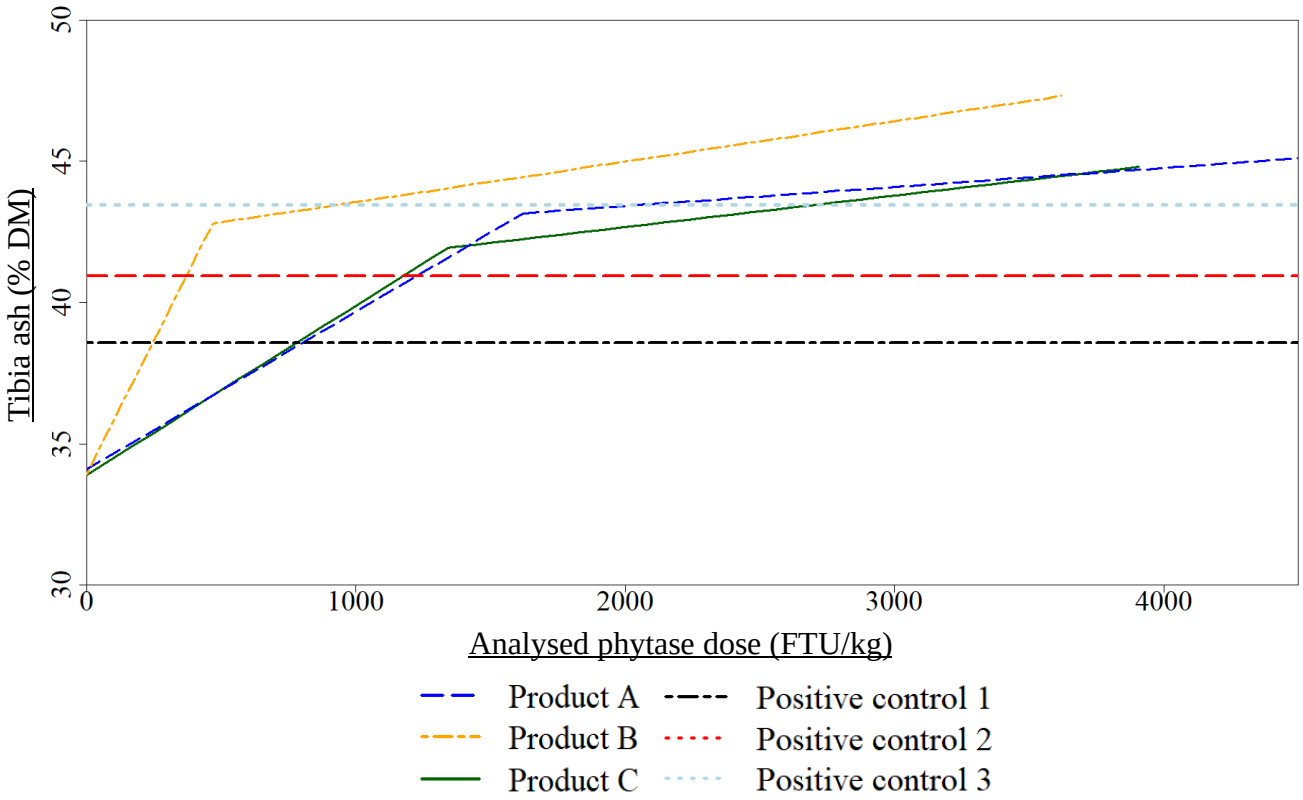


Figure 4.3 Piecewise regression model illustrating the effect of analysed phytase dose (FTU/kg) of three different phytase products on tibia ash (%DM) in 14-day old male broilers

Table 4.8 Analysis of piecewise regression estimates for tibia and metatarsal ash (%) in 14-day old broilers

	Breaking point coordinates				
Phytase product	Y-value (Tibia ash %DM)	X-value (Analysed FTU/kg)	Gradient of 1st line segment	Residual std error	R^2
A	43.23	1723.29	$5.40x10^{-3}$	0.83	0.90
B	42.79	468.48	0.02	1.52	0.93
C	41.89	1309.27	$6.08x10^{-3}$	1.14	0.88
Phytase product	Y-value (Metatarsal ash %DM)	X-value (Analysed FTU/kg)	Gradient of 1st line segment	Residual std error	R^2
A	11.53	661.86	$1.31x10^{-3}$	1.27	0.04
B	13.08	472.90	$5.12x10^{-3}$	1.11	0.11
C	11.52	908.91	$9.46x10^{-4}$	1.34	0.06

Table 4.9 Phytase dose predicted to replace the equivalent amount of calcium and phosphorus in each positive control using tibia ash as the response criteria

Positive control			Phytase Product	Predicted phytase dose (FTU/kg)
Treatment	Added inorganic Ca (g)	Added inorganic P (g)		
PC 1	0.6	0.27	Product A	861.96
			Product B	245.96
			Product C	765.11
PC 2	0.68	0.29	Product A	1297.41
			Product B	370.22
			Product C	1151.64
PC 3	0.72	0.33	Product A	2177.55
			Product B	950.22
			Product C	2701.67

4.4.2 Metatarsal ash

Table 4.7 and Figure 4.5 indicate that Product B exhibited the highest breakpoint value of 13.08% at 472.90 FTU/kg, followed by product B which exhibited a breakpoint value of 11.53% at 661.86 FTU/kg and lastly, product C, which achieved a breakpoint value of 11.52% at 908.91 FTU/kg. As with the reported piecewise tibia ash results, a reason why Product B was able to exhibit a higher breakpoint value of 13.08% at a lower phytase dose compared to the other two products, which in part is due to product B exhibiting a steeper gradient of 5.12×10^{-3} (Table 4.7) compared to product A (1.31×10^{-3}) and product C (9.46×10^{-3}). Due to the NC exhibiting a higher average bone

ash percentage compared to all three positive controlsa comparison of the amount of Ca and P each product could be able to replace is not possible.

