IMPROVING THE EFFICACY OF PHYTASE IN POULTRY DIETS

BY

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Copyright statement

Abstract

Global poultry production faces several challenges, among which ensuring the maximum utilisation of nutrients from plant-based feed materials is gaining importance. Phytate is a major storage form of plant phosphorus (P) which is considered an antinutritional factor due to its adverse effect not only on the utilisation of plant P by poultry, but also other key nutrients. Therefore, it is vital to maximise the efficacy of phytase enzymes in poultry diets to not only release P and other dietary nutrients for use by poultry but also to reduce the use of finite mineral phosphate reserves in feed.

Phytase enzymes have been used for around 3 decades in poultry feed in moderate doses to improve the phytate P utilization to reduce the antinutritional effect of phytate by hydrolysing phytate, but more recently, it has been reported that further increasing the phytase dose ('superdosing') further improves broiler performance. The mechanisms by which phytase superdoses elicit additional performance improvements are not fully understood. Therefore, several factors relating to digestive efficiency need to be explored to get a better understanding of the mechanisms in play to improve digestive efficiency. These factors include how susceptible phytate within feed ingredients is to degradation by phytase and the effects of processing on phytase activity in finished feed. In addition, the use of novel feed ingredients with high mature grain phytase activity as an alternative to external phytase enzymes to improve poultry performance may employ further unexplored mechanisms. Thus, the overarching aim of the present project was to examine the different approaches including superdosing and use of novel feed materials along with other potential factors to improve the efficacy of phytase in poultry diet.

Two broiler trials were carried out alongside a number of *in vitro* studies to investigate different avenues for improving phytase efficacy in poultry diets. The first broiler trial investigated the the effect of super-dosing phytase supplementation on performance, whole body phosphorus (WBP) content and gut health of broilers. Though superdosing of phytase in broiler diets increased the generation of myo-inositol content in gizzard at day 7 and ileum and jejunum at day 21 and 35 through improved phytate degradation, this did not translate to any benefit on performance of broiler compared to other diets. Also, while improved tibia ash percentage has been commonly reported with superdosing of phytase, in this study, the tibia and whole-body Ca and P content were not affected. Interestingly, the WBP to tibia P ratio in broilers was not consistent across the ages assessed, which may indicate that tibia P is not an ideal estimate of WBP content. Finally, from this study, there was no effect of phytase superdosing on caecal microbial diversity compared to other diets, which indicates there is little effect of superdosing on gut microbiome.

The second bird study was conducted to determine the amino acid digestibility of a novel strain of HIGHPHY wheat for broilers. The coefficients of digestibility of some of the essential amino acids (threonine, valine, Isoleucine, leucine, lysine, and histidine) and P digestibility were all higher in HIGHPHY Wheat, suggesting the novel wheat may be a viable alternative to standard wheat not only in terms of improving P but also amino acid digestibility. An additional *in vitro* investigation indicated that phytase activity levels of nearly 58% were retained after pelleting HIGHPHY wheat. While the cost of using HIGHPHY wheat in place of standard wheat with additional phytase is likely too high for broad application, HIGHPHY wheat may have value in formulations of organic feed for poultry.

From the *in vitro* studies, the key finding was that, for all feed ingredients obtained from different countries of origin, there was huge variation in susceptible phytate values between

samples of each ingredient, but overall, it was concluded that the common industry practice of assuming just 35% of dietary phytate from all feed ingredients is hydrolysed by phytase is an underestimate.

To conclude, the findings of the present investigation provide better understanding of the potential mechanisms associated with superdosing phytase in broiler diets. Determination of WBP content is very laborious but future studies are needed to quantify the WBP content together with P digestibility to provide a more complete picture of the P retention in broilers. Also, it would be more precise to formulate diets by taking into consideration the susceptible phytate level of the batches of ingredients present in that particular diet. This would give a more accurate level of plant-derived P available to the bird and reduce the need for excess inorganic P addition. Application of these findings to commercial practice would further improve the environmental sustainability of global broiler production.

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Abbreviations

BWG	Body weight gain
BW	Bird weight
Ca	Calcium
FCR	Feed Conversion Ratio
FI	Feed intake
ICP-OES	Inductively coupled plasma-optical emission spectrometer
NSP	Non-starch polysaccharide
P	Phosphorous
SBM	Soybean meal
SCFA	Short chain fatty acid
AA	Amino acids
ANOVA	Analysis of variance
DCP	Dicalcium phosphate
GIT	Gastrointestinal tract
MGPA	Mature grain phytase activity
WBP	Whole body phosphorous
HPW	High phytase wheat

Contents

List of Tables

List of Figures

CHAPTER 1: Literature Review

1.1. Introduction

Poultry, including meat chickens, laying hens, turkeys and ducks, are key in sustainable food and agricultural production. Poultry are efficient convertors of feed to meat or eggs and enable cycling of nutrients through feed, manure, and soil. Poultry production has increased consistently over recent years due to increased world poultry meat consumption from 11.1 kg/person/ year to 13 kg/person/year from year 2000 to 2009 respectively (DEFRA, 2013). Broilers are considered to be the cheapest source of protein and are important to meet the animal protein requirement of ever-growing population of the world. The low production costs are largely related to the genetic improvement in strains for meat and egg, increased understanding of the fundamentals of poultry nutrition and improved disease control. Modern broiler diets should be protein and energy rich and contain sufficient minerals, particularly calcium (Ca) and phosphorus (P), required for skeletal system formation, general health, metabolic activity, and acid-base balance management.

The poultry industry is in a particularly strong position to support sustainable development around the world and is well positioned to work with the United Nations' 15 -year initiative, Sustainable Development Goals (SDGs). Climate mitigation by limiting emission of greenhouse gases is one of the sustainable goals and among all other livestock, poultry do not produce a significant amount of greenhouse gases such as methane during digestion. Depending on how birds and feeds are raised, poultry production can be carbon neutral. Livestock activities have been estimated to contribute about 18% of total anthropogenic greenhouse gas emissions (Steinfeld *et al.,* 2006). However, poultry is the livestock species with generally the least

impact in terms of land size, water use, environmental stress, and footprints both for poultry meat and eggs (De Vries and De Boer, 2010).

In the past 30 years, broiler genetic development rates and feed efficiency have grown significantly as it takes 35 days by 2009 to gain 2 kg market weight compared to 63 days in 1976 (DEFRA, 2013). These modifications to bird physiology required alterations to nutrient requirements and nutrient management, which boosted the market for low-cost but high-quality feed and raw materials. The phosphorous (P) requirements of this growing broiler chicken cannot be met fully by plant-based diets because two-thirds of P from grain and oil seeds are present in the form of phytate-P (Sandberg, 2002) and this phytate-P is poorly utilized by monogastric animals due to low endogenous phytase activity present in their digestive tract. To meet the 0.45% available P requirement of broiler chicks, inorganic P is added to diets (NRC, 1994). The majority of inorganic phosphates come from expensive and scarce natural rock phosphates. Phosphorus supplements are pricey and using too much of them can increase P excretion in excreta, which is bad for the environment. Due to the lack of efficient endogenous phytases in poultry, phytase enzymes are added to poultry feeds to address these problems. By maximizing the amount of phytate-P available to broilers and decreasing the quantity of P excreted by the birds, phytase lessen the need for inorganic P supplementation.

The superdosing of phytase also has the potential to further improve phytate-P hydrolysis and the extra phosphoric effect derived from phytase superdosing resulting in improving nutrient digestibility and broiler performance (Pirgozleiv *et al.,* 2011, Manobhaban *et al.,* 2016). The mode of action of phytase superdosing on phytate-P hydrolysis through investigating

different physiological parameters in poultry needs to be more extensively evaluated. Additionally, the evaluation of the superdosing effect on whole body phosphorous content and microbiota population within the poultry digestive tract needs to be explored. This would allow for a better understanding of how poultry producer can use superdose of phytase and utilize it to it's greatest potential to enhance not only broiler performance and ensuring better gut health in broilers, but also help to maintain the environment by reducing inorganic P inclusion in the diet.

The efficacy of phytase depends largely on reactivity and susceptibility of phytate within the broiler digestive tract. Therefore, it is crucial to take into account both the susceptible phytate content and the total phytate concentration of diets when considering dietary phytase supplementation. The source, type and volume of phytate in diet, feed processing, structure of phytate, the pH of broiler GI tract, the presence of substrate for mineral binding and many other factors all affect phytate reactivity and response to the external phytase. This project examines some of the factors that influence phytate reactivity and phytase efficacy through the superdosing of phytase and using a novel feed ingredient called HIGHPHY wheat.

1.2. Poultry digestive tract

The avian digestive tract is a continuous tube that opens at either end (beak and vent) to the outside world and consists of a mouth or buccal cavity, oesophagus, crop, proventriculus and gizzard, small intestine, ceca, colon, and cloaca. A specific sequence of digestive events occurs when feed progresses through these organs which includes grinding, acidifying, hydrolysing, emulsifying, and transporting the end products. The digestive tract of the modern chicken has had to adapt to tremendous changes due to intensive breeding for number of eggs for layers and growth rate for broiler chickens. The tremendous amounts of feed handled by commercial poultry breeds require an optimally functioning digestive tract. Functionality of the digestive tract will possibly have a large effect on response to dietary manipulations (e.g., enzyme and pre- or probiotics addition), and therefore needs to be taken into consideration in experimental design and results interpretation. The different parts of the chicken digestive tract are outlined in figure 1.1.

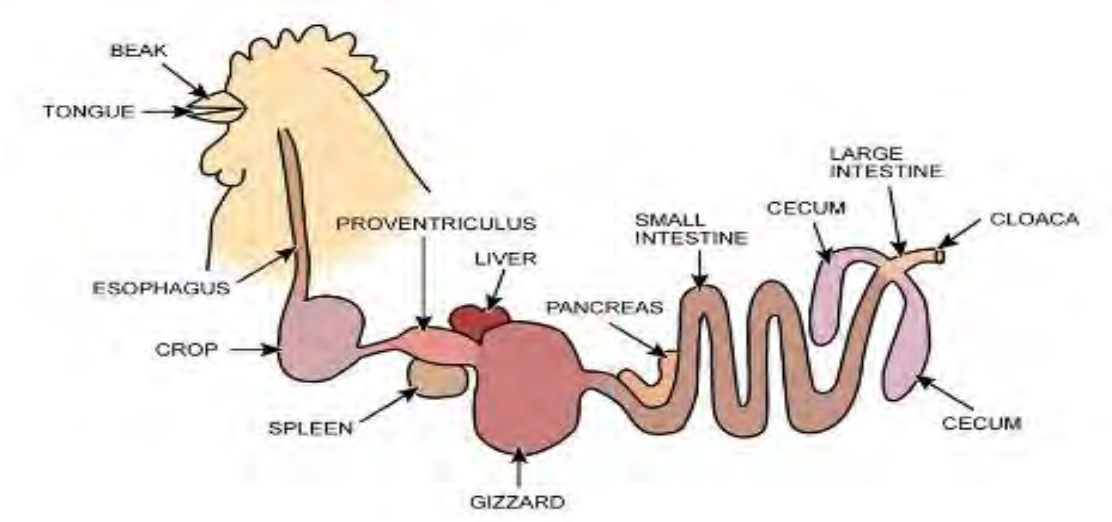


Figure 1.1. The digestive tract of a chicken

1.2.1. Buccal Cavity

In chickens, the opening of the digestive system starts with the beak or buccal cavity. The tongue is situated in lower beak, which is rigid and has very few intrinsic muscles (Duke, 1986). The tongue enables mobility down the oesophagus. The beak, tongue, and larynx of chickens move in cycles during the drinking and feeding processes. Chickens have a low number of taste buds, which results in poor taste perception. This shows that feed consumption in broilers is determined by nutritional levels of the diet rather than taste.

Breakdown of feed and starch digestion start from mouth as bird produce saliva which contains amylase and has got antibacterial properties (Leeson and Summers, 2001). Saliva also contains mucus which keep the feed moist and lubricate the mouth cavity and oesophagus which helps in easy passage of feed.

1.2.2. Oesophagus and Crop

The crop is a transient store for ingested food as after swallowing, the feed can either enter the crop or pass directly to the proventriculous and gizzard when they are empty. The crop is not considered to have any nutritional roles as it does not secret any enzymes and no type of absorption has been reported. However, the crop may aid in grinding and enzymatic digestion further down the digestive tract, as it considerably moistens the feed which can activate any exogenous enzymes and other components. The function of the crop depends largely on feeding systems or feeding behaviour which subsequently will influence dietary effects. It is evident that in ad libitum feeding system the crop is not used in its full capacity as a storage unit, as broilers eat in a semicontinuous manner (Nielsen, 2004), in this feeding system and

the feed bypasses the crop and goes to the proventriculus and gizzard. While *ad libitum* feeding allows a very small amount of feed to enter into the crop, intermittent feeding resulted in significantly increased crop contents (Boa-Amponsem *et al.,* 1991). A large variation in the pH of the crop has been observed, which range from 4.5 to above 6. When feed enters into the crop pH will be similar to the crop as usually the feed pH varies between 5.5 to 6.5 (Ao *et al.,* 2008). However, the pH can change with the retention time as with prolonged retention time considerable fermentation may happen in the digestive tract, resulting in organic acid production and reduced pH (Abbas Hilmi *et al.,* 2007).

1.2.3. Proventriculus and gizzard

The proventriculus and gizzard are considered as the true stomach of birds where hydrochloric acid and pepsinogen are secreted by the proventriculus and mixed with feed contents due to muscular movement in the gizzard. Grinding feed is an important function of the gizzard as it contains strongly myelinated muscles and koilin layer which facilitates grinding due to its sandpaper-like surface (Duke 1992, Svihus 2011). The gizzard and proventriculus are often considered as one compartment in regards to the digestive function because the feed materials flow rapidly through the proventriculus but will potentially be refluxed back into the proventriculus repeatedly during gizzard contraction. As reviewed extensively by Svihus (2011), the addition of a structural component in the diet such as coarse fibre or cereals, increase the size of the gizzard and improve digestive function both through an increase in retention time, a lower pH and better grinding. These positive effects probably combined with better synchronization of feed flow have been shown to improve nutrient

utilization (Svihus, 2011). The gizzard also shows tremendous ability to selectively retain large and tough particles while letting small and soluble particles pass through very rapidly (Rougière and Carré, 2010).

The proventriculus has a pH around 2, as for the gastric juice secreted from proventriculus (Duke 1986). Most of the recent average pH values recorded for broiler chicken gizzard are between 3 and 4 for normal pellet diets (Svihus, 2011). Older data (Farner 1960, McLelland 1989, Riley *et al.,* 1984, Mahagna *et al.,* 1995), however reports a pH value between 2 and 3, although a similar pH has also been reported recently as well (Svihus *et al.,* 2013, Hetland *et al.,* 2002, Sacranie *et al.,* 2012). There are various factors responsible for variation in gizzard pH, such as the amount and chemical characteristics of the feed, retention time etc.

1.2.4. Small Intestine

The small intestine consists of three parts considered as the location for most digestion and practically all absorption of nutrients. The first part is the duodenal loop where the acidic content of the gizzard is mixed with bile and pancreatic juices through gastroduodenal refluxes (Duke, 1986). Duodenal feed retention time is very short, less than 5 min (Noy and Sklan, 1995). Consequently, the process of digestion starts here with the rise of pH to a level above 6 and 95% fat digestion occurs in the duodenum (Sklan *et al.,* 1975).

The adjacent segment after the duodenum that ends at the Meckel's diverticulum, is usually referred to as jejunum. All the major nutrients are to a large extent digested and absorbed here. Meckel's diverticulum, a small projection where the yolk sac was attached during

embryonic development, distinguishes between the jejunum and ileum (Rodehutscord *et al*., 2012). The retention time in the jejunum is only 40 to 60 min and the weight is usually 20 to 50% higher than the ileum (Hetland and Svihus, 2001; Rodgers *et al.,* 2012). It has been evident that the absorption of large extent of the digestion products from fat (Noy and Sklan 1995, Sklan *et al.,* 1975, Hurwitz *et al.,* 1973), starch (Riesenfeld *et al.,* 1980) and protein (Noy and Sklan, 1995; Sklan and Huraitz, 1980) are completed by the end of the jejunum.

The ileum is the last segment of the small intestine which ends at the ileo-ceco-colic junction. The ileum is considered to be the main site for water and mineral absorption along with some digestion and absorption of fat, protein and starch. However, the ileum plays a significant role for digestion and the absorption of starch, as Zimonja and Svihus (2009) reported better starch digestibility of pelleted diet occurs in ileum (81%), compared to other parts of the digestive tract (30% in duodenum and 77% in jejunum). Starch digestibility also increase from 91 to 99% from the anterior third to the posterior third of ileum (Svihus *et al.,* 2004). As most dry matter (DM) been absorbed, the passage rate is much slower in the ileum compared to the jejunum.

1.2.5. Caeca, colon and cloaca

The paired caeca are a unique feature of the poultry digestive tract (except pigeons). The caeca are two blind pouches, about 17cm long in adult birds, at the junction of the small and large intestine. They contain microorganisms that degrade undigested nutrients; the products of fermentation are then able to be absorbed (Gong *et al.,* 2002). The retention time in the caeca is usually long and mostly water and electrolyte absorption occur in the caeca which

makes it quantitively the most important segment of the gut. It was reported that 36% of water and 75% of sodium of renal origin were absorbed from the lower digestive tract where the caeca being the most important organ. There is a possibility that ceca also play a role in recycling renal nitrogen. The functionality of the caeca is greatly affected by diet, especially by fermentable materials in the diet. The caeca are enlarged with increasing amount of fibre in the diet; for example, in turkeys, 25% longer caeca have been observed after adaptation to a high fibre diet (Duke *et al.,* 1984).