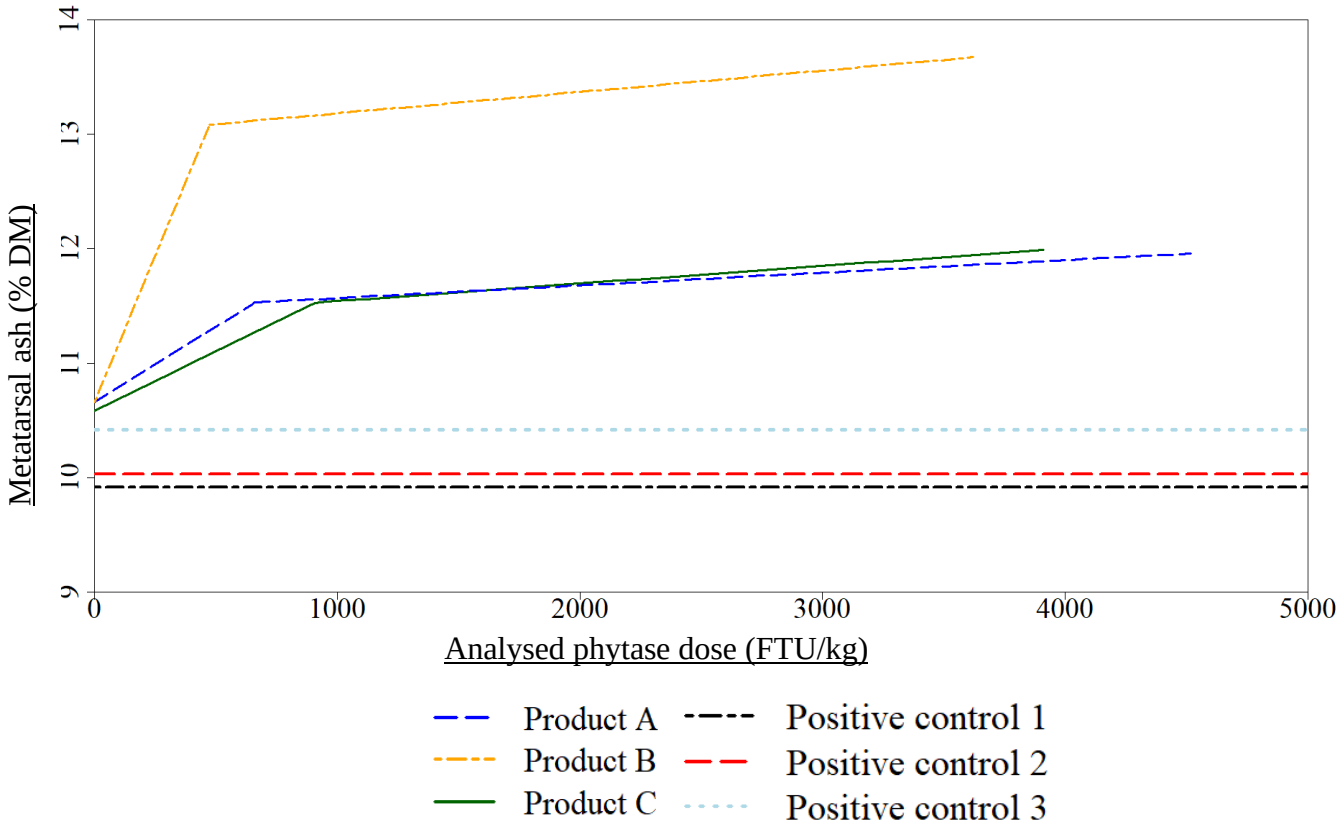


Figure 4.4 Piecewise regression model illustrating the effect of analysed phytase dose (FTU/kg) of three different phytase products on metatarsal ash (% DM) in 14-day old male broilers

4.5 Comparison between nonlinear regression and piecewise regression

The data in Table 4.10 is a summary of the reported goodness of fit values for both regression models used in this study, as well as between phytase product and sampling criterion used. The nonlinear regression model exhibited a larger R^2 value across each product compared to the reported R^2 values for the piecewise regression model. Aditionally, when making use of tibia ash as the selection criterion, the R^2 values were higher than that of the reported R^2 values for metatarsal ash as the selection criterion for both the nonlinear regression model, as well as the piecewise regression model.