The cloaca is the common opening of digestive and urinary system, and the waste materials of these systems pass through this part which opens through vent (Gumus *et al.,* 2004).

1.3. Phosphorous

Phosphorous is an essential mineral required by all living organisms due to its crucial role in maintaining cellular osmotic pressure and acid balance, energy metabolism as a constituent of adenine triphosphate (ATP) and sugar phosphate, and other major physiological functions such as transfer of genetic information and protein regulation via DNA and RNA. It is very important for the maintenance and development of the skeletal system and for fat and carbohydrate metabolism (Julian, 1998). P is very important for bone mineralization (Veum, 2010) and for improved feed utilization and feed conversion efficiency. When the broiler diet is deficient in P, it causes significant health and welfare issues including poor growth, poor bone mineralisation and leg problems such as lameness and tibia dyschondroplasia. In young birds, the symptoms of P deficiency are low plasma P level (<5 mg/100ml) and high plasma Ca level (>10 mg/100ml) along with signs of lameness. Phosphorous can be provided in diet

through feed which is mainly plant based and inorganic phosphate reserve. In plant phosphorous is reserved as phytic acid (myoinositol 1,2,3,4,5,6, -hexakis (dihydrogen phosphate) or InsP6 (Sandberg, 2002; Zeller *et al*., 2015a), which cannot be used fully by the bird due to its anti-nutritional properties. Another source is inorganic phosphate, which obtained from mining of phosphate rock makes it costly and scarce and less sustainable to meet the requirements of growing poultry (Singh, 2008; Beck *et al.,* 2014).

1.3.1. Phosphorus requirement in poultry

The non-phytate phosphorous (nPP) recommendation for broilers stipulated by the National Research Council (NRC, 1994) are 0.45% (0-3 weeks), 0.35% (3-6 weeks) and 0.30% (6-8 weeks). The NRC values for P requirements are based on averages of availability which can result in large errors, partly as biological availability is not constant. Moreover, those values are based on historical research (1952-1982) which may not reflect the requirement of the current commercial broilers, which are more efficient in utilising nutrients due to genetic selection (Havenstein *et al.,* 2003a, b) and this continues to influence the use of nutrients. However, because of the severe effects of phosphorus deprivation in broilers, supplemental inorganic phosphorus is still frequently provided in excess amounts to chicken diets to offer a safety buffer (Waldroup *et al.,* 2000; Leske and Coon, 2002; Dhandu and Angel, 2003). It has been made clear that there is an urgent need to revise the phosphorus needs for broilers (Adedokun and Adeola, 2013; Applegate and Angel, 2014), as excess phosphorous is associated with serious environmental hazards.

1.3.2. Quantitative measurements of P: retained and digestible phosphorus

The general criteria such as bone, blood and growth used for measuring phosphorous availability are qualitative as the values are relative to the criteria chosen (Peeler, 1972). Although the relative bioavailability assays provide comparative data specific to various standards, these are of limited value because of the failure to determine the amount of P retained, and therefore do not properly account for the P excreted (Coon *et al*.,2002 and Leske and Coon, 2002). The commonly used quantitative assays to measure P availability are comparative whole body phosphorous analysis, phosphorous retention studies and pre-caecal digestibility.

1.3.2.1. Whole body phosphorus analysis

The whole-body phosphorous analysis has been examined by several investigators (Nieß *et al*., 2005; Shastak *et al*., 2012c; Van Krimpen *et al*., 2013) to measure phosphorous retention in poultry. The method required the determination of phosphorous concentration in homogenised samples of the whole body and then calculated the difference between the start and end of a feeding period. The advantage of this method is it is precise and does not require metabolic cage. The only disadvantage is the huge laboratory efforts required to get representative samples (Rodehutscord, 2009).

1.3.2.2. Retained phosphorus

The retained phosphorous assay which quantifies ingested and excreted phosphorous has been proposed by Leske and Coon (2002), where they used acid insoluble ash as an indigestible marker in a 5-day bioassay. The values obtained in such a way would provide exact information on the amount of phosphorous retained irrespective of the source of material (both phytate as well as non-phytate P source). Their research said that for a specific phosphorus intake and stage of bird development, retainable phosphorus was defined as the difference between the quantity of phosphorus consumed and the total excreted from the digestive and urinary tracts.

The equation to calculate the retainable phosphorous is following:

Total phosphorus retained = non-phytate phosphorus retained + phytate phosphorus retained

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

Where:

TPI = total phosphorus ingested

TPE = total phosphorus excreted

The main disadvantage of this assay is that it requires metabolic cages to measure the P content in the excreta (Rodehutscord, 2009) and considering the effort of collecting poultry excreta, Shastak and Rodehutscord (2013) thought to use another assay which is precaecal digestibility assay.

1.3.2.3. Precaecal digestible phosphorus

The pre-caecal digestibility assay is an established method for evaluating protein quality of diets in poultry (Ravindran *et al*., 1999). This assay has also been used to evaluate P availability for its simplicity, does not require the use of a metabolic cage (Van der Klis *et al*., 1997) and additionally the values are unaffected by post-ileal microbial activity (Rodehutscord, 2009). It involves feeding birds diets with graded levels of phosphorus which is below the requirement and with an indigestible marker included and collecting digesta from birds' ileum post-mortem. The pre-caecal phosphorus digestibility assay was recommended as a standard protocol to determine phosphorous availability by World Poultry Science Association (WPSA, 2013).

Pre-caecal digestibility (%) is calculated according to the following equation:

$100 - 100 \times [(TiO_2 \text{ Diet} \times \text{P Digesta}) / (TiO_2 \text{ Digesta} \times \text{P Diet})]$,

Where: TiO_2 Diet and TiO_2 Digesta = 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).

1.4. Gut health of Poultry

Gut heath refers to a number of physiological, microbiological and physical functions which are essential to maintain the intestinal homeostasis (Backhed *et al.,* 2005; Kairie *et al.,* 2013; Polansky *et al.,* 2016). The primary function of the gut is effective digestion and absorption of dietary nutrients (Dibner and Richards, 2005; Kairie *et al.,* 2013). The epithelium of the gut acts as a primary barrier to environmental toxins and pathogenic microbes along with nutrient absorption and waste secretion (Turner, 2009). The gut also plays an important role in the immune system by providing a platform for the growth of a diverse microbiota which protects the epithelium from the pathogen colonization and acts as a second barrier (Wigley, 2013; Smith *et al.*, 2014). The gut microbiota regulates immune development and maturation and provides metabolites for host nutrition (Gaggia *et al.,* 2010; Sergeant *et al.,* 2014; Roberts *et al.,* 2015). The gut is also considered to be the largest neuroendocrine organ in poultry which contains a large number of neurons, hormones and secondary messengers regulating a wide range of physiological functions (Neuman *et al.,* 2015; Cani and Knauf, 2016; Weber, 2017).

The gut microbiota plays an important role for maintaining intestinal homeostasis. The microbiota in the GI tract facilitates the digestion and fermentation of the indigestible feed and feed compounds to provide essential amino acids and vitamins to the host (Rinttila and Apajalahti, 2013). The bacteria also produces metabolites like short chain fatty acids (SCFA) which provide energy to the host epithelial cells (Rinttila and Apajalahti, 2013; Sergeant *et al.,* 2014; Cecek and Binek, 2017; Roto *et al.,* 2015; Polansky *et al.,* 2016). The gut microbial community along with the integrity of the gut play an important role in nutrient absorption, immunity, and disease resistance of poultry. Hence, a favourable gut microbiota enhances

the performance of the chicken, and an unfavourable microbiota may promote enteric infections as well as reduce growth and increase mortality of chicken (Jeurissen *et al.,* 2002).

1.4.1. Mucin and gut immunity

Mucin are polymeric glycoproteins that comprise the main component of the mucous layer covering the epithelium of the gastrointestinal tract. The main function of mucus is to protect the epithelium against chemical, enzymatic, physical, and bacterial aggressors present in gut lumen. Increased mucin secretion has been linked to the onset of enteric infection and in response to immune modulation (Linden *et al.,* 2008). Any component, dietary or environmental, that induces changes in mucin dynamics has the potential to affect viscosity, integrity of the mucus layer, and nutrient absorption. Dietary factors such as phytate and fibre have been shown to increase mucin secretion (Montagne *et al.,* 2003; Cowieson *et al.,* 2004). Mucin secretion is increased by anti-nutritional factors through abrasive interactions with the mucus layer and sloughing of mucin into lumen or chime which is poorly digested in the small intestine and could represent an important portion of endogenous protein that reaches the large intestine and is thereby lost for the animal (Montagne *et al.,* 2000). Owing to all these properties, Mucus plays a central role in animal nutrition and health and an intact mucus layer at the surface of the gut epithelium is required for optimal protection and functioning.

However, the thickening of mucous layer on the intestinal mucosa contributes to the reduced digestive efficiency and nutrient absorption. Thinner intestinal epithelium enhances nutrient absorption and reduces the metabolic demands of the gastrointestinal system (Visek, 1978). Increased goblet cell number (GCN) in all the segments of the intestine is attributed to higher mucin production and endogenous protein secretion. Silva and Smithard (2002) suggested

that the absorption of nutrients may be impeded by an increase in the thickness of the epithelium in the small intestine. Fewer GCN in the epithelium of the intestine can be attributed to a less stressful condition that leads to a reduced need for the protective mucus layer.

1.4.2. Microbiota in gastrointestinal tract

The chicken gastrointestinal tract is densely populated with complex microbial communities including bacteria, fungi, archaea, protozoa, and virus (Wei *et al.,* 2013). The most dominant microbes are bacteria, as a chicken GI tract contains ≥500 phylotypes or ∼1 million bacterial genes which equates to 40-50 times the number in the chicken genome (Wei *et al.,* 2013; Sergeant *et al.,* 2014; Oakley *et al.,* 2014). The interaction between the host and the gut bacteria play an important role in bird nutrition, physiology, and gut development (Gerritsen *et al*., 2011).

The GIT's most diverse region, the caeca, is heavily dominated by unidentified microorganisms and is where complex substrates including cellulose, other polysaccharides, and phytate are fermented (Stanley et al., 2014; Choi et al., 2015; Zeller et al., 2015). The groups of microorganisms such as Clostridiaceae, Bacteroidaceae, Lactobacillaceae, and butyrate producers are the most prevalent in caeca (Stanley *et al.,* 2014). The favourable environment of ceca enhances the diverse microbial community to assimilate nutrients from food, produce vitamins and amino acids (Zhu *et al.,* 2002; Sergeant *et al.,* 2014), and defend the host against pathogens (Stanley *et al.,* 2014).

The caeca contained a significant number of bacteria from the order Clostridiales, which involves in SCFAs metabolism and regarded as a sign of healthy hens (Choi et al., 2015). Through its nutritive, immunomodulatory, and regulatory actions, SCFA have an impact on the physiology of the host. By lowering the pH, SCFAs promote the proliferation of epithelial cells, boost mineral absorption, and prevent the growth and adhesion of harmful microbes (Walugembe et al., 2015).

The ceca are often chosen as a site for microbial investigation because of their importance in metabolism and immune maturation. The cecum harbours a more diverse, rich and stable microbial community including anaerobes compared to the ileum (Videnska *et al.,* 2013).

The richness and diversity of bacterial community in cecum increase from hatch to 6 weeks of age and the bacterial community shifts from Proteobacteria, Bacteroides and Firmicutes to almost entirely Firmicutes by 3 weeks of age (Oakley *et al.,* 2014, Kogut & Oakley 2016). However, Kumar *et al.* (2018) reported Firmicutes are the most abundant from 0 to 42 days in both caeca and ileum except at day 42 Bacteroides are the majority of all phylum in ceca. Certain classes of Fermicutes (such as Lachnospiracecae or Ruminococcaceae) and Bacteroidetes plays an important role in fermentation of indigestible polysaccharides and to produce short chain fatty acids which provide up to 10% of metabolizable energy in chicken (Zozefiak *et al.,* 2004). These short chain fatty acids can eliminate *Salmonella spp.* which are foodborne pathogens (Ricke 2015).

The Firmicutes: Bacteroidetes (F/B) ratio can be of certain interest as report showed some conflicting evidence of affecting metabolism and feed conversion ratio (FCR) in chickens.

Report suggested that birds with a poor FCR had lower (F/B) ratio while birds with a better FCR had higher (F/B) ratio, which indicates increased metabolic efficiency can be achieved with a higher abundance of Firmicutes in the gut (Singh *et al.,* 2012). However, Stanley *et al.* (2013) reported no effect on apparent metabolizable energy or FCR has been found despite of the differences in (F/B) ratio between two flocks of chicken.

Faecalibacterium prausnitzii, a butyrate producing member of Ruminococcacecae is considered to have an anti-inflammatory effect as a lower count has been observed in various inflammatory diseases in human and mouse (Sokol *et al.,* 2008). Improved inflammation and intestinal barrier function is also observed in mouse by the addition of Faecalibacterium *prausnitzii* (Sokol *et al.,* 2008). Another member of Firmicutes, *Christensenellaceae* has been reported to have positive effect on gut heath and lower BMI in humans (Goodrich *et al.,* 2014).

Lachnospiraceae, Ruminococcaceae and *Bacteroidaceae* colonization is also very important to reduce the abundance of *Enterobacteriaceae* as the growth of this bacterial family is linked to the level of SCFAs like butyrate, propionate, and acetate (van der Wielen *et al.,* 2000). *Enterobacteriaceae* is very important in poultry not only because it is a pathogen, but also, they are the carriers of genetic factors responsible for antimicrobial resistance.

Another caecal microbe is Bifidobacteriaceae which plays important role in pathogen exclusion and intestinal barrier function. Exopolysaccharide, which is a complex carbohydrate has been produced by Bifidobacterium used by other bacteria as a substrate and facilitates the growth of other bacterial populations.

The development and maturation of these bacterial populations and differences in bacterial composition can be expected due to the differences in nucleic acid extraction protocol, primers, sequencing approach, environmental factors, dietary treatments, breed and geographical conditions. Among these factors dietary treatments has certain importance and therefore, understanding of dietary factors and their roles in microbial population is very necessary for improving the poultry GI microbiome.

1.5. Overview of phytate

Phytic acid is the major storage form of phosphorous (P) in plants, as 80% of the P in the cereal seeds is present in the form of phytic acid. Most poultry diets are primarily composed of plant-based ingredients of which P from phytic acid is poorly available to poultry (Applegate and Angel, 2005). Cowieson *et al.* (2006a) reported, as little as 10% of phytate-P is digested by poultry because they lack in effective endogenous phytases needed to release the phosphate from inositol ring of phytate and also, the formation of insoluble phytate-mineral complexes in the small intestine at pH 5-7 reduces the bioavailability of minerals as phytate-mineral complexes are only soluble at low pH (<3.5) (Simon and Igbasan, 2002). Therefore, phytic acid is considered as an anti-nutritional factor in monogastric animals not only due to its low phosphorus digestibility in the gastrointestinal tract but also it reduces bioavailability of minerals, protein, and starch through complex formation in the gastro-intestinal tract (Schlemmer *et al.,* 2009).

1.5.1. Chemical characteristics of phytate

Phytate consists of six phosphate groups which are negatively charged and bound to 12 hydrogens in an inositol ring. Each of the 12 protons has two proton dissociation sites or reactive sites per phosphate group, among them six sites are acidic (pKa < 1.5), two are weekly acidic (pKa approximately 6) and four are very weekly acidic (pKa between 9.0 to 11.0) (Costello *et al.,* 1976; Erdman, 1979). A proton dissociation site is a site where H+ can be released leaving it negatively charged. At pH>1 phytate has a neutral charge or mostly unreactive, at acidic pH (1-2), 6 of the 12 protons dissociate and become negatively charged and can react with proteins and at neutral and basic pH, the protons dissociate. This means phytate will carry a strong negative charge within the gastrointestinal tract and feeds where pH range is wide. These negative charge sites can bind di and trivalent cations such as Zn2+, Cu2+, Ni2+, Co2+, Mn2+, Fe2+, and Ca2+ in very stable complexes (Wise 1983; Persson *et al.,* 1998; Maenz *et al.,* 1999; Wise 1995; Kaufman and Kleinberg, 1971; Vohra *et al.,* 1975; Maddaiah *et al.,* 1964) as well as protein residues and starch. Thus, phytate reduces the availability of different nutrients as well as the dietary phytate-P of the animals (Pallauf and Rimbach, 1997).

1.5.2. Impact of phytate on the poultry industry and bird health

Monogastric diets mainly consists of plant-based feedstuff such as grains in which 60-80% of phosphorous (P) is stored in the form of phytate. In a typical corn and soybean meal diet, 8-9 g of phytate-P present per kg of feed (Cabahug *et al*., 1999). The utilization and digestion of these plant phytate-P is quite poor in poultry due to their inadequate endogenous phytase activity and denaturation of intrinsic plant phytases in the stomach and during feed

manufacturing, which ended by adding inorganic phosphate to meet birds' P requirement (Cowieson *et al*., 2006; Wendt and Rodehustscord, 2004).

Phytate is considered not only as a nutrient and antioxidant but also as an antinutrient. The antinutritional effect of phytate is mainly related to its ability of chelating with minerals and binding nutrients as well as increasing endogenous losses, hampering nutrient digestions which resulted in poor performance of the birds (Selle *et al.,* 2000; Cowieson *et al.,* 2004, Woyengo and Nyachoti, 2013).