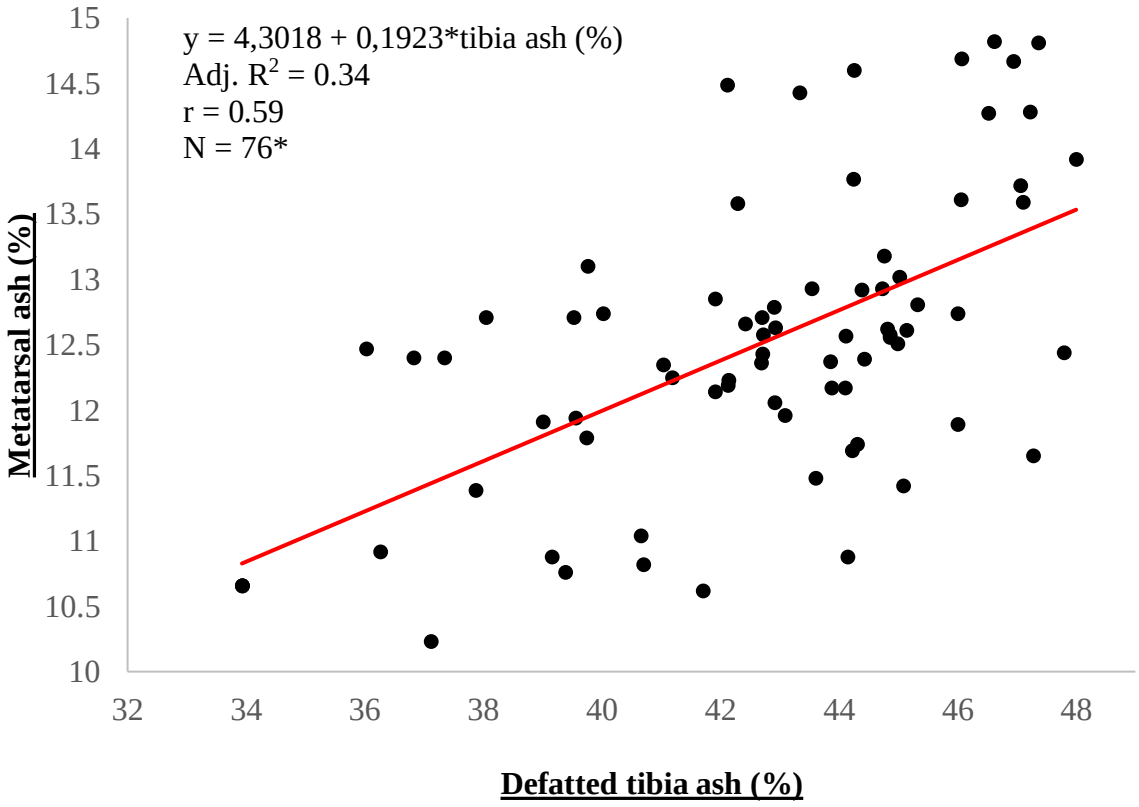
Table 4.10 Comparison of best fit between two different regression models

Model	Non-linear regression			Piecewise regression		
	R^2 value					
Phytase product	A	B	C	A	B	C
Tibia analysis	0.92	0.95	0.89	0.90	0.93	0.88
Metatarsal analysis	0.32	0.38	0.12	0.04	0.11	0.06

4.6 Correlation between defatted tibia ash and metatarsal ash

Metatarsal ash was plotted against defatted tibia ash according to the following regression equation: *Y = 4,3018 + 0,1923*tibia ash (%)* in order to determine the reported correlation (r) value of 0.59 and R^2 value of 0.34 (Figure 4.5). The interpretation of the strength of the relationship between defatted tibia ash percentage and metatarsal ash percentage was based on guidelines outlined by Cohen (1998); were a weak relationship is defined as r = 0.10 to 0.29, medium relationship r = 0.30 to 0.49 and strong relationship r = 0.50 to 1. The strong positive r value of 0.59 indicated that as tibia ash increases so does metatarsal ash. However, as indicated by the poor R^2 value of 0.34, metatarsal bone mineralisation can only be partially explained by defatted tibial bone mineralisation.

As seen in Figure 4.6 all three phytase products exhibit fairly similar correlation values with Product A displaying the strongest correlation of 0.59, followed by Product B (0.49) and Product C (0.46). All three products displayed similar poor R^2 values, with product C having the worst R^2 value of 0.17, followed by Product B (0.21), with Product A displaying the largest R^2 value of 0.31. These results indicate that the phytase product could not fully explain the correlation between defatted tibia ash (%) and metatarsal ash (%) and that other variables such as the Ca and P levels, as well as sampling method, could be influencing the correlation between tibia and metatarsal ash.



*Positive control values for both criterion were not included in final results

Figure 4.5 Correlation scatter plot of relationship between metatarsal ash and defatted tibia ash