Phytate affects energy and protein digestion by binding energy generating nutrients (lipids and carbohydrates) and endogenous enzymes and increasing endogenous losses (Cowieson *et al.,* 2006; Selle *et al.,* 2000; Selle and Ravindran, 2007; Thompson *et al.,* 1987; Cosgrove 1980). Excretion of endogenous compounds including amino acids was also increased with ingestion of Inositol phosphate-6 (IP6) in growing broilers (Cowieson *et al.,* 2004). The adverse effect of phytate on N and amino acid ulilization may depend on various mechanisms, one of them is phytic acid which may change protein solubility or alter protein structure by interacting with dietary protein and reducing the activity of endogenous proteases because of steric hindrance. Another mechanism is that IP6 impaired the ability of endogenous enzymes and their co-factors to digest dietary protein by binding to or interacting with them, which can be indicated by hypersecretion of digestive enzymes in a similar manner to the effects seen with protease inhibitor (Clarke and Wiseman, 2003). Another possible mechanism is that IP6 may increase enterocyte turnover, mucin secretion or both by interacting with GI tract (Cowieson *et al.,* 2004). Increasing the turnover of protein in the form of cells, mucin or enzyme production results in the increase of the endogenous N loss and

eventually increase maintenance energy requirements. Another mechanism includes the excretion of N in the form of uric acid by increasing catabolic process influenced by IP6. In an experiment it was evident that sulfer digestibility was influenced by phytate which is an indication of methionine and cysteine metabolism was affected by phytate (Cowieson, Acamovic and Bedford, 2006). However, Cowieson *et al.* (2006) reported that IP6 impaired the protein digestion in vitro to a similar extent of as in vivo, so the contribution of endogenous amino acids to the detrimental effect of IP6 is relatively minor. They also reported the addition of 1g IP6 to casein reduced the true amino acid digestibility coefficient by 12%. Phytate interacts with the -NH2 group and side groups of basic amino acids (arginine, histidine, lysine) (Selle *et al.,* 2000).

Amino acid digestibility was reduced from 30 to 20% by synthetic phytate supplementation and reduction in digestibility was varied with the type of amino acid and level (Cowieson *et al.,* 2006a) and source (Onyango *et al.,* 2009) of phytate in the diet. Liu *et al.* (2009) reported, in broilers the activity of different amino acids such as pepsin, trypsin & alanyl amino peptides were reduced in the gizzard, duodenum and jejunum respectively on day 21 by phytate from corn germ and rice bran. Ravindran *et al*., (2006) also reported that the ileal digestibility of protein and amino acids and energy utilization was impaired by increasing the concentration of phytase in broiler diet.

Phytate impedes efficient growth rate as evidence shows that broiler body weight and FCR was increased 2% and 4% by feeding low-phytate corn compared to that of normal phytate corn (Li *et al*., 2000). The antinutritional effect of phytate may suppress appetite and hamper

nutrient availability which adversely affect feed consumption and depress growth performance in broilers (Selle and Ravindran, 2007; Cowieson *et al*., 2011).

1.5.3. Impact of phytate on the environment

Phytate utilization and digestion (only approximately 4%) is quite poor due to inadequate endogenous phytase activity in the poultry digestive tract (Harland and Oberleas, 2010). To meet the P requirement of broilers, inorganic phosphate is supplemented in the poultry diet, which is not only costly, but also alarming for the environment when excreted. In 1990 the adverse effect of high-P in animal excreta came into public view as huge amount of fish deaths were reported in US (Lei *et al.,* 2013). The adverse effects of excess inorganic P and phytate P are soil run off, post soil saturation and eutrophication of surface water and exhaustion of global raw phosphate resources (Rodehutstcord, 2009). To reduce these adverse effects, legislation has been designed to reduce phosphorous pollution which ultimately increased the interest of using phytase in poultry diet (Ravindran *et al.* 2006). Researchers agree that concentration of litter P can be reduced by decreasing P supplementation in the diet and 0.1% of dietary P can be replaced by supplementing phytase in broiler diets.

However, phytase supplementation is very costly and sometimes phosphorus (P) over-supplementation (through use of combined phytase and mineral phosphates) leads to excesses of 20-100% over the published requirement (Applegate and Angel, 2008), which can cause excess P excretion in the poultry waste (Leske and Coon, 2002). The scale of global poultry production (Global broiler meat production rises 84.4 to 89.5 MMT from 2013 to 2017

reported by USDA, 2017) meaning that small improvements in the efficiency of phosphorus utilisation can profoundly impact on the economic and environmental cost of poultry production. The efficiency of phytase enzyme activity is greatly affected by factors such as pH and substrate availability, but little is known about the exact level of influence of these factors exerts in the gastrointestinal environment. Therefore, it is necessary to understand some important modes of action of phytase as well as searching for alternative options to meet the P requirements of growing poultry. An alternative solution could be reducing the level of phytic acid and increasing the level of bioavailable P in cereal seeds or increasing the level of phytase in seeds.

1.5.4. Economic effects of phytate

Phytate can affect broiler mortality because higher mortality of the birds resulted when phytate P was not available to the chicks to the level needed to fulfil their requirement (Simons *et al.,* 1990). Scientists have reported that birds not fed phytase had significantly higher mortality compared to bird fed phytase at 300 FTU/kg during week 5 (28 to 35 days) (Simons *et al.,* 1990; Sohail and Roland, 1999).

When phytate P is not available from the feed, phosphorous need to supplement from the finite source which is very costly. The price of the inorganic phosphorous is increasing day by day as it needs to mine out from the phosphate rocks which is decreasing and the demand of inorganic phosphate is increasing as chemical fertilizers (80%) and animal feed and other applications (20%) (Graham *et al*., 2003). Li *et al. (*2015) reported the price of dicalcium phosphate is increased by huge margin (£770/tonne) compared to approximately £150/tonne

in 2007. The use of phytase enzyme (approximately £1.50/tonne at 1000 FTU/kg) on the other hand is cheaper compared to that of dicalcium phosphate and can replace 0.18% phosphorous (Lei *et al.,* 2013). So, the demand for phytase enzyme has been increased both for its economic benefit and environmental benefit as it reduced the pressure on the finite phosphate source.

1.6. Overview of phytase

Phytases are phosphatases which are responsible for stepwise dephosphorylation of phytate (Angel *et al.*, 2002) and produce myoinositol phosphates from IP5 to IP1, inositol and inorganic P. Usually, phytases are supplemented according to their activity in standard condition (pH 5.5, 37°C, 5 mmol/L sodium phytate) (Greiner and Bedford, 2010).

Typically, phytase was supplemented in traditional level at 500 FTU/kg. Supplementation of phytase not only improves broiler growth and feed efficiency (Cowieson *et al*., 2009), but also it improves bone mineralization in broiler (Walters *et al*., 2019). While some studies showed the beneficial effect of supplementing phytase at lower levels, others showed further benefit can be achieved by superdosing of phytase in poultry diets. Wu *et al*. (2004) reported higher feed efficiency in broilers when phytase was supplemented at 2000 FTU/kg compared to lower dose of phytase.

This section examines different types of phytases and how they are classified. It also includes the overall effects of phytase not only on broiler performance but also on gut physiology and

microbiota of broilers and how different other factors were associated with the efficacy of phytase.

1.6.1. Phytase classification

Two types of phytases are classified on the position of the phosphate group they hydrolyse first, those are 3-phytases and 6-phytases. According to the International Union of Biochemists, 1979 and Kies *et al.* (2001), 3-phytases and 6-phytases start dephosphorylation of ester bonds at the 3 and 6 positions respectively. The activity of these two phytases differs in the number of phosphates they remove from the phytic acid molecule, the location sequence of phosphate removal (Woodzinski and Ullah, 1996), and by their activities at different pH levels (Selle *et al.,* 2000). The sequential hydrolysis of phosphate groups following initial attack is in numerical order; 6-phytases attack only at position 1 following initiation at position 6, and 3- phytases attack at position 4 then 5, 6 and 1 following initiation at position 3. The 3- phytase has two pH optima which are 2.5 and 5.5 when derived from *Apergillus niger* (Kies *et al.,* 2001) and 2.5 and 5 from *Aspergillus ficuum* respectively (Gibson and Ullah,1990). On the contrary the 6-Phytases have only one pH optimum at 5.5 (Kies *et al.,* 2001) and between 4.5 and 5 (Lassen *et al.,* 2001) when derived from wheat *and Peniophora lycii* respectively.

Phytases are also classified based on their origin; fungal or bacterial, and whether they are coated to protect from high temperature. Bacterial phytases are considered to be more efficient than fungal phytases, as phytases derived from *Escherichia coli* releases more P (Auspurger *et al*., 2003) and more active Oyango *et al*. (2005) in the small intestine compared to two different fungal phytases and phytase derived from *Peniophora lycii* respectively. Additionally, Greiner (2006) and Greiner and Bedford (2010) state that phytases can be

classified based on their catalytic mechanism or divided into acid or alkaline phytases based on their optimum pH range.

1.6.2. Phytase activity

Phytase activity is measured in phytase enzyme units (FTU) and a phytase enzyme unit is defined as "the quantity of enzyme that catalyses the liberation of 1 Mol of inorganic P per minute from 0.0051 mol/L sodium phytate in pH 5.5 buffer at 370C" (Kriseldi *et al.,* 2021). An estimated 300-600 phytase activity units/kg of diet result in the release of about 0.8 g of digestible phosphorous, having the ability to replace 1.3 g/kg of P from dicalcium phosphate.

Types and thermostability of phytases affect phytase activity. Those phytases which are heat stable while feed processing, cheap to produce and able to release phosphate in the upper digestive tract (crop and gizzard) are the most effective ones (Greiner and Bedford, 2010). Microbial phytases are more heat stable compared to plant phytases because microbial phytases have a wide optimal temperature range, ranging from 35-145°C compared to plant phytase which is 45-60°C (Wodzinski and Ullah, 1996). Coating the phytase with a thermo-tolerant material or adding stabilising chemicals is one method to improve heat stability (which are patented process), but the performance of the enzyme may suffer as a result of the coating which delay release of phytase enzyme in the intestine. Most of the commercial phytases (e.g., Quantum Blue) were selected for heat stability, those were not coated (European Food Safety Authority (EFSA), 2018).

1.6.3. Phytase in poultry diets

1.6.3.1. Phytase effects on performance

To reduce the adverse effects of phytate which considered as an anti-nutritional factor, several methods have been practiced among which the use of exogenous phytase has gained much interest. Phytase improves the efficiency of utilization of amino acids and energy by reducing anti-nutritional factors and improves growth in broilers (Cowieson *et al.,* 2009). Scientists have also recently suggested the generation of myo-inositol through a phytase-initiated enzymatic cascade is responsible for a part of the beneficial effect of microbial phytase in poultry (Walk *et al.,* 2014). Supplementation of phytase also improves the availability of phytate P in poultry (Ravindran *et al.,* 2000). Apparent P utilization was also improved in young turkeys by supplementation of either 3 or 6-phytase (Ledoux *et al.,* 1995).

Typically, phytase has been supplemented at conventional levels (≤500 FTU/kg) in broiler diet which improved digestibility of P and other nutrients bound to the inositol ring through dephosphorylation (Selle *et al.,* 2000; Ravindran *et al.,* 1999; Ravindran *et al.,* 2008; Powell *et al.,* 2011). Lower doses of phytase have been supplemented to better utilize plant P, improve animal performance, decrease inorganic P inclusion and environmental P pollution (Lenis and Jongbloed, 1999; Kumar *et al.,* 2015; Selle and Ravindran, 2007; Bedford, 2000).

Supplementation of phytase is expected to improve the nutritive value of feedstuffs and reduce the negative effects such as atrophy of the intestinal villi, enlarged digestive organs and increased size of gastrointestinal tract (Ravindran *et al.*, 2001). The inclusion of phytase

has greater impact on nutrient utilisation with increasing age of the birds, as in young bird the impact of phytase is very low on energy and few amino acids compared to that of older birds where energy and all the amino acids are influenced (Walters *et al.,* 2019). Similar findings have also been reported by Li *et al.* (2015) where amino acid digestibility was more pronounced with phytase inclusion in day 19 to 21 broilers compared to broilers at day 7-9. Moreover, with phytase supplementation some amino acids showed higher responses such as cysteine, threonine, serine, glycine, and valine; while some showed lower responses such as arginine, glutamine, and methionine (Cowieson *et al.,* 2017). These increase in response to phytase may be due to the degradation of phytate complexes resulting in increased liberation of proteins and amino acids for digestion and absorption (Onyango *et al.,* 2004, Selle *et al.,* 2000).

Phytase improves the energy digestibility in birds which is well documented (Ravindran *et al.,* 2001; Selle *et al.,* 2007; Santos *et al.,* 2008). Increasing phytase concentration up to 12000 U/kg, resulted in linearly increased energy utilization (Shirley and Edwards, 2003). Logarithmic increase in ileal digestible energy (IDE) from 4.55 to 10%, with increasing doses of phytase have also been reported by (Walters *et al.,* 2019). The improvement in energy utilization may be associated with the increase in protein absorption or improved digestibility of starch and lipid through hydrolysis of phytate complexes (Camden *et al.,* 2001). Another mechanism of increasing metabolizable energy by reducing endogenous losses by phytate and reducing energy required for maintenance and sparing this energy for growth (Wu *et al.,* 2015). Santos *et al*. (2008) reported inclusion of increasing levels of phytase (500, 750, and 1000 U/kg) improved metabolizable energy from 65 to 195 Kcal/kg through increasing protein, starch, and fat digestibility. Ravindran *et al*. 2001 found an increase in energy from 96-120 kcal/kg

when phytase have been supplemented at 400 or 800 FTU/kg in the diets with different levels of phytic acid.

Tibia bone ash has been used as an ideal indication for evaluating phytase efficacy on mineral utilization and storage in broilers (Tang *et al.,* 2012). Several scientists have reported tibia ash and P content increased in a linear and quadratic manner when increasing phytate level in the diet (Dilger *et al.,* 2004; Taheri *et al.,* 2015; Walters *et al.,* 2019). The addition of phytase improved P availability in the GI tract which resulted in greater P digestibility and higher P deposition in bones (Rousseau *et al.,* 2012).

1.6.3.2. Phytase effects on intestinal microbiota

Intestinal InsP6 hydrolysis occurs in the GI tract of birds and is the result of a combination of endogenous and microbiota phytase (Lu *et al.,* 2003). Among different part of the digestive tract, the highest phytase activity has been observed in caeca (Marounek *et al.,* 2010). The InsP6 hydrolysis is very low in proventriculus/gizzard for its acidic environment and resulted in higher accessibility of phytate in the posterior segment, mainly duodenum-jejunum where intense hydrolysis of InsP6 has been observed in broilers fed a basal diet. The increase in InsP6 hydrolysis may be a result of substrate-induced InsP6 hydrolysis by microbiota or endogenous mucosa phytase where phytate-induced phytase production was documented for several bacteria (Greiner *et al.,* 1997; Lan *et al.,* 2002).

A broad pattern of InsP isomers was also observed in the caeca which indicates that caeca contain a highly diverse microbial population producing several phytate-degrading enzymes.

Phytate degrading activity has been described for different bacteria in the chicken digestive tract such as *Lactobacillus spp. (*Raghavendra and Halami, 2009), *Enterobacter spp.,* (Yoon *et al.,* 1996), *E. coli,* (Greiner *et al.,* 2000), *Klebsiella pneuminiae* (Escobin-Mopera *et al.,* 2012), *Bacillus spp.* (Greiner *et al.,* 2002), *Bifidobacterium spp*. (Palacios *et al.,* 2008), and *Pseudomonas aeruginosa* (Sasirekha *et al.,* 2012). The lower molecular weight esters of InsPs show a lower mineral-binding strength compared to that of InsP6 or InsP5 (Persson *et al.,* 1998) which improves the solubility of these esters in the small intestine and allowed access to them by the endogenous phytase or phosphatases (Cowieson *et al.,* 2011). In the lower ileum the activity of the supplemented phytase was significantly reduced due to increasing pH and/or proteolytic degradation (Zeller et al., 2015). So, the importance of endogenous phytase either from microbiomes and/or from intestinal mucosa is very important for hydrolysis of InsP6 in the lower part of the digestive tract.

In broiler diets, supplementation of phytase tends to reduce lactic acid bacteria and significantly reduce *E coli* count in the ileal digesta (Aydin *et al.,* 2010) where both bacteria are considered to produce phytate-degrading enzymes. The decrease in a phytase producing bacterial population may be related to the decrease in the quantity of substrate to the intestinal microbiota (Aydin *et al.,* 2010). Moreover, phytase supplementation has also been reported to decrease intestinal mucosal phytase activity in chickens (Abudabos, 2012).

Broilers and their microbiota have a high capacity to hydrolyse InsP6 in the intestine and more experimental works are required to investigate the differentiation between InsP6 hydrolysis products of endogenous or microbiota phytases and their contribution to InsP6 hydrolysis in different segment of GI tract. The main InsP6 degradation products of *Aspergillus* and *E coli*

phytases are formed in the crop and proventriculus/gizzard which indicates that phytase supplementation is more effective in the anterior than in the intestinal segment of broiler digestive tract (Zeller *et al.,* 2015). When *E coli* phytases were supplemented in the diet, InsP4 accumulated in the crop, whereas InsP3 accumulated when Aspergillus phytases were supplemented. So, it is apparent that, the hydrolytic cleavage of the first phosphate group is not the only limiting step in phytate degradation in broilers.

The effects of phytase and coccidiostats on nutrient digestibility can be of significant relevance for phosphorus and protein-reduced feeding concepts if confirmed in further experiments. Some microorganisms are known as phytase producers, many of them belonging to *Lactobacillus* species, such as *L. salivarius*, *L. brevis, L. plantarum* and *L. pentosus* (Leytem *et al.,* 2008; Schlemer *et al.*, 2001; Kemme *et al*., 2006). *L. salivarius* is often found to be part of the gut microbiota of broiler chickens, specifically in the crop (Pontoppidan *et al.,* 2012; Rodehutscord, 2008). Supplementation of antimicrobial products to poultry diets might change the proportion of phytase producing bacteria in the microbial community. A decrease of *L. salivarius* has been detected in the caecal content of broiler chickens fed with diets supplemented with the antibacterial agent zinc bacitracin (Abelson, 1999). Phytase is well studied and explored, however, little is known about its effects on the microbial ecology of the gastrointestinal tract.

1.6.3.3. Phytase effects on mucin and gut immunity

Phytase may alter the secretion of mucin through the destruction of phytate which results in limited secretion of mucin and endogenous amino acids as mucin is relatively rich in threonine,

serine, and proline (Cowieson *et al*., 2004; Onyango *et al*., 2008; Cowieson and Ravindran, 2008). The effect of phytase on mucin has not been extensively studied which needs evaluation in future studies.

1.6.3.4. Phytase effects on the environmental impact of poultry meat production

The total P, soluble P and reactive soluble P concentration in broiler litter was reported to be reduced by 22.6%, 11.7% & 14.3% respectively when 600 FTU/kg phytase was supplemented in broiler control diet and total P was reduced by 21.5% when phytase was supplemented in Low CaP diet (Powell *et al.,* 2008). Reduction of total P in poultry litter by supplementation of phytase was also reported by Applegate *et al.* (2003) and Shelton *et al.* (2004).

1.6.3.5. Effect of non-phytate phosphorus (nPP) on phytase efficacy

The P which comes from sources (inorganic phosphate) other than diet is called non-phytate phosphorous (nPP). There are several factors which are associated with the effect of phytase supplementation on broiler diets, one of those factors is in the level of dietary P fed. Research has been shown that positive effect of phytase supplementation can be achieved with lower levels of non-phytate P. Powell *et al.* (2008) reported average daily gain (ADG) and G: F (growth: feed intake) was decreased in broilers when supplemented with phytase in control diet compared to that when supplemented with low non-phytate CaP diets.

The nPP is very Important in broiler diets as reducing the nPP level negatively influenced nutrient digestibility, bone mineralization and growth performance in broilers compared to adequate P level in the diets. When phytase was supplemented in increasing levels in P deficit diets, it improved all the performance parameters in broilers (Walters *et al.,* 2019). Moreover,

when birds fed diets deficient in P, they experienced higher mortality which is documented by several scientists (Walters *et al.,* 2019, Waldroup 1999, Vieira *et al.,* 2015). The inclusion of phytase in the low-p diet regardless of dose, improved mortality of broilers similar to that of diet with adequate P (Walters *et al.,* 2019). The positive effect of phytase on growth performance of broiler has been well documented (Santos *et al.,* 2014; Singh, 2008; Onyango *et al.,* 2004; Selle and Ravindran, 2007; Ravindran *et al.,* 2008, Powell *et al.,* 2011). Bone mineralization (tibia ash, Ca and P) was also adversely affected when birds were fed a diet with low nPP level (Lan *et al.,* 2012, Panda *et al.,* 2007). Reduced feed consumption and poorer growth rate in broiler are also well documented, when birds were fed with low-P diet (Bradbury *et al.,* 2014; Ceylan *et al.,* 2012; Wu *et al.,* 2004).

The ileal digestibility of Ca is also affected by dietary nPP level, where Ca digestibility has been reported to improve with low nPP diets (Walters *et al.,* 2019; Dilger *et al.,* 2004, Sommerfeld *et al.,* 2018). Various responses have been found by supplementation of the diet with phytase, both improvements in Ca digestibility (Akter *et al.,* 2018; Bradbury *et al.,* 2018; Ravindran *et al.,* 2006) and reduction in Ca digestibility (Hamdi *et al.,* 2018; Amerah *et al.,* 2014) has been documented. The possibility is that phytase improves feed intake which modulate the absorption of Ca in small intestine thus lowering the overall Ca digestibility. Pansu *et al.* (1981) suggested that greater consumption of dietary Ca downregulated the Ca absorption while the lower consumption upregulated it in rats.

1.7. Phytate reactivity

How the feed phytate react with the phytase affects its efficacy. Other than reducing phosphorus digestibility, phytate also decreased energy metabolism and absorption of vitamins and amino acids in broiler gut (Cowieson et al., 2011; Maenz, 2001; Nahm, 2007). The effectiveness of phytase is determined by the solubility and susceptibility of phytate. Different feedstuffs degrade phytate in different ways. For instance, Leske and Coon (1999) discovered that a fungal phytase improved phytate breakdown by 38% in soyabean meal but by only 15% in rice bran.

Phytase efficacy and phytate reactivity depend on the inherent characteristics of the enzyme, the concentration of phytate and phytase in the diet, the age of the bird, and the composition of the diet. The gastrointestinal tract's various regions affect solubility and retention duration in different ways. Next section examines factors that dictate phytate reactivity, including location of phytate in ingredients fed to poultry, position of phosphate groups in phytate molecules and gastrointestinal pH.

1.7.1. Location of phytate in poultry feed ingredients

Wheat phytate localized in the aleurone layer of the grain (Peers,1953) and because of the location of this phytate fraction is less accessible to the supplemental phytase within the retention time of the crop which is responsible for lower InsP6 hydrolysis in wheat-based diet compared to that of a maize-based diet (Zeller *et al.,* 2015). In maize 90% of phytate is present in the germ and in wheat over 80% present in the aleurone layer and outer brans (O'Dell *et al.,* 1972). Blaabjerg *et al.* (2007), reported the effect of phytase supplementation on phytate

hydrolysis was greatest in soybean meal, intermediate in wheat/soybean meal and nondetectable in wheat alone during in-vitro incubation. The position of phytate in the grain is considered to be the differentiating factor as the thick aleurone layer and other surrounding structure might hinder the access of phytase to hydrolyse phytate in wheat, whereas in germs the phytate in maize and soybean meal are more accessible by the phytase (Zeller *et al.,* 2015). The efficacy of the supplemental phytase in the crop depends on the accessibility of the phytate which differs in different gains and the particle size between maize and wheat meal could also have contributed to the accessibility of phytate.

1.7.2. Degradation of phytate

It is not well known which positional inositol phosphate (InsP) isomers are formed by different phytases in the GI tract of birds. Some findings in pigs described the formation of different positional InsP in different segments of GI tract (Schlemmer *et al.,* 2001, Kemme *et al.,* 2006, Pontoppidan *et al.,* 2012), but because of differences in GI tract physiology and anatomy between pigs and birds, caution must be taken when considering applying the findings of pigs for broilers. So, an understanding of rate of degradation of InsP6 to different positional InsP isomers is essential for better understanding of bird P requirements and increasing the potential to avert potential planet P crisis (Abelson, 1999).

The origin of different phytases and their properties such as P^H optimum, proteolytic stability, kinetic efficiency may differ in effectiveness to degrade phytate with transit as the conditions along the GI tract change. A high rate of InsP6 hydrolysis (76%) and P net absorption (57%) was observed in the lower ileum in studies which used low Ca and low P diets (Leytem *et al*., 2008; Tamim *et al*., 2004; Tamim & Angel, 2003). Without supplementation of phytase, the

majority of InsP6 hydrolysis occurred by the end of duodenum/jejunum, but hydrolysis continued in ileum and caeca as the greatest endogenous mucosa phytase activity was found in the duodenum (Abudabos, 2012; Maenz & Classen, 1998). But with the supplantation of phytase, major hydrolysis occurred in the crop and proventriculus/gizzard (Zeller *et al*., 2015). The temperature optimum is higher for *E. coli* compared to *Aspergillus* phytases (Brüning, 2009; Igbasan *et al*., 2000). Moreover *E. coli* phytases are more resistance to pepsin and pancreatin and show a higher activity at pH 3 which is close to proventriculus or gizzard pH (Igbasan *et al*., 2000; Garrett *et al*., 2004; Elkhalil *et al*., 2007). This could be the reason why the differences between phytases noted in the crop disappeared in the duodenum/jejunum. Moreover, during incubation in digesta of the proventriculus, *E. coli* and *Aspergillus* phytases showed a residual activity of 93% and 60% respectively (Igbasan *et al*., 2000).

The retention time of the feed in broiler GI tract also influences the degree of InsP6 hydrolysis and phytase efficacy in the bird, as higher InsP6 hydrolysis have been reported with more retention time of the feed in the crop of broiler (Svihus *et al*., 2010).

1.7.3. Gut pH

Phytase presence and activity is affected by gut pH and the optimum pH of the enzyme. Phytate is most susceptible to attack by phytases in the crop (at pH of 4-7), proventriculus (at pH of 0.5-4) and gizzard (at pH of 0.5-4). Similarly, pepsin in poultry has an optimum pH of 2.5-3 and becomes denatured at pH 3.9 (Crévieu-Gabriel *et al*., 1999), so protein solubility and utilization are reduced if the pH shifted away from pH optimum. Furthermore, phytates complex with protein and reduce protein digestibility which results in increased intact proteins in the tract and instigates increased secretion of HCl and pepsin (Cowieson *et al*.,

2008). Because of this increased acidity, more bicarbonate ions are released and increased intestinal pH, which reduces phytate-protein complexes solubility. Morgan *et al*. (2016) reported that, in the presence of phytase, gastric pH was closer to the optimum pepsin activity and phytate-complex degradation. Considering the importance of pH influence on phytase and pepsin activity, it is important to investigate the factors influencing pH to improve the efficiency of phytase utilization.

1.7.4. Mineral presence

Phytic acid has chelating potential and at neutral pH it forms different variety of insoluble salts with di and trivalent cations (Vohra *et al*., 1965; Oberleas, 1973). Each phosphate group of phytate molecule has two different acid dissociation constants and in total there are 12 acid dissociation constants which are negatively charged both in low and high pH. The probability of phytate-cation reaction is increased because of increasing negative charge with rising P^H along the GI tract and stable salts formed which precipitate out of the solution. Phytate makes complexes with di and trivalent cations and Maenz *et al* (1999) suggested the ranking of minerals based on ability to inhibit phytate hydrolysis by phytate at neutral P^H is Zn2+>Fe2+>Mn2+>Ca2+>Mg2+. Monovalent cations may not be subjected to the anti-nutritional effects of phytate as sodium is not affected by phytate and the bond between them may be weaker compared to that of multivalent cations and phytate.

So, the dietary mineral level is an important factor which influences phytate P utilization (Sandberg and Andersson, 1998). Phytate has a net pKa charge of -3 at pH 1.5 rising to -8 at pH 7.5, can make complexes with multivalent cations and the solubility and stability of those

phytate mineral complexes are pH-dependant (Maenz *et al*., 1999; Selle *et al*., 2000). At low pH (<3.5) most phytate-mineral complexes are soluble and between pH 4 and 7 the insolubility is maximum (Selle *et al*., 2000). In broilers, the pH of the small intestine is between 5.5 and 6.6 which affects the solubility of phytate-mineral complexes as well as the availability of phytate P and mineral cations (Shafey *et al*., 1991). So, The P^H of the small intestine has also influenced the ability of phytase to hydrolyse phytate-P which requires the phytate complex be soluble (Reddy *et al* 1982; Wise, 1983).

The extent of phytate degradation is proportionate to the amount of phosphorus liberated, but the amount of calcium liberated varies and often exceeds that of phosphorous.

1.7.4.1. Calcium

Calcium (Ca) is the mineral added at the highest concentration in typical broiler diet which has the greatest impact on phytate P availability. Decreased phytate P hydrolysis have been reported by increasing Ca level in broiler chicks' diets (Nelson and Kirby, 1987; Selle *et al.,* 2007). Reducing dietary Ca level (>5g/kg) improved digestibility of phytate-P by up to 70% (Tamim and Angel, 2003; Tamim *et al.,* 2004). Applegate *et al*., 2003 also found reduced intestinal phytase activity and apparent ileal phytate-P hydrolysis when the dietary Ca level was 0.9% (commonly used), compared to with a lower level of Ca 0.4%. To maximise phytase efficacy without impairing skeletal development, dietary calcium should be kept to a minimum. Salt production and precipitation take place when calcium and phytate concentrations go above a threshold level. As a result, there is less calcium that may be absorbed in the intestine. Ca commonly oversupplied in broiler diets as it is a relatively cheap dietary component. However, recent research has shown that excess Calcium inhibits the

efficacy of phytase (Zanu et al., 2020). So, the reduction of Ca should be practiced in a level that doesn't hamper the performance and facilitate phytase activity.

The impact of P^H on phytase activity and phytase P utilization is greater when Ca is present. Even though, the phytase activity is highest at pH 5.5 and 4.5, but the solubility of Ca-phytate complex deceases at a pH higher than 4 (Selle *et al*., 2000). The insoluble complexes are precipitated for which the enzyme cannot hydrolyse it and consequently the effectiveness of phytase decreases. In instance, a rise in pH decreases susceptibility to the actions of phytase, hence dietary limestone may be utilised as a technique to modulate phytate activity by modulation of digesta pH. By reacting and making complex with either phytate or protein which are more soluble at extremely acidic pHs, calcium can reduce the amount of protein-phytate complex formation (Selle et al., 2009).

The Ca to total P ratio in the diet has been reported to affect microbial phytase efficacy as the ratio increases, phytase activity decreases (Qian *et al*., 1996, 1997). An increase in dietary Ca from 0.6 to 1.5% reduced intestinal alkaline phosphatase activity by 52% and phytase activity by 75%. When dietary calcium levels are reduced, caution must be exercised because doing so below what is necessary can change the Ca: P ratio and interfere with the body's ability to utilise phosphorus (Cowieson et al., 2011). To maximise phytase efficacy, diets should be designed using available calcium rather than total calcium.

1.7.4.2. Vitamin D3

Vitamin D3 functions as a precursor for 1,25-dihydroxycholecalciferol and is crucial for controlling calcium metabolism. It has been demonstrated that vitamin D3 increases the activities of endogenous intestinal phytase and alkaline phosphatase. The metabolism of phosphorus is directly impacted by vitamin D3 deficiency (Harrison and Harrison, 1961).

1.7.4.3. Protein-phytate complexes

Phytate reduce protein digestibility by making complex with them. There are factors affecting the strength of these protein-phytate complexes, among which the isoelectric point of protein, the P^H of the GI tract and the concentration and solubility of phytate etc are major ones. One g of phytate may bind roughly 10g of protein (Cowieson *et al.,* 2009) and majority of these complexes formed in the onset of broiler GI tract. Phytate rapidly binds to proteins with a high isoelectric point (5–6), binary and tertiary complexes are formed at acidic and near neutral pH respectively (Selle and Ravindran, 2007). The isoelectric points of poultry will vary because different poultry diets contain different amounts of amino acids.

Phytate increases the endogenous loss and reduces the digestion and reabsorption of amino acids by reducing protein solubility (Cowieson *et al.,* 2006; Cowieson and Ravindran, 2007). Cowieson and Ravindran (2007) reported phytate mostly affects glycine, serine, threonine, and proline. Protein phytate complexes increase the secretion of pepsin and HCl as the complexes are resistant to them, which resulted in increased mucin secretion and amino acid loss in broiler GI tract. Few studies suggested improved amino acid digestibility by

supplementing phytase (Rutherfurd *et al.,* 2012) in the diet, while some suggested no effect on digestibility (Liao *et al.,* 2005).

1.7.4.4. pH profile of pepsin

According to Crévieu-Gabriel et al. (1999), pepsin in poultry has an optimum pH range of 2.5 to 3.0 and denatures at a pH of 3.9. If pH is off the ideal range due to differences in the digesta pH of individual birds or the presence of dietary limestone, protein solubility and utilisation are decreased. Phytate binding to the activating peptide prevents pepsinogen, the zymogen of pepsin, from being transformed into pepsin (Liu and Cowieson, 2010). This indicates yet another way in which phytate interferes with the utilisation of protein and implies that altering amino acids to increase pepsinogen activation may be a viable strategy for combating the antinutritional effects of phytate on protein.

1.8. Methods of measuring amino acid digestibility

1.8.1. Introduction

Accurate evaluation of amino acid (AA) digestibility in poultry feed is very important for estimation of AA requirement while formulating diet for poultry. For poultry, small intestine is considered to be the main site for AA absorption than that of large intestine where AA absorption is negligible and the microbial activity in hind gut can modify digesta AA profile (Laplace *et al.*, 1985; Nyachoti *et al.*, 1997a; Ravindran *et al.*, 1999). So, digesta sample need to be collected from end of ilium for accurate evaluation of AA digestibility and ileal AA digestibility has been an important research topic in last few decades (Stein *et al.*, 2007).

The most applied method of quantifying amino acid digestibility is 'apparent ileal digestibility'. This approach relies on the concept that the digesta collected from the ileum consists of dietary undigested material and endogenous protein and amino acids (Laplace *et al.*, 1985). Hence the calculated ileal digestibility of AA is defined as Apparent Ileal Digestibility (AID) because it does not consider endogenous AA losses. The equation for calculation of AID is:

AID, % = [(AA intake − ileal AA output)/AA intake] × 100

However, when total collection of ileal digesta is not possible the quantity of the ileal AA output must be estimated by an index marker. Thus, the equation for AID calculation is:

$$AID, \% = [1 - (M_{diet}/M_{ileal}) \times (AA_{ileal}/AA_{diet})] \times 100. \quad (2)$$

Where M_{diet} and M_{ileal} are the concentrations of index marker (g/kg, DM basis) in diet and ileal digesta, respectively; AA_{ileal} and AA_{diet} represent AA (g/kg, DM basis) concentrations in diet and ileal digesta, respectively.