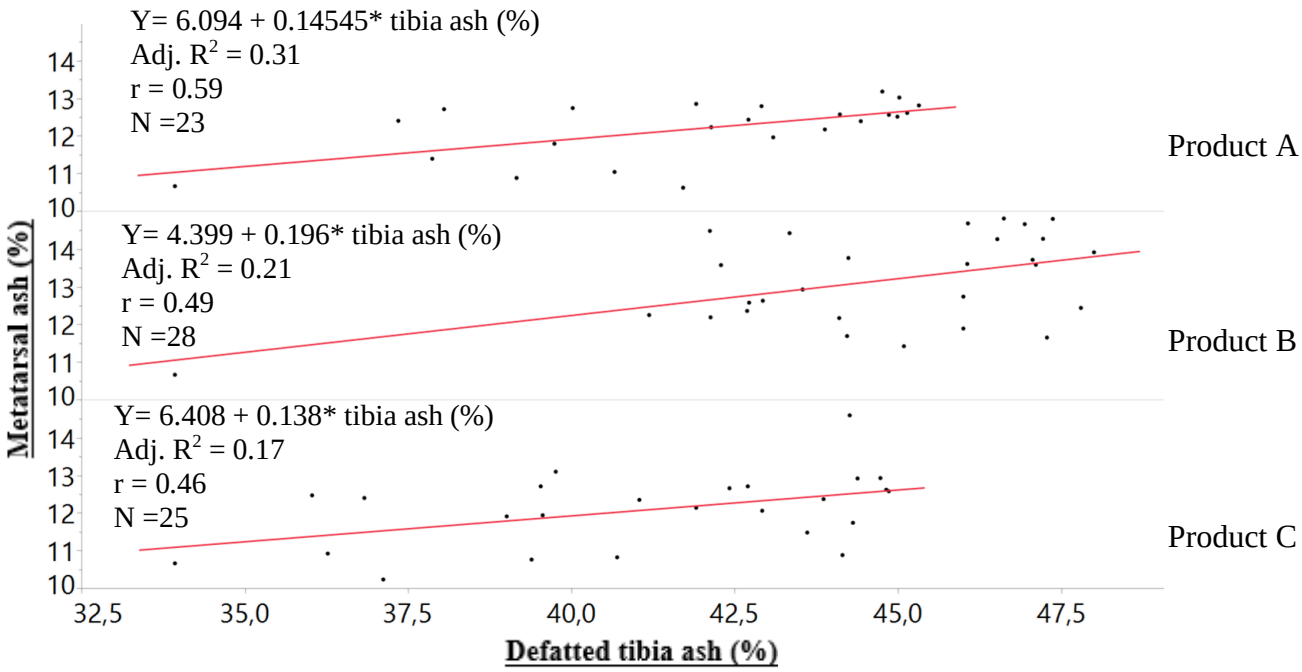


Figure 4.6 Correlation scatter plot of relationship between metatarsal ash and defatted tibia ash for each phytase product

CHAPTER 5: Discussion

5.1 Diet analysis

The study made use of diets formulated to be deficient in total P and Ca to examine if the asymptote at which bone ash is maximised, differs between three different phytase products. It is common knowledge that in the absence of supplemented phytase, high levels of dietary Ca have been shown to chelate with phytate, reducing the amount of P available to the bird (Selle *et al.,* 2009). On average the analysed Ca percentage was 18.33% higher across the three phytase product diets compared to the formulated. According to Walk *et al.* (2021), the higher than formulated results may be due to several factors such as the actual phytase activity being higher than expected, variation in sampling and mixing as well as errors in the assay used to analyse the phytase activity. Similarly, the analysed tP across all three phytase products was more than double that of the formulated tP levels. A similar trend could be seen when comparing the analysed Ca and P values of the three diets to that of their formulated values. These inflated values help to explain some of the results that will be discussed in this chapter as it has been well documented that dietary Ca levels have a detrimental effect on P digestibility (Tamim *et al.*, 2004; Plumstead *et al.*, 2008; Walk *et al.*, 2012). The higher-than-expected Ca values and low analysed tP could also have influenced phytase efficacy and subsequent bone mineralisation. Research by Létourneau–Montminy *et al.* (2008) showed that an excess of Ca may have a detrimental effect on ash results in diets deficient in available phosphorus (aP). Driver *et al.* (2005) showed that phytase exhibited a greater return in diets formulated with high Ca levels and low nPP levels (0.86% Ca and 0.20% nPP) versus diets containing low Ca levels and low nPP levels (0.47% Ca and 0.24% nPP).

Another factor influencing the efficacy of each product was the type of limestone and, more specifically, the rate at which the limestone solubilised. Looking at the dynamic solubility profile of the limestone used for this study (section 3.7.2), one can see that at 5 minutes that 81% of the test limestone had already been solubilised indicating that the limestone used was highly soluble. A limestone with a high rate of solubility as seen by the limestone used in this study has been shown by Kim *et al.* (2019) to be detrimental to P digestibility and phytase efficacy compared to a slower solubilising limestone. This is because a more rapid solubilising limestone results in an influx of Ca ions in the blood, leading to an increased potential for chelation to occur between ionized Ca and phytic acid in the gizzard and proventriculus of the bird, preventing the phytase enzyme from hydrolysing the phytate and thus rendering both Ca and P unavailable to the bird.

5.2 Performance

5.2.1 Body weight and body weight gain

In this trial, significant differences in reported BW and BWG at day 14 were observed across the three phytase products. As expected and demonstrated by Shirley & Edwards (2003) and Dersjant-Li & Kwakernaak (2019), all three phytases exhibited marked improvements in final BW and BWG versus the negative control, as phytase dose increased. The relationship between phytase dose and BWG for all three phytases was curvilinear. This resulted in final BW exhibiting a diminishing curvilinear increase as phytase dose increased. Published information on the growth performance effects of all three phytases used in the study at the higher phytase doses (>2000 FTU/kg) is limited. However, literature does exist for the lower phytase doses (<2000 FTU/kg). Shirley & Edwards (2003) reported BWG values in 16-day-old, mixed sex, Cobb broilers of 424, 459 and 481 g/bird when supplementing a modified *Aspergillus niger* phytase at 750, 1500 and 3000 FTU/kg diet. Dersjant-Li & Kwakernaak (2019) reported BWG values in male Ross 308 broilers from 5 to 21 days-of-age of 848 and 876 g/bird when supplementing a *Buttiauxella sp.* phytase, similar to that of Product A at 887 FTU/kg and 1046 FTU/kg, respectively. Furthermore, in the same study they showed that supplementing an *E. coli* phytase at analysed phytase levels of 1505 and 1811 FTU/kg provided BWG of 848 and 844 g/bird. It must be noted that any direct comparison with available literature should be viewed with caution. The reason for this being as already discussed in the review of literature and summarised by Dersjant-Li *et al.* (2015) and Wealleans *et al.* (2016), is that numerous factors such as the age of the bird, Ca and P levels in the diet, phytase product used and level of phytase dosed in the diet can all have an influence on phytase efficacy, leading to differences in reported performance values.

5.2.2 Feed conversion ratio

Apart from ensuring an adequate and balanced nutrient intake, feed intake (FI) has been suggested as the single-most important factor in determining the growth rate of broilers (Ferket & Gernat, 2006). Idan *et al.* (2020) stated that the particle size of the grain and feed form affect bird performance, especially during the early stages of growth. In this trial, there was no significant differences in FCR between treatments, which contrasts with research done by Dersjant-Li & Kwakernaak 2019). The authors reported a significant reduction ($P<0.05$) in FCR as the dose of each phytase increased. Although the same trend was numerically seen in this current trial the potential

reason for the lack of significance in reported FCR values between treatments could be partially explained by the higher than formulated Ca values. Another reason could be due the low feed intake from day 4 to 14 seen across all treatments. The low feed intake could be a result of the form of feed given to the birds during the trial and the amount of feed wastage observed. For the duration of this trial the birds were fed a mash diet. Jahan *et al.* (2006), suggested that mash is less palatable to broilers compared with crumbles, thus reducing feed intake. One of the challenges faced in the trial was the amount of feed wastage due to birds flicking feed out of their feeders. A common practice to correct for feed spillage is to weigh the amount of feed spilled and account for it in the final feed intake calculations. That was not possible for this trial due to the proximity of individual feed troughs, making it difficult to distinguish from which trough the feed came from.

5.3 Bone ash analysis

Phytase is typically included in broiler diets with the principal aim of liberating phytate-bound P from phytate, thus improving the digestibility of P (Driver *et al.,* 2005; Dersjant-li & Kwakernaak, 2019). To determine overall phytase activity, tibia ash concentration is often measured to estimate the degree of bone mineralisation in broilers. Bone mineralisation is sensitive to the bioavailability of minerals within a diet and is directly correlated to phosphorous and calcium deposition with increasing bone ash being associated with increased amounts of available P (Viveros *et al.,* 2002; Hall *et al.,* 2003; Shastak *et al.,* 2012a). Research from various authors have shown that phosphorus availability, which is essential for growth and skeletal development in chickens, can be increased via the use of phytase enzymes (Shaw *et al.,* 2010, Li *et al.,* 2015). The reason 14 days was chosen as the duration for this study is because work done by Olukosi *et al.* (2007) and Majeed *et al.* (2020), showed that phytase supplementation is most effective for the first 14 days of the bird's life. Olukosi *et al.* (2007) stated that one of the reasons why phytase supplementation is so effective during this time, is because young chicks have a lower amount of endogenous phytase compared to older birds, leading to lower Ca and iP bioavailability.

In many biological processes the response of a variable changes at a critical value. In the case of microbial phytase supplementation the response to phytase supplementation can be described as a nonlinear diminishing returns function (Shirley & Edwards, 2003). A diminishing response would be expected with increasing phytase inclusion level until a critical, or more commonly referred to asymptotic, value is reached as demonstrated in the dose response trial by Dersjant-Li & Kwakernaak (2019). It is of economic interest to the broiler industry to know at what dose level this asymptotic

value is achieved as dosing beyond that point could become less feasible from a feed cost point of view. Conversely, dosing at below the asymptotic value would be considered as unutilised potential. Research by Sherley & Edwards (2003) showed that at conventional levels (≤500 FTU/kg) this asymptotic value is not achieved and that super-dosing phytase (levels exceeding 1500 FTU/kg) offers an opportunity to further increase phytate degradation and improve bone mineralisation (Walters *et al.,* 2019). Both tibia ash percentage and metatarsal ash percentage exhibited a diminishing curved linear increase as phytase dose increased across all three treatments. The reason for this increase in bone mineralisation according to Gautier *et al.* (2017) is due to the increase in availability of minerals because of the hydrolyses of phytate mineral complexes when phytase is supplemented in the diet.

5.3.1 Tibia ash analysis

Majeed *et al.* (2020), states that the most influential factors in determining the degree of bone mineralisation is the level of Ca and iP in the diet. It was noted in this trial (Figure 4.2) that an incremental increase in Ca and iP in the three positive control diets increased tibia ash percentage. The supplementation of incremental levels of MDCP into the three positive controls (PC1, PC2, and PC3) increased tibia bone ash by 14%, 23%, and 27% compared to that of the NC. These results were expected as Ca and iP contribute significantly to bone mineralisation and increasing their levels would result in greater availability, leading to improved tibia ash content. This is in line with work done by Majeed *et al.* (2020) and Driver *et al.* (2005), where both authors reported improved tibial bone ash percentage when making use of diets higher in Ca and iP levels compared to diets with lower Ca and Pi levels in 14 and 16-day-old broilers, respectively. Furthermore, Driver *et al.* (2005) showed that tibia ash is maximised when dietary Ca levels are set at 1%. Additionally, birds fed the NC diet exhibited significantly poorer bone mineralisation, as indicated by the low tibia ash percentage, which may have resulted from insufficient levels of P and too high levels of Ca in the NC diet. The results of this trial are in line with those achieved by Walters *et al.* (2019) in which they showed that a reduction in nPP by 0.25% compared to the positive control diet of 0.43%, nPP decreased tibia ash percentage by 7.83% in 14-day old broilers.