A negative feature of AID is that the method underestimates the AA digestibility and lack of additivity may occur for multiple protein containing diets (Fan *et al.*, 1994,) specially feed ingredient containing small amount of protein (Stein *et al.*, 2005; Xue *et al.*, 2014). So, ileal endogenous AA losses (IAA_{end}) should be taken into consideration while calculating Ileal AA digestibility accurately. This quantification method is defined as True Ileal Digestibility (TID) (Stein *et al.*, 2007). The equation to calculate TID is

The TID and SID calculation are:

TID, % = [(AA intake − (ileal AA output − IAA_{end}))/AA intake] × 100;

1.8.2 Endogenous AA losses and standardised ileal digestibility of AA

The endogenous AA losses can be consisted of protein that are synthesized and secreted in animal GI tract lumen which is not reabsorbed (e.g., digestive enzymes, mucin protein, and serum albumin), sloughed intestinal epithelial cells and bacterial protein from hind gut (Nyachoti *et al*., 1997a). According to McDonald *et al*. (2011), The IAA_{end} can be divided into two parts- basal and specific endogenous losses. The basal loss is the inevitable losses inside the poultry GI tract, which is not diet dependant, only it influenced by the DM intake of birds (McDonald *et al*., 2011), while Specific endogenous loss is the type which is diet specific and induced by dietary factors such as protein level, fibre type and anti-nutritional factors (Cowieson and Ravindran, 2007; Stein *et al*., 2007). A high level of protein inclusion can increase the secretion of digestive enzymes resulted in higher specific endogenous losses (Nyachoti *et al*., 1997b; Hodgkinson *et al*., 2000; Adedokun *et al*., 2008b). Fibre content and type in the diet can change the viscosity and digesta passage rate in the GI tract which can impact mucin and epithelial cell turnover and eventually influence the endogenous losses (Parsons *et al*., 1983; Mosenthin and Sauer, 1991). Dietary anti-nutritional factors such as phytate can also influence endogenous AA losses and inclusion of phytase can ameliorate this ani-nutritional effect which is documented by Cowieson and Ravindran (2007). This explains in an extent how phytase improves AA digestibility in poultry.

So, when only basal endogenous losses considered, the AA digestibility can be calculated more accurately and it is defined as Standardised Ileal Digestibility (SID) (Stein *et al.*, 2007). The equation for calculating SID is:

SID, % = [(AA intake − (ileal AA output − basal IAA_{end}))/AA intake] × 100

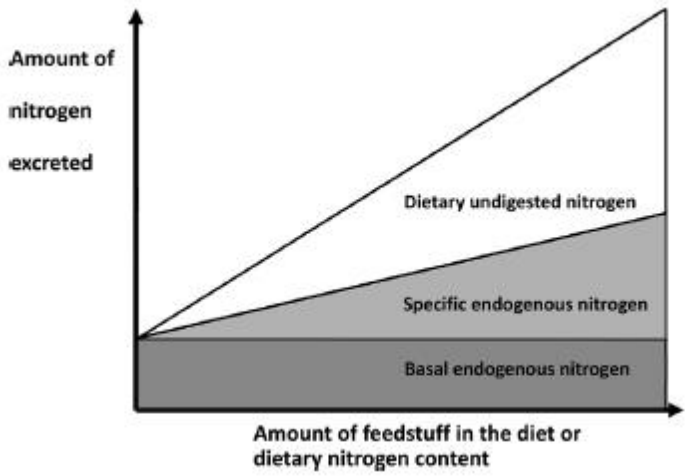


Fig.1.2. Partition of ileal nitrogen flow [adapted from McDonald *et al.* (2011)].

1.8.3 Methods for the determination of basal endogenous AA losses

There are several methodologies which were developed for last few decades to measure endogenous losses. These include the use of

1. nitrogen-free diet (NFD) (Adedoken *et al.*, 2011)

2. Fasting method (Green *et al.*, 1987),

3. highly digestible protein diet (Adedokun *et al.*, 2007a),

4. enzyme hydrolyzed protein diet (Yin *et al.*, 2004),

5. Regression method (Eklund *et al.*, 2015),

6. homo-arginine diet (Nyachoti *et al.*, 1997b), and

7. ^{15}N isotope marked technique (Hess *et al*., 1998).

The equation for basal IAA_{end} estimation is:

basal $IAA_{end} = AA_{ileal} \times (M_{diet}/M_{ileal})$.

Each of this methodology has advantages and disadvantages. The main concern of the NFD - method is bird welfare (de Lange *et al*., 1989) and as they fed N free diet, the bird may degrade its own body protein to maintain its normal biological functions, which will lead to higher secretion of amino acid specially proline and glycine, in the ileal AA flow (Jansman *et al*., 2002). However, when the NFD method is applied, it has been noted that the basal IAA_{end} are high in proline and glycine (Kim *et al*., 2009; Urriola *et al*., 2009a; Zhai and Adeola, 2011). This phenomenon sometimes results in SID estimates that are above 100% for those two AA. The IAA_{end} determined by this method may vary between labs and even within the same lab among studies. So, it is practical to include NFD in every AA digestibility experiment.

The highly digestible protein diet method was developed in response the disadvantages of NFD diet, it has been suggested to include low level of highly purified and digestible protein e.g. casein should be added to NFD to maintain normal physiological status of the bird (Adedokun *et al*., 2007a). Thus, the digestibility of casein needs to be measured within each study to ensure that the endogenous losses are completely animal origin. Different scientists have observed different SID for AA of casein (96% and 100%) when measured with different techniques (direct method and regression analysis), Cervantes-Pahm and Stein (2010) Jezierny *et al*. (2011); Eklund *et*

al. (2015). The IAA_{end} in an animal fed casein might be increased with the inclusion of casein.

Similarly, to the highly digestible protein diet, the enzyme hydrolysed protein approach can be also used as an alternative to casein in NFD (Golian *et al.*, 2008). This hydrolysed protein are mainly peptides of a known and low molecular mass, it can easily be separated from the endogenous protein. However, considering the potential stimulation of specific endogenous losses by the additional peptide, this method may overestimate the basal IAA_{end}.

Regression method is another improvement to the NFD where a series of diets with increasing concentration can be used (Fan and Sauer, 1997; Eklund *et al.*, 2015). The regression equation, which is a variation of Eq. (6) is:

$$AID_C = (SID_C/100) \times AA_{diet} - \text{basal } IAA_{end}. \quad (8)$$

Where AID_C and SID_C are the content of digestible AA in experimental diet. AID_C and AA_{diet} are the dependent and independent variables, respectively. The slope provides an estimate of standardised ileal digestible AA in diet, and the intercept stands for basal IAA_{end}.

However, the regression method requires more dietary treatments and labours compared to NFD method. Moreover, the intercept which is obtained by extrapolation may not be statistically reliable due to the interval or the regressor. In order to reduce the time and costs associated with amino acid digestibility determination, another method was developed for estimating IAA_{end} using a fasted

caecectomised rooster model. However, this model underestimates the IAA_{end} and express in g/d rather than DM intake (Parsons, 1986; Adedokun *et al*., 2008a; Adedokun *et al*., 2009).

Basal endogenous AA losses vary considerably between studies (Adeola *et al*., 2016). For instance, basal endogenous AA were found to be influenced by the ingredient composition of nitrogen-free diets (Kong & Adeola, 2013b) and by other unknown factors (Adeola *et al*., 2016; Moter & Stein, 2004). Moreover, Different techniques have been shown to lead to great differences in the estimate of endogenous losses (for example, Cremers *et al*., 2001; Jansman *et al*., 2002), and all techniques are subject to certain limitations and criticism (Sauer *et al*., 2000). Thus, approaches that do not depend on a separate determination of endogenous losses appear advantageous for the purpose of feed evaluation. One of those methods is the regression approach, where the linear regression of the increment of the amount of pc digested AAs and their incremental intake represents the AA digestibility (Rodehutscord *et al*., 2004). The assumption of linearity between the intake and digested amounts of AA is based on previous studies (Rezvani, Kluth, & Rodehutscord, 2008; Rodehutscord *et al*., 2004). The regression approach implies that basal endogenous losses are excluded in the way AA digestibility is calculated; thus, this is the approach with the highest accuracy (Ravindran *et al*., 2017).

When regression analysis is applied to quantitative data for both AA intake and AA flow at the terminal ileum (Table 4), the slopes describe the proportions of incremental intake (in this case from RSM) which did not reach the terminal ileum. The difference in slope from 1.0 contains both the unabsorbed part of the AA from RSM and the specific endogenous loss

caused by RSM. Thus, the slopes can be interpreted without corrections as pc digestibility of the AAs from RSM. Using this approach, the basal endogenous loss is contained in the intercept and hence does not need to be further accounted for. By regression analysis, the partial digestibility of the AAs from RSM are separated from the digestibility determined for the entire diet, where the RSM contributed only part of the total protein. A separation into unabsorbed AAs and specific AAs secretion is not possible by regression analysis.

In theory, digestibility may be influenced by dietary factors that modify the basal endogenous losses. Such effects were not relevant for explaining the present result because the approach implied that endogenous losses in the gastrointestinal tract are not contained in the measure of digestibility. From these reports, it appears the linear regression and slope analysis approach offers the most accurate means to determine amino acid digestibility in poultry.

1.9. Endogenous Phytase

Recent studies have indicated that Insp6-P is highly available for broilers (Leytem *et al*., 2008), but the origin of phytase activity in poultry digestive tract is controversial. Some authors suggested high InsP6 hydrolysis occurred by endogenous mucosal phytase of the small intestine (Tamim and Angel 2003, Tamim *et al*., 2004) and others suggested higher InsP6 hydrolysis by microorganism present in the small intestine and particularly in the caeca (Leytem *et al*., 2007). The impact of endogenous phytase on IP6 and IP5 degradation however is lower than that induced by microbial phytases in the same pH range (3-5.5) (Brejnholt *et al.,* 2011).

1.9.1. The role of endogenous phytase: In feed ingredients

The feed materials fed to poultry naturally contain phytase (Greiner and Egli, 2003) and different feed materials have varying levels of intrinsic phytase activity. The effect of endogenous feed phytase on phytate-P hydrolysis is controversial. Brejnholt *et al.* (2011) reported that endogenous wheat phytase significantly hydrolysed phytate in wheat. InsP6 hydrolysis was negligible in the crop when a maize-based diet without detectable intrinsic phytase activity was fed to birds, however, InsP6 hydrolysis was high (59%) when a wheat-based diet with intrinsic phytase activity of 623 U/kg was fed to the birds (Roadhurstcord *et al*., 2015). Zeller *et al*. (2015b) also found low InsP6 hydrolysis in the crop of the broiler when fed diet based on maize-soybean meal without phytase supplementation. Nelson (1976) also found grains which have low phytase concentration have lower phytate-P hydrolysing capacity compared to that of grains with high endogenous phytase concentration. Hydrolysis of phytate-P was just 3% when 9-week-old birds were fed only corn-based diet whereas 13% hydrolysis was reported when birds were fed diet consisted of wheat and maize (Nelson, 1976). Phytase concentration in maize is about less than 100 FTU/kg, in wheat and rye it is 12000 FTU/kg and 5000 FTU/kg respectively (Brejnholt *et al*., 2011; Viveros *et al.,* 2000). On the other hand, despite a sixfold difference in dietary phytase activity, the phytate-P digestibility was similar in broiler fed diet with maize and normal barley respectively (Leytem *et al*., 2008). One of the important factors that affect the ability of endogenous phytase of grain is the processing temperature, as phytase loses activity at approximately 60°C. So, during pelleting the efficacy of phytase is greatly reduced because of high steaming temperature (80°C) of the pellet (Ullah 1988). Further research needed with diets typically used in commercial poultry production to find out more about endogenous phytase activity

of cereal grains and its effect on overall availability of P from the diet because previously it has been overestimated.

A synergistic effect of wheat phosphatase and supplemented E coli phytase on the hydrolysis of Ins (1,2,5,6) P4 or its enantiomer in the crop has been suggested when diet with high intrinsic phytase activity observed with more rapid degradation of (1,2,5,6) P4 in the crop. A higher release of myo-inositol in-vitro when phytases added to wheat-based diet compared to maize-base diets, also indicates a synergism of intrinsic grain phytase with supplemented phytases in InsP degradation (Zyla *et al.*, 2013)

1.9.2. Endogenous phytase: In the bird

Several authors concluded that microbiota and mucosa associated phytases have a greater influence on InsP6 hydrolysis than do intrinsic plant phytases (Leytem *et al*., 2008; Shastak *et al*., 2014). They suggested InsP6 hydrolysis associated with the phytases generated from microbiota and mucosa might compensate from the lack of hydrolysis in the crop in absence of intrinsic plant phytases and the degree of InsP6 hydrolysis measured in ileum was higher when a wheat-based diet was fed compared to maize-based diet (Shastak *et al*., 2014).

There are not sufficient studies about the contribution of bush border phytase activity to the hydrolysis of dietary phytate-P. So, there is a common misconception that poultry cannot utilize dietary phytate-P due to lack of effective endogenous phytase, but it is the fact that both phytase and phosphatase are present in intestinal mucosa (Cowieson *et al.,* 2006), liver and blood of poultry (Cowieson *et al.,* 2011). Cowieson *et al., (*2011) suggested that the

dietary phytate-P utilization by endogenous phytase is negligible not for lack of effective endogenous phytase but mainly for poor solubility of these phytate in small intestine which related largely to cation concentration, particularly Ca.

1.10. Phytate susceptibility to the effects of phytase

it's crucial when formulating with phytase to consider both the total phytate content of the diet and the relative solubility, which is largely determined by where the phytate is located in the grain. By adding H+ ions to the weak acid phosphate groups, phytate is changed from phytase-resistant mineral-complexed forms to being sensitive to phytase's actions. With the help of mineral chelators like EDTA and citrate, this conversion to phytase-susceptible form can be facilitated. Soluble mineral complexes that reduce mineral binding to phytate materialise in the digesta and can either be dissociated and rebound to intestinal brush border mineral-binding sites or absorbed as whole complexes (Maenz et al., 1999). A part of the phytate that has been bound to divalent cations may become resistant to phytase; a chelator may prevent cations from binding to the phytate, increasing the amount of phytate that is susceptible.

The accessibility of wheat for supplemental phytases seems to be lower than maize due to a different storage location of phytate in the respective grains resulting in limited hydrolysis of wheat phytate compared to that of maize (Zeller *et al.*, 2015). Early evidence (Morgan *et al.*, 2016), indicates that phytate susceptibility to degradation by phytase may vary both between feed materials and between batches of material. It is therefore important to collect and analyse the commonly used poultry feed materials from different geographical origin to examine the variation in total and susceptible phytic acid content of feed materials. Morgan

et al. (2016) has reported broilers fed diet with high susceptible phytate content had significantly better Feed Conversion Ratio (FCR) and Body Weight Gain (BWG) compared to broilers fed diet with low susceptible phytate content.

1.11. Superdosing phytase

Supplementation of phytase in a dose higher than 1500 FTU/kg in diet is called superdosing. Supplementation of conventional dose of phytase (≤500 FTU/kg) is a very common practice in poultry diet due to its beneficial effect to ameliorate the anti-nutritional effect of phytate through dephosphorylation. The dephosphorylation of phytate by phytase supplementation results in improving the digestibility of P and other nutrients bound to the inositol ring (Selle *et al*., 2000; Ravindran *et al*., 1999; Ravindran *et al*., 2008; Powell *et al*., 2011). Due to rising cost of poultry feed ingredients and to reduce the use of inorganic P and lower enzyme prices, superdosing of phytase has become a growing opportunity for poultry producers around the world. Superdosing of phytase has shown to further improves phytate degradation and increase P availability and perhaps generates myoinositol which has vitamin-like/lipotropic effects (Shirley and Edwards 2003; Cowieson *et al*., 2011; Pirgozliev *et al*., 2011). The most important effect of superdosing which increase its potential to the poultry producer is its extra- phosphoric effect which are associating with further improvement of nutrient availability other than P such as amino-acids, starch and other minerals and translate to improve BW gain, feed intake and feed conversion ratio of poultry beyond that of traditional doses (Shirley and Edward, 2003; Pirgozleiv *et al*., 2011; Cowieson *et al*., 2006; Augspurger and Baker 2004; Manobhaban *et al*., 2016; Kies *et al*., 2006, Pirgozliev *et al*., 2008). Pieniazek *et al*. (2017) reported further increase in amino acid digestibility, feed intake (9.1%) and heavier bird (142g), when broiler diet supplemented with 2000 FTU/kg phytase compared to

conventional dose. An increase in phytase supplementation in a logarithmic manner from doses 150 to 24000 FTU/kg in low-P diet, also showed greater improvements in nutrient utilization, feed intake and weight gain in broiler (Cowieson *et al*., 2006).

One possible mechanism behind the observed improvements with superdose of phytase is likely to be increased production of soluble lower inositol-phosphate esters which have lower capacity to chelate divalent cations and can be solubilized in the small intestine and increase mineral release. Low myo-inositol eaters also have the capacity to stimulate lipid breakdown by generating myo-inositol with lipotropic effects (Cowieson *et al.,* 2011), which needs further investigation. As a result of their lipotropic actions, superdosing has also been demonstrated to protect against fatty acid syndrome and decrease the risk of vitamin E insufficiency. This shows that exogenous phytases' function is more to stop higher esters from passing through the stomach phase than it is to fully dephosphorylate phytate into inositol and free phosphates.