Table 4.3 and Figure 4.2 showed tibia ash percentage improved ($P<0.05$) with increasing levels of supplemented phytase for all three products until their respective asymptotic values were reached. This is in line with research published by Shirley & Edwards (2003), in which they demonstrated that tibia ash percentage increased in a nonlinear diminishing manner as phytase dose

increased. To better understand this increase in bone mineralisation, one must realise that P is not only an essential nutrient in numerous metabolic pathways but more importantly approximately 80% of P from the diet can be found in the skeleton making bones an important selection criterion for estimating P availability (De Groote and Huyghebaert, 1997). Thus, it would stand to reason that the more P and Ca that is available to the bird, the greater the bone mineralisation (Qian *et al.*, 1996). Qian *et al.*, (1996) reported on the histology of tibiae from chicks fed phytase-supplemented, tP-deficient diets. Their findings indicate that higher levels of dietary phytase increased the availability of dietary Ca and P, thereby allowing for improved bone growth and mineralisation. This is confirmed by work done by Walters *et al.* (2019), in which the addition of phytase improved the availability of P in the gastrointestinal tract, resulting in improved digestibility of P and increased availability of minerals required for increased bone mineralisation.

5.3.2 Metatarsal ash analysis

One of the disadvantages of determining tibia ash is the laborious process involved in dissecting and cleaning the bones as stated by Cambell *et al.* (1945). This made it of interest to explore the use of metatarsal ash as a criterion for phytase efficacy due to the reduced amount of time and labour required to obtain samples. The metatarsal ash results reported in Figure 4.2 showed no significant differences within phytase or across phytase products compared to results obtained from the tibia ash (Figure 4.1), which showed significant differences in dose response within products as well as across products. Although no significant differences were reported for metatarsal ash (Table 4.5) Product B exhibited the highest metatarsal ash asymptotic value (13.36) followed by Product A (12.80) and Product C (12.45), which is slightly different to the ranking reported for tibia ash asymptotic values with Product B showing the highest value (46.40), followed by Product C (45.59) and Product B (45.51). Not only were there no significant differences in metatarsal ash percentage but results were also highly variable, with the NC diet outperforming all three PC diets.

A possible reason for the NC diet outperforming the positive control diets could be the excess amount of analysed Ca in the diet, which could have had a detrimental effect on subsequent P utilisation, as the excess Ca would have bound with dietary phytate in the absence of supplemented phytase, resulting in poor bone mineralisation due to the lack of mineral availability. Although this effect of phytate chelating with Ca to render dietary P unavailable to the bird is well documented (Tamim *et al.*, 2004; Plumstead *et al.*, 2008), this effect was not seen when reviewing the tibia ash results (Figure 4.1). We can only, therefore, hypothesise that the reason for the NC diet outperforming

all three PC diets is due to sampling error when removing the metatarsals during sampling. Combined with the sensitive nature of the criterion when being analysed, could have led to variation in the reported metatarsal ash percentage values. Shastak *et al.* (2012a) stated that there is no fixed methodology as to which metatarsal or joint metatarsal should be removed, leading to ambiguity when it comes to sampling and interpreting results. Due to the relatively small size, and subsequent low weight, of the metatarsals, any variation in sampling technique can lead to large standard deviation in reported results (Scholey & Burton, 2017).

5.4 Comparison between nonlinear regression and piecewise regression

When comparing the equivalancy values obtained from both statistical methods as seen in Table 4.6 and Table 4.9. One can see that the statistical methogology used has a impact on the reported equivalance values. To this author's knowledge, this is the first study comparing the use of nonlinear regression and piecewise regression in broilers using bone ash percentage as the selection criterion. Even though literature is scarce, similarities and meaningful conclusion can be drawn from looking at other fields of study.

One way of comparing how well a model fits the data is by looking at the goodness of fit value. The nonlinear regression model exhibited higher R^2 values across the three products compared to that of the piecewise regression model (Table 4.10). This indicated that when ash percentage is used to analyse the effects of increased phytase supplementation, a non-linear regression model would be better suited than a piecewise regression model, keeping in mind that any regression model is only as good as the data. A potential reason for the lower reported R^2 values when making use of the piecewise regression model, could be due to the model's inability to explain the relationship between phytase dose and bone mineralistation. The response to phytase supplementation can be best described as a nonlinear diminishing returns function (Shirley & Edwards, 2003). As with all enzymes, phytase is limited by the amount of substrate available (i.e., phytate), explaining the issue when making use of segmented piecewise regression. Linear, broken-line regression presumes that the response to phytase dose is linear when it is not, as the rate of change in bone ash percentage decreases as the amount of substrate is used up (Robbins *et al.*, 2006). Adding to the issue is the method used to estimate the breakpoint value; in this study the value was unknown and needed to be estimated by calculating the mean square error value for each dose. A study by Parr *et al.* (2003), evaluated the isoleucine requirements of growing pigs and concluded that the use of a straight broken-line model, like the one used in this study, tended to underestimate the isoleucine requirement of the

pig, which also has a curvilinear response. Robbins *et al.* (2006) argued that because broken-line analysis uses a straight-line, single-breakpoint model, this tend to underestimate the point (asymptote) at which a response is no longer elicited. This is claim by Robbins *et al.* (2006) is supported by the results obtained from this study as the point at which a response is no longer elicited is lower when making use of piecewise regression as opposed to nonlinear regression for both tibia and metatarsal ash percentage. The authors further suggested that data exhibiting a curvilinear response, should make use of a model utilising a quadratic component.

5.5 Comparison between phytase products

The results reported in this study showed that Product B has a greater efficacy compared to Product A and C, regardless of sampling method and regression model used. Product B exhibited a significantly greater mean asymptotic value compared to the other two products when making use of nonlinear regression for tibia ash (46.40%), however, product B only exhibited a numerically higher mean asymptotic value (13.36%) when analysing metatarsal ash. Furthermore, Product B exhibited a numerically better mean asymptotic value for all three performances parameters measured (BW = 390.89, BWG = -218.07 & FCR =1.375). One of the major factors influencing phytase efficacy is the absolute concentrations of Ca and P in the diets (Driver *et al.*, 2005; Amerah *et al.*, 2014; Li *et al.*, 2016). In this study, the dietary Ca and P values for each product were similar due to the mixing process implemented when mixing the treatment diets (Table 4.1). An interesting observation from this study was, that although the analysed activity levels varied among the treatment diets, with Product A and C showing on average marginally higher analysed phytase activity compared to that of Product B, these higher analysed values did not necessarily translate into Product A and C having improved bone ash percentage values when compared to that of Product B (Table 4.1). This highlights the fact that analysed values of phytase activity cannot be used as a predictor of phytase efficacy when measuring bone ash percentage in the bird.

There are numerous factors that influence the efficacy of phytase enzymes. If the differences in reported ash percentage values cannot be attributed to analysed phytase activity (i.e., products exhibiting similar analysed phytase units), then there must have been other factors at play. A detailed explanation of such factors can be found in the review of literature done by Dersjant-Li *et al.* (2015). This review highlighted the fact that apart from dietary factors such as Ca and P levels, other factors, such as biochemical properties, can influence the efficacy of phytase enzymes. Such biochemical properties include pH profile, stability under digestive tract conditions, temperature stability, kinetic

constants, and substrate specificity. All these properties can be influenced by the donor organism of the phytase being used. All three phytase products tested in this study differed in their donor organism. The difference in phytase efficacy between the three different products can thus be partially explained by the differing biochemical properties of each product when looking at the tibia nonlinear regression graph (figure 4.1) and table 4.4. One reason why Product B resulted in a statistically higher asymptote ($P<0.05\%$) compared to the other two products could be due to the improved biochemical properties of Product B. Even though the properties of each product could be compared after consulting with each manufacturer, this would be a frivolous task since each manufacturer makes use of different assay conditions to determine their product's respective enzymatic properties. This highlights the importance of making use of standardised methodology when evaluating phytase activity from multiple enzyme sources (Shaw *et al.*, 2010). Work done by Meneze-Blackburn *et al.* (2015), in which the performance of seven commercial phytases was compared *in vitro,* concluded that due to the vast number of biochemical properties that contribute to phytase efficacy, there is no unique property responsible for a better performance *in vivo* but rather a combination of all properties. The comparison of these three phytase products should not be on which one is best overall product, but rather which one is best under the current feeding conditions. Driver *et al.* (2005) concluded that to make any meaningful decisions regarding the efficacy of different phytases, the concentrations of Ca and tP used need to be as close to those fed under commercial conditions.

5.6 Correlation between metatarsal and defatted tibia ash

Although the reported correlation value of 0.59 for this study can be classified as strong according to the criteria set out by Cohen (1998). The R^2 value of 0.32 for this trial is considerably lower than the R^2 values reported in literature, implying that defatted metatarsal ash percentage is a poor substitute for defatted tibia ash percentage. Mendez *et al.* (1998), and Yan *et al.* (2005) both reported a strong positive correlation between tibia ash and metatarsal ash of 0.82, 0.88. One possible reason for the low reported R^2 value observed in this trial could be the criteria used to measure metatarsal and tibia ash as well as sampling method used. In this study, tibia ash percentage was used to determine bone mineralization. Work done by Hall *et al.* (2003), in which ash weight was compared to percentage bone ash percentage reported the R^2 value was higher for bone ash weight compared to that of bone ash percentage (0.92 vs. 0.57). The authors go on to suggest further that the amount of tibia ash is the more sensitive indicator of bone mineralisation. This finding concurred with work published by Li *et al.* (2015), in which their results suggested that ash weight better reflects the amount of bone mineralisation compared to ash percentage, and that using ash percentage may lead

to an underestimation of phytase efficacy. Li *et al.* (2015) further explains that bone ash percentage is a value derived from the ash weight of a bone relative to the total bone weight, where bone weight can be defatted or not. In contrast, ash weight reflects the absolute amount of mineral contained in the bone after being ashed and can be influenced by both the size of the bone and degree of bone mineralisation. During this study, while examining the tibia bones after defleshing, the author noted that some of the epiphysial plates had been damaged, or section of the plates was missing. The reason for this damage may be due to the Ca and P deficiency observed in the first trial, resulting in the bones becoming extremely brittle leading to epiphyseal damage whilst removing the tibia bone during sampling. Due to the sensitive nature of the analysis, the damaged epiphysial plates could negatively influence the weight of each tibia, leading to subsequent variation in reported tibia ash percentage. Although multiple authors (Mendez *et al.*, 1998; Yan *et al.*, 2005) agree that there exists a strong positive correlation between tibia ash and metatarsal ash, Scholey & Burton (2017) disagreed, claiming that they observed no correlation when comparing metatarsal ash and tibia ash. According to Scholey & Burton (2017), one of the reasons they claim for the lack of correlation observed, is in part due to the lack of a standardised sampling method when it comes to the mechanical excision of the metatarsal bone, leading to errors in sampling, which is further magnified by the small sample weight leading to disproportionately large errors during analysis.