The early signs of the positive effect of superdosing have been shown by Nelson *et al.* (1971), where Phytate-P disappearance was increased from 38.9 to 94.4% by supplementation of phytase from 950 and 7600 FTU/kg (*Aspergillus ficuum*) and 59% improvement in bone ash content was noticed with higher phytase level. However, despite these early findings, interest in superdosing did not increase until the commercialization of phytase as feed additives in 1991 (Cowieson *et al.,* 2011). Initially the cost of phytase was relatively high so the dose of phytase used constrained to 350-500 FTU/kg, but in recent years the phytase cost has been significantly reduced which means currently 500 FTU/kg phytase can be fed for considerably

less cost. The reducing in enzyme price also gives the poultry producer the flexibility of using higher phytase dose to get the extra benefits of superdosing yet in reasonable cost.

Scientists reported that while higher doses of phytase doses (750 and 1000 FTU/kg) produced BW like that of positive control (PC) diet but superdosing (2000 and 3000/1000 FTU/kg) further increased feed intake and body weight of broilers beyond that of PC diet (Walters *et al*., 2019). Though birds fed stepdown phytase program had shown highest feed intake, heaviest body weight and lowest FCR among all the phytase supplemented diet in starter phase but in grower phase phytase inclusion reduced to 1000 FTU/kg resulted in a lower magnitude of improvement in performance at the end of the trial compared to superdose 2000 FTU/kg fed throughout the trial. So, Higher doses of phytases should be fed through to, market age in order to maximise performance response. They have also observed superdosing of phytase further improved amino acid digestibility beyond that of 500 FTU/kg. This further improvement in digestibility could be related to the increased hydrolysis of phytate releasing bound nutrients and/or the reduction in endogenous amino acid secretion. The effect of phytase superdosing on energy utilization has also been investigated, some scientist found superdosing has additional effect in ileal digestible energy such as 153 and 152 kcal/kg when supplemented 2000 and 3000/1000 FTU/kg phytase respectively beyond that of standard dose; while others did not find any additional effect of superdosing (>2000 FTU/kg) on energy utilisation beyond traditional phytase dose (Pirgozliev *et al*., 2008).

The highest P digestibility was also found with superdose of phytase compared to other doses in broiler trial with further improvement in 32.4 and 17.3% respectively compared to the conventional dose. Superdosing of phytase leads to grater destruction of phytate structure

resulting the release of additional phosphate molecule for absorption (Cowieson *et al*., 2011). Superdosing of phytase has little effect on Ca digestibility compared to conventional doses (Manobhaban *et al*., 2016). Superdosing of phytase has greater effect on bone mineralization as highest tibia ash, P and Ca contents are observed with superdose of phytase due to potential liberation of greater portion of mineral (Walters *et al*., 2019). Additional work by Auugspurger and Baker (2004) and Shirley and Edwards (2003) also found further increase in tibia ash with superdosing (1500 to 12000 FTU/kg) compared to conventional dose. In further experiment conducted by Manobhaban *et al*. (2016) observed that superdosing of phytase by 2500 and 5000 FTU/kg improve tibia ash by 7.1 and 12.9%; tibia Ca by 9.3 and 14.3 % and tibia P by 11.5 and 17.9% respectively, compared to that of 500 FTU/kg.

There are important factors which needed special consideration when supplement broiler diet with high doses of phytase. Scientists believe that the improved effect of high phytase on digestible P can only be expected to improve bird performance if P is limiting. Angel *et al.* 2002 highlighted the potentially negative effects of phytase as when they added phytase to the diet sufficient in P level, found increased amount of soluble P excreted in faeces. Żyła *et al. (*2013) also suggested in P deficient diet, superdosing may stimulate and increase feed intake and increase the presence of inositols which have potential growth promoting effect. In P deficient diets P release from phytate instigates increased intake until the P requirements are met, but then continues to increase intake even further beyond this possibly because phytate is an appetite suppressant and superdosing with phytase reduce this antinutritional effect (Cowieson *et al.,* 2011).

1.12. Combining phytase with other enzymes

Protein solubility and broiler performance can both benefit from phytases used in combination with carbohydrases like xylanase and proteases (Cowieson and Adeola, 2005). This is most likely since phytate is found in protein structures and cell walls, thus carbohydrases and proteases release it from the cell matrix and expose it to phytase for hydrolysis (Ravindran *et al.,* 1999). In the absence of phytases, carbohydrases and proteases may have a negative impact on the performance of birds because they promote the synthesis of phytate-protein complexes, according to the solubilization of phytate in the presence of these enzymes.

Xylanase when combined with phytase and supplemented in poultry diet, it has an additional effect on InsP6 hydrolysis (Zeller *et al*., 2015). The heat treatment has been applied in wheat there is a possibility that it changes the grain structure and increase the accessibility of arabinoxylans (major storage part of phytate in wheat) by xylanase (O,Dell *et al*., 1972). As a result, the plant cell wall hydrolysis by xylanase might have been improved which eventually increase the accessibility of phytate for phytase and increase InsP6 hydrolysis. Cowieson *et al.* (2005) suggested high conditioning temperature (>800C) might make structural arabinoxylans more susceptible to hydrolysis as increased released of encapsulated non-starch-polysaccharides from diet has occurred with high-temperature treatment.

1.13. Overview of HIGHPHY wheat

Through genetic approaches, increased level of phytase has been found in some grains such as transgenic soybean and canola, which overexpressing phytase have been shown to reduce phosphate discharge from pig and poultry by 50%. Thus, a new variety of wheat has been developed called HIGHPHY Wheat (Madsen CK *et al*., 2013), might have the potential to do so and research is needed to find out its effect on poultry, which may drastically reduce or avoid the use of synthetic phytase to poultry diet as well as may be beneficial for environment.

Mature grain phytase activity (MGPA) of cereal grains could play an important role in hydrolysis of phytate to make the phytate P available to birds, so scientists are trying to increase the MGPA through in planta expression of microbial phytase in transgenic crops (Brinch-Pedersen *et al*., 2002). Microbial HAP (Histidine Acid Phosphatase) phytases are considered the main group favoured for increasing MGPA through transgene overexpression (Lei *et al*., 2007). But scientists have recently developed a cisgenic wheat mutant (HIGHPHY) holding extra copies of purple acid phosphatase phytase (PAPhy) through which increased MGPA (up to 3500 FTU/kg) has been achieved (Brinch-Pedersen *et al*., 2012; Holme *et al*., 2012). In the HYPHY mutant PAPhy's are considered to attribute to the bulk of MGPA rather than conventional HAP phytases (Dionisio *et al*., 2007 and 2011). This increase in MGPA has increased the interest of using this cultivar in non-ruminant diet as an alternative to exogenous phytase supplementation.

1.13.1. Use of HIGHPHY wheat in poultry diet

The use of HIGHPHY wheat in poultry diet has not been explored extensively. Only one article has been published so far where the scientists explore the Ca and P digestibility while using HIGHPHY wheat in broiler diet. In that trial, it has been reported that replacement of conventional wheat with HIGHPHY wheat significantly improved Ca & P digestibility over the diet supplemented with exogenous phytase (Scholey *et al.*, 2017). The FCR was improved incrementally with increasing inclusion of HIGHPHY wheat and better than that of control diet with standard wheat. Though there was no statistical significance in body weight gain, but numerically highest body weight gain was recorded in diet where standard wheat was replaced 100% with HIGHPHY wheat. The mortality of the bird also assessed during this trial and no effect of mortality shows there was no detrimental effect of HIGHPHY wheat on bird health.

HIGHPHY wheat containing diets had no effect on tibia bone length, width, strength, or tibia mineral content. The scientist suggested to use a lower level of P provision in the experimental diet to reveal the full potential of the diets. Ileal Ca and P digestibility has been increased by 22.9% and 33.6% when 100% standard wheat has been replaced by HIGHPHY wheat. The higher phytase activity of 100% HIGHPHY wheat diet (5925 FTU) compared to that of control diet (605 FTU), considered to be beneficial to improve Ca and P digestibility in the ileum o broilers.

The differences in protein and P levels between standard wheat and HIGHPHY wheat might show beneficial effect over longer feeding period on other nutrient digestibility and broiler

performance. The high protein content of the HIGHPHY wheat is worthy of further investigation via amino acid digestibility assessment.

1.14. Conclusion

Phytase enzyme supplementation in conventional level has been practiced extensively in poultry diet due to its beneficial effect on hydrolysing phytate-P and increase its availability in poultry. Superdosing of phytase is not practiced routinely in poultry but the beneficial effect of superdosing over the conventional dose has a great potential for poultry producers to reduce inorganic P inclusion in poultry diet and supplying more efficient diet to poultry. The key novel aspect of this project is that it explores phytate superdosing and its effect on the whole-body mineralization of broiler and the microbial population in the caeca. This project focuses on variation in susceptible phytate content not only between feed ingredients fed to poultry, but also feed ingredient of different origin and between batches of ingredients which has yet to be explored extensively. Investigation into the mechanisms of endogenous phytase contribution in a novel variety of feed to phytate degradation and nutrient utilization, particularly in starter birds, is a further novel aspect featured in this project.

The implications of this project for the poultry industry are deeper understanding of how we can use phytase more efficiently in poultry diet either by superdosing of external phytase or introducing novel feed ingredient with higher mature grain phytase activity through proper investigation of all the physiological parameters within broiler digestive tract; or of phytate reactivity in the different sections of the poultry gastrointestinal tract.

1.15. Aims and Objectives

The overall aim of this project is to investigate the impact of different biological factors within the gastrointestinal tract of poultry on the action of phytase in broiler diets in and to examine the impact of these factors on a new variety of wheat with extremely high expression of endogenous phytase.

The specific objectives are-

1. Examine the effect of super-dosing phytase supplementation on performance, inositol esters, and whole-body phosphorus of broiler
2. Examine the effect of super-dosing of Phytase on gut heath of broiler including P^H and mucin content in the gastro-intestinal tract and gut microbiota of broiler
3. Investigate the variation in susceptible phytate content among feed ingredients from different country of origin and effect of pelleting on phytase activity of HIGHPHY wheat
4. Evaluate the effect of a novel variety of wheat called HIGHPHY Wheat on inositol esters, amino acid digestibility and growth performance of broilers

CHAPTER 2: Materials and Methods

2.1. Introduction

This chapter describes the general materials and methods used in this thesis and how they correspond to subsequent thesis chapters. A total of three studies involving two bird trials were conducted as summarised below in table 2.1. Bird trial 1, at the Old Poultry Research Unit (Nottingham Trent University, Brackenhurst) was conducted to investigate how the efficacy of phytase in poultry diet can be improved by superdosing the diet with phytase enzyme and compared it with the standard phytase dose. Bird trial 2, at the new Poultry Research Unit (Nottingham Trent University, Brackenhurst), was conducted to investigate the effects of a novel variety of wheat named HIGHPHY wheat containing higher Mature grain phytase activity (MGPA) on amino acid digestibility in broilers and investigating the use of this wheat as an alternative to external phytase in broiler diets. MGPA is the intrinsic phytase activity of individual feed which is explained in section 1.9.1.

Table 2.1: Description of individual studies conducted

Studies	Areas Investigated	Chapter
Study 1	The effect of standard (500 FTU) and super-dosing (3000 FTU) of phytase on performance and whole body phosphorous content of broiler	3
Study 2	Comparison of amino acid digestion and physiological response of broilers fed diets containing graded levels of a standard wheat or a novel strain of wheat containing high levels of phytase	4
Study 3	Comparative investigation of the phytate content of seeds from Australia and the UK and Bangladesh and their susceptibility to denaturation with phytase enzymes & In vitro study to investigate the effect of processing on phytase activity of HIGHPHY wheat	5

Study 3 was a combination of *in-vitro* studies to investigate how pelleting affects the Phytase activity of HIGHPHY wheat and how the susceptible phytate content varies in feedstuffs from different countries of origin and between batches of feed materials.

2.2. Birds and Management

Institutional and national NC3R ARRIVE guidelines for the care and use of animals (Kilkenny *et al.*, 2014) were followed and all experimental procedures involving animals were approved by the School of Animal, Rural and Environmental Sciences Ethical Review Group and logged as ARE488 (bird trial 1) and ARE460 (bird trial 2). All bird trials used Ross 308, day old broiler chicks, supplied within 24 hours of hatching by PD Hook, Cote Hatchery, Oxfordshire and transported to the trial locations by NTU poultry research staff.

For trial 1, day old male and female Ross 308 birds (250 males and 250 females) were ordered from a flock aged approximately 40 weeks to optimize bird quality and have a target weight range of approximately 37-43g. Flock of 40 weeks was selected because they were in mid lay and more uniform eggs hence more uniform day old chicks were obtained.Upon arrival at the Poultry Research Unit (PRU), day old male and female Ross 308 were weighed before random allocation to 48 mesh sided pens bedded on wood shavings (3 cm thick) and there were 10 broilers per pen. The broilers were not identified individually, they were weighed per pen basis (one pen=one replicate). Additional shavings were added as required throughout the trials. Feed and water were available ad libitum, with care taken to ensure the birds ate and drank as soon as possible after arrival. Temperature was set at 32°C on day 1 and reduced by approximately 1°C per day to 21°C by day 21. The room was then maintained at 21°C for the remainder of each trial. Lighting was controlled with 1 hour of darkness from day 1, which

was then increased by 1-hour increments until day 6. Six hours of darkness was then maintained until the end of the trial with 15-minutes dusk and dawn periods. Temperatures were recorded at bird head height from 2 positions in the room with one thermometer positioned adjacent to the door and the other positioned in the far corner under an outside vent. Health checks were made twice daily, and heating and ventilation adjusted depending on bird behaviour. Ventilation was set at 5 minutes fan on per 3 hours, from D5; then 5 minutes per hour from Day 12; then 5 minutes per 30 minutes from D16. Fan speed was adjusted to maintain room temperature and humidity. Bird mortality was recorded daily in addition to the bird weight and reason if culled (for example due to deformities or illness).

For trial 2, day-old male-only Ross 308 birds were ordered from a flock aged approximately 43 weeks. On arrival, all the broilers were group raised and fed a commercial crumb starter diet until day 14. After day 14, 384 male Ross 308 were weighed before random allocation to 48 mesh sided pens sized approximately 0.64m^2 and bedded on wood shavings (3 cm thick). This trial was conducted in the new Poultry Research Unit (PRU) and birds were raised in a thermostatically controlled poultry room. The temperature and lighting regime to the same as previously described for trial 1.

2.3. Diet Formulation and Feed Preparation

For trial 1, four diets were manufactured as a pellet for starter phase by a commercial collaborator and crushed to a crumb on-site by PRU staff. Another four diets were also manufactured as a pellet for grower phase by the commercial collaborator. Diet specification and calculated nutritional content are presented in the relevant chapters. One kg samples of each diet treatment were reserved for proximate analysis and stored at -20°C.

For trial 2, a commercially available starter crumb was fed to all birds from day 0 – 14. Six test diets were fed as a mash from d15 to day 20. Test diet formulation was undertaken by a commercial nutritionist and diets manufactured by NTU. Thirty-five kg of each mash diet were made and individually mixed according to diet manufacture sheets. Titanium dioxide was added to all diets at 5g/kg inclusion as an inert marker for digestibility measures. Grab samples were taken immediately post mixing for analysis.

2.4. Sampling and Preparation Method

2.4.1. Bird Performance Measurements

In all studies, feed was weighed into pre-weighed bags that were labelled with designated pen number. Each pen of birds was fed from the designated bag. Additional feed of the same formulation was added if required throughout the trial. For trial 1, feed intake was recorded weekly, by tipping back remaining feed into the original bag and weighing. Feed intake per bird was calculated from the difference between the starting amount and the amount remaining on each weigh day divided by the number of birds in the pen to give an average feed intake per bird. For trial 2, 5.5kg of diet was weighed out into one bag per pen to allow feed intake to be measured. Pens were fed from their designated treatment diet from d15-20 and, leftover feed were weighed back to measure feed intake prior to study procedures on d20.

In both studies, birds were individually weighed at the start of trial to ensure equal distribution between treatments. For trial 1, birds were then weighed by pen at the start of the study (d0) and then weekly to calculate average bird weight (BW) (total pen weight/ number of birds). Body weight gain (BWG) and feed conversion ratio (FCR) were calculated weekly for trial 1. BWG was calculated by subtracting average bird weight at the start of the

week from average bird weight at the end of the week for each pen. FCR was calculated by dividing average FI of each bird with BWG. For trial 2, birds were weighed by pen at the start of the study at day 15 and then again at the end at day 20, then calculated average bird weight (total pen weight/ number of birds). BWG (average bird weight at day 20-average bird weight at day 15) was calculated at the end of trial in trial 2.

Birds were sampled according to trial needs, (as detailed in chapter 3 and 4) on days 7, 21, 28 and 35 for trial 1 and on day 20 for trial 2. Prior to sampling birds were humanely euthanized via cervical dislocation by trained operatives in a separate room in line with the guidelines of the Scientific procedures Act (ASPCA, 1986). Birds were selected around the mean BW per pen and weighed individually. Feed conversion ratio (FCR) was determined by pen by dividing the total feed intake by the total weight gain for trial 1.

2.4.2 pH measurement

To ensure sufficient gut fill for analysis of pH and no alterations in gastrointestinal pH resulting from periods of empty tract, birds were fed sequentially (ensure each pen got feed). Immediately upon euthanasia by cervical dislocation, pH was recorded from the gizzard, ileum, and crop of 2 birds per pen where the birds were selected based on average BW per pen. Gizzard and crop were removed intact and a digital pH meter (Mettler-Toledo, UK) with a spear tip piercing pH electrode (Sensorex, California, USA) was directly inserted into the digesta in the lumen of the crop and proximal gizzard (proventricular opening) and pH was measured and recorded in 6 times for each site (Morgan *et al.*, 2014). The probe did not touch the wall of any site and was rinsed with ultra-pure water and dried before taking the reading from another site. The pH was measured for the ileum by inserting the probe into the opening

made from removing ileum from the ileo-caecal-colonic junction. The mean of the six readings per section of tract was then calculated and recorded.