CHAPTER 6: Conclusion

6.1 Conclusion and recommendations

Results from this study showed that no single efficacy value can be ascribed to various phytase products, as the asymptote of phytase response at which tibia ash is maximised (FTU/kg feed) is affected by the source of phytase. Based on the R^2 value for each model, nutritionists should make use of a nonlinear regression model when evaluating the effect of phytase dose and keep in mind that the strength of accurately predicting the response of bone mineralisation is strongly influenced by the selection criterion used. Previous studies have reported that there is a strong correlation between metatarsal ash and tibia ash, which was not evident in this study. A potential reason for the lack of correlation between metatarsal ash and tibia ash in this study could be due to the lack of standard methodology regarding the removal of metatarsals, which could of lead to the large variation in metatarsal ash results seen in this study. Furthermore, the use of bone ash percentage in this study could have led to further variation and that bone ash weight should rather be used for evaluating phytase efficacy. The results from this study suggest large differences in the *in-vivo* efficacy of newly developed phytases based on differences in the gradient and asymptote of the response in bone ash, BW, and FCRm. Lastly, the results suggest that when comparing the efficacy of different phytases, that it is rather better to make use of tibia ash percentage due to its improved accuracy than compared to that of metatarsal ash percentage. Current industry practice is to make use of tibia bones when using bone mineralisation to evaluate phytase efficacy. The laborious task of having to extract and process the tibia bone has led scientist to explore alternative sampling criterion. One such criterion is to make use of the bird's metatarsal, with research suggesting a strong positive correlation between tibia ash and metatarsal ash. However, from this study that strong positive correlation was not observed. Potential reasoning for this lack of correlation could be due a lack of standardised sampling method used for removing the metatarsal as well as the final units used to measure bone ash with literature recommending that bone ash weight be used rather than bone ash percentage.

6.2 Critical review

The current trial gave insight into areas that could be refined as well as methodology that could be better explained. This refinement could aid in helping to reduce the amount of variation in bone ash results observed and subsequently improving the quality of reported results for future studies. Recommendations to improve the accuracy of reported results in future studies includes:

1. Improved methodology is required when obtaining the metatarsal samples. The current lack of standardised methodology when it comes to sampling metatarsal bone could have contributed to variation of metatarsal ash results within treatments. Furthermore, the current lack of methodology makes it difficult to compare current results to previous literature. It is the author's recommendation that any future methodology must include a detailed explanation describing at what joint the metatarsal must be removed.
2. Instead of making using of ash concentrations (i.e., ash percentage) as an indicator of bone mineralisation, results should rather be interpretated as the quantity of ash in the bone (i.e., mg/g of bone) as this would better take into consideration tibia bones of different lengths and sizes.
3. To improve feed intake, as well as subsequent bone mineralisation this author recommends paying close attention to the form of feed being fed. A crumble diet should be used during the prestarter and early starter phase (0-10 days) and mini pellets from day 11-14, rather than providing a mash diet for the duration of the trial (14 days). This change in feed form would aid in preventing selection of feed by the bird. Furthermore, placing feed guards on each trough would aid in preventing any unnecessary feed spillage. Not only will this aid in reducing the amount of feed wastage but also allow for more accurate feed intake results.

Opportunities to expand on current research:

1. Studies to date have only compared alternative measurements (i.e., metatarsal and tibia ash) using young chicks; further studies are needed to determine if the sampling techniques used are suitable for use with older birds. As bird age could have possible implications for which bone should be chosen when evaluating differences in dietary phosphorus and calcium uptake.
2. Currently this study determined the optimum phytase dose by using bone ash as the selection criterion. However, the optimum dose at which phytase is used is influenced by multiple factors such as the price of the feed as well as the phytase and subsequent phytase matrix being used. Therefore, there exists the potential to determine the optimum phytase dose using a value-based approach, which would take into account not only performance parameters such as bone mineralisation but also economic parameters.

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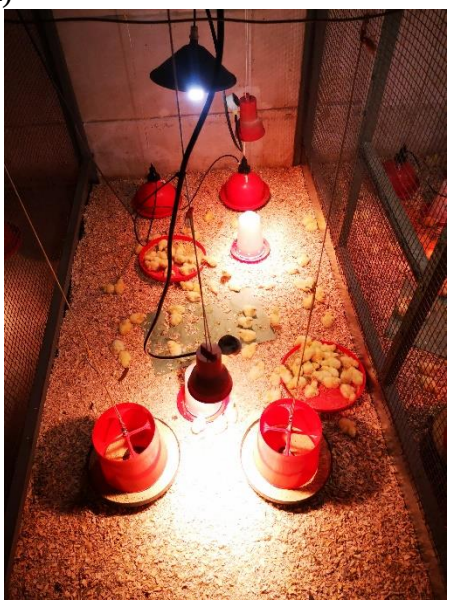
Chapter 8: Annexures

A)



B)

A) An example of 1 of 30 pens used for the adaptation phase. Each pen consisted of two bell drinkers and two feeders.

B) An example of 1 tier of 20 battery units used for the test phase. Each tier consisted of 6 metabolic cages.

C)

D)

C) Image of mash test diet being fed to the birds.

D) Image of Soxhlet apparatus used to defat tibia and metatarsal samples. Each apparatus was able to hold 4 organza bags at a time containing 8 thimbles per an organza bag.

E)

F)

E) An example of day-14 tibias after being defleshed, dried and defatted.
F) An example of day-14 metatarsals after being dried and defatted.

G)

H)

G) A picture illustrating the oven used to dry the tibia and metatarsal samples.
H) An example of metatarsal samples after being ashed.