2.4.3. Digesta Collection and digestive tract weight

Prior to digesta collection, birds were sequentially fed to stimulate eating and had access to feed for 1 hour before sampling to ensure adequate gut fill. For study 1, after euthanasia, the whole digestive tract was collected from 1 bird per pen and weighed and after that digesta was collected by careful digital pressure along the distal end of the small intestine (identified as the portion of gut between the Meckel's diverticulum and the ileal-caecal-colonic junction) and the empty digestive tract was reweighed again. Ileal and jejunal digesta was pooled per pen (when multiple birds sampled) into labelled pots and weighed. Crop and gizzard contents were also collected into labelled pot per pen. All digesta samples were stored at -20° until required. Samples were later freeze dried (LTE Scientific, Oldham, UK) for 7 days before being re-weighed and then ground manually with a pestle and mortar to homogeneity fine powder. Ground samples were analysed for Ca and P content described later in this chapter. Both caeca from each bird were excised post-mortem and collected separately in clean labelled bags. The caeca were weighed before being snap frozen on dry ice. Caeca were then stored at -20^{o}C.

For study 2, on day 20 post hatch, birds were humanely euthanized by cervical dislocation before removal of the small intestine from the Meckel's diverticulum to the ileal-caecal junction. Ileal digesta was removed by gentle digital pressure and pooled into one pot per pen. Digesta was stored at -20^{o}C prior to freeze drying and grinding. All digesta were freeze dried and ground and split into 2 pots. One set of Ileal digesta and crop digesta pots were sent

to Markus Rodehutscord for IP ester analysis. The remaining ileal digesta were analysed by NTU for amino acid content, Ca and P content and Ti.

2.4.4. Ileal tissue sample collection

A 1cm section of ileum with digesta flushed out using cold H_2O) was taken from the middle of the flushed section and analysed for mucin adherent layer thickness.

2.4.5. Bone samples

Tibia bones were separated from the feet at the tibio-tarsal junction, and from the femur at the tibio-femoral junction. The femur was separated from the hip by carefully dislocating it with the aid of a scalpel where necessary. Both the feet were collected as is. The bones were individually stored in sealed plastic zip-lock bags at -20^{0}C until further processing. Prior to further analysis, bones were fully thawed to room temperature to prevent false readings from bones being partially frozen and therefore more brittle.

2.4.6. Whole bird preparation

At processing, birds were initially weighed to determine live bird weight. After humanely euthanized of the bird, the digestive tract was removed. After which the organs were rinsed with ultrapure water. Right and left bones (for each bone type studied) were removed from the related bird for onward processing as described in Section 2.4.5. The carcass of each bird (whole bird excluding bones) was then cut into small pieces and stored at -20^{o}C until further processing. At processing, the carcass of each bird was thoroughly minced from slightly frozen using an electric mincer (Andrew James, Durham, UK) 4 times until a homogeneous mix was obtained. The mincer was thoroughly cleaned after each bird was minced to prevent carry over between bird samples. Minced birds were stored at -20^{o}C until further processing.

2.5. Analytical Procedures

2.5.1 Dry matter of feed and raw materials

Dry matter content of the diet was analysed by accurately weighing approximately 5-10 g of finely ground sample into pre-weighed crucibles. The crucibles were then dried in a drying oven set at 105°C for approximately 4 days, until a constant weight was reached. The dried samples were cooled in a desiccator and reweighed. Dry matter content was then calculated with the following formula.

$$\text{Moisture content (\%)} = \frac{\text{fresh sample weight (g)} - \text{dry sample weight (g)}}{\text{Fresh sample weight}} \times 100$$

Dry Matter (%) = 100 – moisture content (%)

2.5.2 Crude Protein

Protein content of each diet used in studies 1 and 2 was analysed using the Dumatherm Nitrogen Analyser (Gerhardt, UK). The instrument works on the principle of Dumas method which is a quick combustion of liquid or solid samples in a pure oxygen atmosphere, followed by analysing the resulting gases. The measurement of the thermal conductivity with a TCD-detector gives a signal which corresponds to the amount of nitrogen in the combusted sample. Results are expressed as mg of nitrogen, then converted into percentage of protein present in the sample.

Protein content was calculated by nitrogen content x 6.25 (standard multiplier)

$$\%\ crude\ protein = 6.25\ x\ \%\ Nitrogen$$

To run the analysis 0.5 g of the sample was weighed in a tin foil. The tin foil was then placed in the shaping tool provided with the instrument. The tin foil along with the sample was

compressed in the form of an airtight tablet by pressing and turning the closing cap clockwise as shown in figure 2.3. The tablets thus made were placed in the sample tray which is then inserted in the sample loader in the Dumatherm.

2.5.3 Crude Fat (ether extraction) - Study 1 & 2

The Soxtherm fat extraction system (Gerhardt, UK; fig 2.4) is based on the same principles as the conventional Soxhlet fat extraction. Clean dry extraction flasks with boiling stones were accurately weighed at the start of the fat extraction process. 5 g of dried diet was accurately weighed and inserted into extraction thimbles which were then placed in fat extraction beakers. The fat extraction process took a total of 2 hours and constituted of the following programmable steps:

a. Hot extraction phase: 170ml petroleum ether (CAS 64742-49-0; Fisher Scientific, UK) was poured into the extraction flask containing dried samples and brought to boil at 150oC for 30 minutes. Fat was liberated from the sample during this process.

b. Evaporating phase A: the level of the solvent was lowered below the extraction thimble. Excess solvent was collected in the rear solvent recovery tank.

c. Extraction phase: petroleum ether was refluxed to further extract fat from sample for 1h.

d. Evaporating phase B: the remaining solvent was distilled and collected in the rear solvent recovery tank.

e. Evaporating phase C: a further recovery of the remaining solvent which was distilled and collected in the rear solvent recovery tank.

The extraction flasks with remaining petroleum ether and boiling stones were placed on a hot plate to evaporate off the solvent. Flasks were then placed in an oven for 2h set at 105°C until constant weight was reached. Flasks including contents (fat and boiling stones) were weighed after cooling down in a desiccator. Fat was determined using the following formula:

$$\% \, extractable \, fat = [(M2 - M1) \div MO] \, x \, 100$$

Where:

M0 = sample weight (g)

M1 = weight of flask + boiling stones (g)

M3 = weight of flask= fat + boiling stones (g)

2.5.4 Ash Content

2.5.4.1. Diets and digesta

Ash content of diets and digesta were determined by weighing 5 - 10g of dried samples into pre-weighed crucibles. These were then placed into a muffle furnace (Nabertherm, Germany) for 24h at 650°C. Ashed samples were cooled in a desiccator and re-weighed. Ash weight was determined by weighing the ash residue accurate to 4 d.p using an analytical balance (Sartorius, UK).

2.5.4.2. Bones

Ash content of bones was determined after the tibia and femur bones were defleshed and dried at 105°C for 24h. Fat was extracted from the dried bones in Study 1 by refluxing petroleum ether for 1 - 6h, using a conventional Soxhlet apparatus as described in the particular studies. The Soxtherm fat extraction apparatus was used to extract fat from bones due to its rapid processing time and solvent recovery features. Feet were dried at 105°C for 24h without any prior fat extraction. Following fat extraction, the bones were dried at 105°C for 24h, cooled in a desiccator, weighed, and placed in into pre-weighed crucibles. These were then ashed in a muffle furnace (Nabertherm, Germany) for 24h at 650°C. Ashed samples were cooled in a desiccator and reweighed to determine the ash weight.

Ash % was determined using the following formula:

% Ash = [(W3-W1) / (W2-W1)] x 100

Where, Original weight of crucible = W1

Weight of fresh sample plus crucible = W2

Weight of ashed sample plus crucible = W3

Results were corrected for dry matter.

2.5.5 Titanium Dioxide Analysis – study 2

Titanium dioxide was added at 0.5% to all diets as an inert marker for digestibility measures. TiO_2 was measured in the diets and digesta using a UV spectrophotometer following the methodology of Short *et al.,* (1996). Standard titanium solution (0.5mg/ml) was made by dissolving 250mg Ti02 in 100ml concentrated H_2SO_4 (Fisher Scientific, UK) before adding

500ml of distilled water. 1-10ml of the Ti02 standard was placed in pre-labelled 100ml volumetric flasks. Concentrated H_2SO_4 was then added to each flask to bring the total volume to 10ml. 10ml of 30% hydrogen peroxide (Fisher Scientific, UK) was added before the flasks were brought to volume with distilled water. Samples were measured on a UV spectrophotometer (Cecil, CE 1011, USA) at 410nm to confirm standard curve. The standard solution was stored in amber bottles in darkness until required.

0.3-0.5g of feed or digesta (freeze dried) were weighed in triplicate (diet) and duplicate (digesta) into ceramic crucibles and ashed in a muffle furnace (SNOL, Germany) for 14 hours at 650°C. Once cooled the samples had 10ml 7.4M H_2SO_4 added and were boiled on a hotplate for 2 hours. An additional 5ml 7.4M H_2SO_4 was added to each crucible and the samples were continually heated until completely dissolved. Once cooled the samples diluted with 10ml of distilled water then quantitatively filtered into 100ml volumetric flasks through Whatman 541 hardened ashless filter papers. 10ml of 30% hydrogen peroxide was added to each flask which was then brought to volume with distilled water. The absorbance of the samples was measured after thorough mixing at 410nm using a UV spectrophotometer (Cecil, CE 1011, USA). A standard curve was measured each day from the standard solutions previously described. The coefficient used to determine TiO_2 concentration was derived from the regression analysis of the standard curve.

The amount of TiO_2 in the solutions was calculated by:

$$TiO2/mg = \frac{\text{Absorbance} \times 100}{\text{Coefficient} \times \text{sample weight (mg)}}$$

2.5.6. Calcium and Phosphorous Determination Using Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES) - Study 1 & 2

Diet and digesta samples were analysed for Ca and P by Inductively Coupled Plasma mass spectroscopy with Optical Emission Spectrometry (ICP-OES) (ICP-MS model PQ Excell, VG Elemental, USA). Prior to the assay, all glassware was acid washed for a minimum of 12 hours, rinsed with ultra-pure water and dried, to ensure there was no cross contamination. Approximately 0.5 g of sample was weighed in duplicate into 50 ml conical flasks. The samples were then incubated for a minimum of 16 hours with 10 ml of aqua regia (1 part nitric acid and 3 parts hydrochloric acid) before heating until dissolved (approximately 90 min) in a fume cupboard. If necessary, an extra 5ml of aqua regia was added and an additional 30 min of heating was carried out to ensure complete dissolution. One blank flask containing just aqua regia was prepared for each 5 samples. The samples were then cooled before the flask contents were diluted with ultra-pure water and filtered into 50 ml volumetric flasks through Whatman 541 hardened, ashless filter papers. The volumetric flasks were then brought to volume with ultra-pure water, and the contents were mixed and transferred into 15 ml, duplicate tubes per sample. ICP-OES standards were prepared with differing levels of Ca and P (dependent on the predicted levels of the sample being analysed) using 1000 ppm ICP-OES grade standards (Fisher Scientific, Loughborough, UK) diluted in ultra-pure water. The samples were analysed on the ICP-OES, set to analyse Ca at wavelength 317.933 nm and P at wavelength 213.617 nm. The readings on the ICP-OES are presented as concentration in mg/L; and the following equation used to convert to g/kg:

$$Ca\ or\ P\ in\ sample\ (g/kg) = Ca\ or\ P\ in\ sample\ (mg/L) * volume\ of\ sample\ (ml) \div weight\ of\ sample\ (g)/1000$$

2.5.7. Gross Energy - Study 1 & 2

Gross energy (GE) of the feed was sent to a commercial lab and measured using bomb calorimetry using sucrose as a standard.

2.5.8. Amino acid determination - Study 2

On day 20, two birds per pen were euthanized by cervical dislocation after an hour feed to ensure sufficient gut fill. Ileum digesta was collected 5-10 cm from Meckel's Diverticulum to the ileo-caecal-colonic junction by flushing with cold water, pooled together and weighed. The digesta samples were then freeze dried (LTE Scientific, UK) for 5 days, reweighed and then ground. 500 mg of the digesta samples were oxidised with hydrogen peroxide/ formic acid/ phenol mixture. Excess oxidation reagent was decomposed with sodium metabisulphite and hydrolysed with 6 M HCl acid for 24hrs, the hydrolysate was adjusted to a pH of 2.2, centrifuged and then filtered. Amino acids were separated using ion exchange chromatography and determined by reaction with ninhydrin using photometric detection at 570 nm (440 nm for proline) (Rutherfurd *et al*., 2007; Rochell *et al*., 2013). The concentration of amino acid is expressed as gram per Kg sample and calculated as follows.

$$\text{g amino acid/ Kg sample} = \frac{A \times MW \times F}{W \times 50000}$$

A – Concentration of hydrolysate obtained by the instrument (ISTD-nmol/50 µl)

MW – molecular weight

E – concentration of standard in µmol/ml or nmol/µl

W – gram sample

F – ml total hydrolysate

TiO_2 in the digesta was determined as documented in 2.5.5 and read in a UV spectrophotometer at wavelength of 410 nm. The amount of TiO_2 / mg in the solutions was calculated as follows.

Absorbance / Coefficient x sample weight (mg)

The digestibility coefficients (DCs) of the AAs and the crude protein for each diet were calculated, on a pen basis, according to the following equation:

$$DC_{AA\ Diet} = 100 - \{(TiO_{2Diet} * AA_{Digesta}) / (TiO_{2Digesta} * AA_{Diet})\}$$

where TiO_{2Diet} and $TiO_{2Digesta}$ are the respective concentrations of TiO_2 in the diet and digesta samples (g/kg), and AA_{Diet} and $AA_{Digesta}$ the respective concentrations of the AAs in the diet and digesta samples (g/kg) (also for crude protein).

The quantitative daily intakes of the AAs and crude protein were calculated as feed intake (g/d) * analysed AA (or crude protein) concentration in the diet (mg/g). The quantity of AA digested up to the terminal ileum was calculated as AA intake (mg/d) * $DC_{AA\ Diet}$. The partial digestibility of an AA from the test wheat was obtained by calculating the linear regression between the quantitative AA intake and the amount of AA digested up to the terminal ileum.

2.5.9. Nutrient Digestibility – Study 2

Titanium dioxide concentration in the diets and digesta was determined by UV spectrophotometry as described in section 2.5.5, after the method of Short *et al*., (1996). Ca and P content of diets and digesta was determined by ICP-OES as described in the previous section. The following calculations were utilized to determine an ileal digestibility coefficient and quantity of nutrient digested, following the methodology of Mutucumarana *et al.,* (2014).

The apparent ileal digestibility coefficient = 1 - $\frac{\text{(Ca or P in digesta x } TiO_2 \text{ in diet)}}{(TiO_2 \text{ digesta x Ca or P diet)}}$

Ca or P digested = apparent ileal digestibility coefficient x Ca or P in diet

2.5.10. Gastrointestinal pH – Study 1 & 2

Birds were fed sequentially to ensure adequate gut fill and no differences in intestinal pH resulting from periods of empty tract prior to measurement of gut pH. Immediately post-mortem birds were dissected and a digital pH meter (fully calibrated to manufactures calibration specifications) (Mettler-Toledo, UK) with piercing tip attachment was inserted into the ileum at the Meckel's diverticulum and into the centre of crop and gizzard, in the method described by Morgan *et al.*, (2014). The measurement was repeated in triplicate for each site. In-between birds, the tip was rinsed in ultra-pure water to reduce cross contamination. The mean of the three measurements per section of tract was calculated.

2.5.11. Mucin Layer Thickness Analysis - Study 1 & 2

The thickness of the mucous adherent layer was estimated by a modification of Corne's method (Corne *et al.*, 1974) which was further described by Smirnov *et al.,* (2004). Briefly, a 1 cm^2 piece of intestinal tissue was incubated in 10 g/L Alcian blue (AB) dye solution in buffer containing 160 mmol/L sucrose and 50 mmol/L sodium acetate, pH 5.8; excess dye was washed, and absorbed dye was extracted from the tissue by incubation in 10 g/L docusate sodium salt solution. Samples were cleared by centrifugation at 700 g and optical density was

measured at 620 nm using AB solution as a standard. The amount of absorbed dye is given in g AB/cm^2 of intestinal tissue.

2.5.12. Viscosity- Study 2

Viscosity of feed was measured using a Brookfield cone and plate viscometer (Brookfield Engineering laboratories, INC. USA) maintained at chick body temperature (41^oC) using a circulating water bath. About 5 g of each diet was weighed and 10 ml of water was added to the diets and properly mixed. The pH of the mixture was taken, and an aliquot of the feed samples were centrifuged at 13000 rpm for 5 minutes. The cone and plate viscometer were set up and calibrated, the gap space needed for measurement was also adjusted according to manufacturer's instructions prior to measurement. The temperature of the water bath was set at 41^oC. 0.5 ml of the supernatant was placed in the center of the sample cup with the help of a pipette with and without liquid xylanase and ß-glucanase enzymes (Ronozyme Wx (A) and Ronozyme Multigrain (B)) at 10 µl each. The motor was turned on, the viscosity values were displayed in centipoise (cP) and recorded. The sample cup was cleaned with distilled water after each sample.

2.5.13. Total Phytase Activity- Study 1, 2 & 3

Phosphate stock solution (50 mmol/l potassium dihydrogen phosphate dissolved in 0.25 mol/l acetate buffer (sodium acetate trihydrate dissolved in water and adjusted to pH 5.5 with 25 % mass fraction hydrochloric acid) with 0.01 % mass fraction polysorbate 20) (Fisher Scientific, Loughborough, UK) was diluted to concentrations of 25, 12.50, 6.25 and 3.125 µmol/ml with

0.25 mol/l acetate buffer containing 0.01 % mass fraction polysorbate 20. For the phosphate standards, 360µl 0.25 mol/l acetate buffer with 0.01 % mass fraction polysorbate 20 and 40µl phosphate standard solution was transferred into 2ml microfuge tubes. For the blanks, 400µl 0.25 mol/l acetate buffer with 0.01 % mass fraction polysorbate 20 was transferred into 2ml microfuges. STOP reagent was prepared by mixing together 1 volume ammonium vanadate reagent (ammonium monovanadate dissolved in dilute hydrochloric acid and water), 1 volume ammonium heptamolybdate reagent (ammonium heptamolybdate tetrahydrate dissolved in 25% mass fraction ammonia solution and water) and 2 volumes dilute nitric acid (Fisher Scientific, Loughborough, UK). 0.8ml phytate substrate solution (dodecasodium phytate with inorganic phosphorus content of 0.1 % mass fraction dissolved in acetate buffer and 25% mass fraction hydrochloric acid) (Fisher Scientific, Loughborough, UK) and 0.8ml STOP reagent was added to all the microfuges. The contents were then mixed, maintained at room temperature for 10 minutes, and centrifuged for 3 minutes at 13000g. Each standard and blank were made in triplicate, and optical density was measured using a UV-VIS spectrophotometer (Unicam Helios, USA) set to 415nm. The standard curve was formulated by plotting the optical density against the phosphate concentration (µmol/ml).

50ml of ultra-pure water and 0.5ml of 10% mass fraction polysorbate 20 was added to approximately 5g of diet or ileal digesta and mixed on a magnetic stirrer for 45 minutes. 2ml of the extract was then transferred into a microfuge tube and centrifuged for 3 minutes at 13000g. A phytase level control was included for each batch of samples; phytase stock standard solution with a known activity was diluted to a final activity of 0.15 U/ml to 0.25 U/ml, and the exact activity was determined.

For the phytase level controls, 360µl 0.25 mol/l acetate buffer with 0.01 % mass fraction polysorbate 20 and 40µl phytase level control was transferred into a 2ml microfuge. For the feed sample, 300µl 0.25 mol/l acetate buffer with 0.01 % mass fraction polysorbate 20 and 100µl of feed sample was transferred into a 2ml microfuge. The samples were mixed and pre-incubated for 5 minutes at 37°C. For the phytase level controls and feed samples, 0.8ml of phytate substrate solution was added to the sample and it was incubated at 37°C for exactly 30 minutes. 0.8ml of STOP reagent was then added; it was maintained at room temperature for 10 minutes and then centrifuged for 3 minutes at 13000g. For the blanks, immediately after the pre-incubation step, 0.8ml of STOP reagent and 0.8ml of phytate substrate was added to the sample; it was maintained at room temperature for 10 minutes and then centrifuged for 3 minutes at 13000g. Optical density of the phytase level controls, feed samples and blanks were measured at wavelength 415nm. Each phytase level control, feed sample and blank was measured in triplicate and the results were averaged. An example standard curve is presented in Figure 2.1.

Phytase activity was calculated by:

*ΔD(415)*Vd/ k*m*t*

ΔD (415)= net optical density at 415nm (ΔD (415)t-ΔD (415)b) (where ΔD (415)t is the value for the test portion and ΔD (415)b is the value for the blank)

K= slope of the standard curve

Vd= volume corrected for dilution

For phytase level control= 25000 (100ml extraction volume x 25 (dilution of stock solution) x 10 (40µl diluted stand solution + 360µl buffer))

For digesta= 2000 ml (500ml extraction volume x 4 (100µl extract + 300µl buffer))

m= mass in grams

t= incubation time

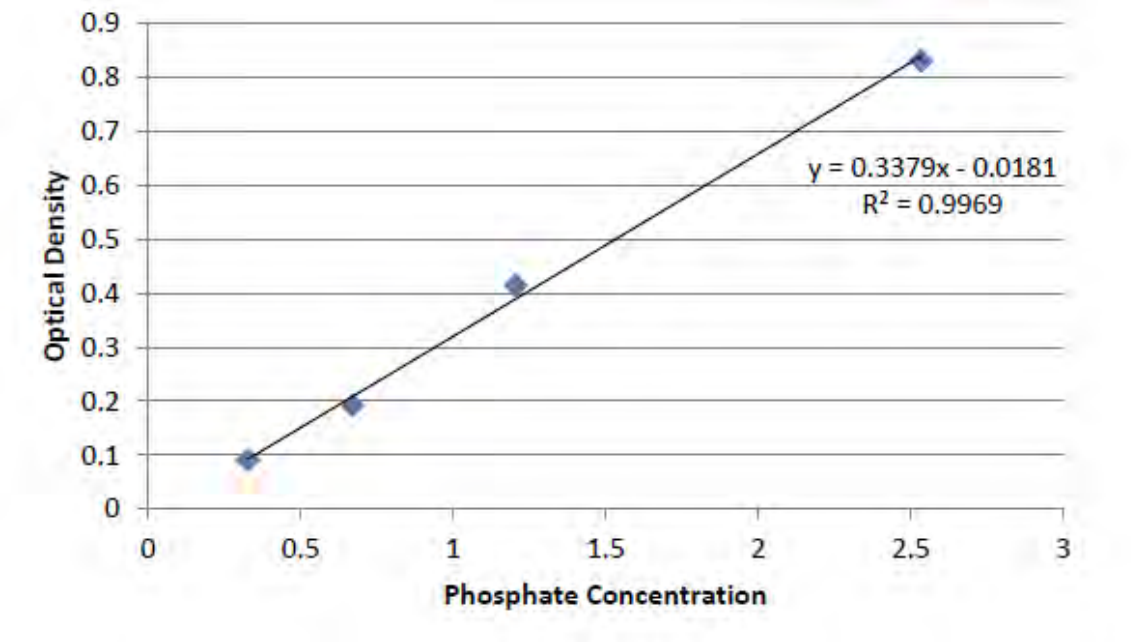


Figure 2.1.: Example of standard curve for phytase activity analysis

For study 3, the phytase activity was determined by little modification of the direct incubation method or quantification of the liberated phosphate method described by (Greiner and Egli, 2003). Samples (0.1 g) of dry-milled cereal grains were suspended in 20 mL of 100 mM sodium acetate buffer, pH 5.0, containing 100μmol of sodium phytate preincubated at 37 and 45 °C, respectively. After certain time intervals at the given temperature, 400 μL portions of the incubation mixtures were removed, and the liberated phosphate was measured by the

ammonium molybdate method (Heinonen and Lahti, 1981) with some modifications. Added to the assay mixture were 1.5 mL of a freshly prepared solution of acetone/5 N H_2SO_4/10 mM ammonium molybdate (2:1:1 v/v) and 100 μL of 1.0 M citric acid. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. To calculate the enzyme activity, a calibration curve was produced over the range of 5-600 nmol of phosphate. Blanks were run by addition of the ammonium molybdate solution prior to adding the enzyme to the assay mixture. Activity (units) was expressed as 1 µmol of phosphate liberated per minute.

2.5.14. In-vitro evaluation of Protein Solubility - Study 2

For study 2, a factorial *in-vitro* trial aimed at determining the main and interactive effects of pH and feed substrates on protein solubility. pH ranges were 1.0, 2.0 and 3.0, feed substrates were HIGHPHY wheat and standard wheat.

Each substrate (feed sample) was weighed (5.0 g) into tubes with 0.2 g pepsin. Solutions of 0.1 M HCl (pH1), 0.01 M HCl (pH 2) and 0.001 M HCl (pH 3) were prepared, warmed to 41˚C (Leader 98C027) and 25 ml was added to the tubes according to the specified pH. The tubes were shaken at 41˚C in a water bath (Stuart SBS40) for 30 minutes. An aliquot was taken and centrifuged (SIGMA D-37520) at 13,000 rpm for 5 minutes, after which it was diluted to 100 µl of sample to 900 µl of distilled water. About 10 µl of standards and the diluted samples were placed in the wells of a microplate in duplicates using a micropipette, then 200 µl of working reagent (using Pierce™ BCA protein assay kit) were added to the wells of the microplate. The microplate was incubated (Genlab 12G14) at 37˚C for 30 minutes and then read in a plate reader (Thermo Scientific Multiskan FC) at 560nm against standard curve. The mean and standard deviation were recorded (Thermo Fisher, 2014).

2.5.15. Inositol phosphates Analysis - Study 1 & 2

For trial 1 samples, Inositol phosphates were extracted from freeze-dried digesta and processed for HPLC according to Lu *et al,* (2019). Milled, dry feed, or digesta (100 mg) were extracted with 5 mL, 100 mm NaF, 20 mM disodium EDTA, pH 10, for 30 min with shaking, followed by 30 min in a bath sonicator at approximately 10 °C and a further 2 h standing at 4 °C. The extract was centrifuged at 9,000 × *g* for 15 min at 4 °C and an aliquot of the supernatant filtered through a 13-mm, 0.45-µm pore-size PTFE syringe filter (Kinesis, UK).

Inositol phosphates were analyzed by anion-exchange HPLC on a 3mm x 250mm CarboPac PA200 column, fitted with a guard column 3mm x 50mm of the same material. Samples of 50 uL volume were injected. Inositol phosphates were eluted at a flow rate of 0.4ml/min with a gradient of methanesulfonic acid and detected by the post-column addition of ferric nitrate in perchloric acid, measured at 290nm (Whitfield *et al*., 2018). Individual peaks of inositol phosphates were integrated with Jasco ChromNav v2 software. Integrated peak areas of different isomers classified by number of phosphates, e.g. InsP2, were summed and reported as 'InsP2, InsP3, InsP4, InsP5 and InsP6'. Calculations assumed equal detector response for the different classes of inositol phosphate and were calibrated against standards of InsP6 dodecasodium salt (Merck Millipore - Calbiochem Cat:407125-25mg, Lot: 2663470). A set of standards for identification of the different classes was obtained by acid hydrolysis of phytate (Sigma P8810) (Madsen *et al*., 2019).

For trial 2 samples, the extraction and measurement of InsP3–6 isomers in feed and digesta were carried out using the method of Zeller *et al*. (2015a) with slight modifications (Sommerfeld *et al*. 2018). By using this methodology, separation of enantiomers is not possible and, therefore, we were unable to distinguish between the D- and L-forms.

Briefly, 1.0 g sample were extracted twice with a solution of 0.2 M EDTA and 0.1 M sodium fluoride (pH 8.0; 4◦C) for 30 min under agitation and centrifuged after each extraction at 12,000 × g for 15 minutes. The respective supernatants were combined, and a 1-mL sample was centrifuged at 14,000 × g for 15 min, and then filtered before being centrifuged again at 14,000 × g for 30 minutes. Filtrates were analyzed using high-performance ion chromatography and UV detection at 290 nm after post-column reaction with $Fe(NO_3)_3$ in $HClO_4$ using an ICS-3000 system (Dionex, Idstein, Germany). Some InsP3 isomers could not be identified because the specific standards were unavailable. A clear discrimination between the isomers Ins(1,2,6)P3, Ins(1,4,5)P3, and Ins(2,4,5)P3 was not possible because of co-elution, and therefore the term InsP3x will be used for these InsP3 isomers of unknown proportions. InsP6 was used for quantification, and correction factors for differences in detector responses for InsP3–5. For the analysis of the InsP1–2 isomers that were analyzed solely in the ileum digesta, an extraction was performed with 0.2 M sodium fluoride at pH 8.0, and otherwise carried out as previously described for InsP3–6 isomers. Filtrates were analyzed by high-performance ion chromatography and conductivity detection using an ICS-3000 system (Dionex, Idstein, Germany). A clear discrimination between the isomers Ins(1)P1 and Ins(2)P1 was not possible because of co-elution, and therefore the term InsP1x will be used for the

InsP1 isomers of unknown proportions. All samples were analysed in duplicate. The InsP concentration is reported on a DM basis.

InsP6 hydrolysis in the digestive tract (y) were calculated for each pen based on the ratio of InsP6 and Ti according to the generally accepted equation:

$$y(\%) = 100 - 100 \times \{\text{Ti in the diet (g/kg DM)}/\text{ Ti in the digesta (g/kg DM)}\} \times \{\text{InsP6 in the digesta (g/kg DM)}/\text{ InsP6 in the diet (g/kg DM)}\}$$

2.5.15. Total Phytate determination - Study 3

Feed ingredients in study 3, were analysed for total phytic acid, using the MegazymeTM K-PHYT assay (Megazyme International Ireland Ltd., UK). The procedure was carried out as per manufacturer's instructions which are further described below:

A. Sample extraction: 20ml of 0.66M hydrochloric acid was added to 1g of feed or freeze-dried digesta and was stirred on a magnetic stirrer for a minimum of 3 hours. 1ml of solution was then transferred into a 1.5ml microfuge and centrifuged at 9,500 x g for 10 minutes. 0.5ml of the resulting supernatant was then transferred into a fresh 1.5ml microfuge, and 0.5ml of 0.75 M sodium hydroxide was added to neutralise the sample.
B. Enzymatic Dephosphorylation reaction: To test for free phosphorus, 0.62ml of ultra-pure water, 0.2ml of an acidic buffer solution (pH 5.5 and sodium azide (0.02 % w/v) and 0.05ml of the neutralised sample was pipetted into a fresh 1.5ml microfuge. To test for total phosphorus 0.60ml of ultra-pure water, 0.2ml of the acidic buffer, 0.05ml of neutralised sample and 0.02ml of phytase was pipetted into a fresh 1.5ml microfuge. All the microfuge tubes were then mixed and incubated at 40°C for 10 minutes. 0.02ml

of ultra-pure water and 0.2ml of an alkaline buffer (pH 10.4, $MgCl_2$, $ZnSO_4$ and sodium azide (0.02 % w/v) was then added to the free phosphorus samples, and 0.02ml of alkaline phosphatase and 0.2ml of the alkaline buffer was added to the total phosphorus samples. All the microfuge tubes were then mixed and incubated at 40°C for 15 minutes. 0.3ml of trichloroacetic acid (50% w/v) was then added to each tube to stop the reaction, and the microfuge tubes were centrifuged at 13,000 rpm for 10 minutes.

C. Colorimetric determination of phosphorous and preparation of phosphorous calibration curve: 1ml of the supernatant was then carefully pipetted into a fresh 1.5ml microfuge tube. 0.5ml of colour reagent (ammonium molybdate (5 % w/v) to ascorbic acid (10 % w/v) / sulphuric acid (1 M) in the ratio 1 part ammonium molybdate to 5 parts ascorbic acid) was added to each of the samples.

Standards were prepared by adding phosphorus standard (24 ml, 50 µg/mL) and sodium azide (0.02 % w/v) to 15ml tubes as followed: Standard 1 (0ml), Standard 2 (0.05ml), Standard 3 (0.25ml), Standard 4 (0.5ml) and Standard 5 (0.75ml), made to total volume of 5ml with ultra-pure water. 1ml of each of these standards and 0.5ml of colour reagent was pipetted into 1.5ml microfuge tubes in duplicate.

All the microfuges prepared for colorimetric analysis, including the standards, were incubated at 40°C for 1 hour. They were then mixed by vortex, transferred 1 ml into cuvettes, and read on a UV-VIS spectrophotometer (Unicam Helios, USA) set at 655nm within 3 hrs. The absorbance of standard 0 was subtracted from the other standards to obtain phosphorus concentration. Mean value of phosphorus standards (µg/phosphorus) was calculated by:

$$\frac{\text{Mean STD1 + Mean STD2 + Mean STD3 + Mean STD4}}{4}$$

The absorbance of the free phosphorus samples was subtracted from the absorbance of the total phosphorus samples, thereby obtaining phosphorus concentration. The concentration of phosphorus was calculated as:

$$\text{Phosphorus} \times \frac{\text{mean value of phosphorus standards (µg/phosphorus)} \times 20 \times \text{dilution factor}}{10{,}000 \text{ (conversion µg/g to g/100g)} \times \text{sample weight (g)} \times \text{sample volume (ml)}}$$

To calculate phosphorus concentration (g/100g):

$$\text{Phosphorus} \times \frac{\text{mean value of phosphorus standards} \times 20 \times 55.6}{10{,}000 \times 1 \times 1}$$

=Mean value of phosphorus standard x 0.1112 x phosphorus

The calculation of phytic acid content assumes that the amount of phosphorus measured is exclusively released from phytic acid and that this comprises 28.2 % of phytic acid (Kumari *et al.*, 2014). These calculations were simplified by using the Megazyme Mega-Calc™ downloaded from the Megazyme website (Megazyme, 2019).

To calculate phytic acid content:

$$\frac{\text{Phosphorus (g/100g)}}{0.282}$$

2.5.16. Susceptible phytate content - Study 3

The susceptible phytate content of the feed ingredients was analysed by a modified version of the Megazyme K-Phyt™ assay described above. Acetate buffer was produced by adding

2.5M acetic acid to 2.5M sodium acetate until pH 4.5 was reached. This buffer was warmed to 37°C in a water bath and then 25 ml of the warmed buffer was added to 5 g of diet or feed sample. The samples were then incubated at 37°C for 5 minutes. 2ml of sample was then immediately transferred into a microfuge tube and centrifuged at 9,500 x g for 10 minutes. 0.5ml of the resulting supernatant was then transferred into a fresh 1.5ml tubes and neutralised with 0.5ml 0.25M NaOH. The pH was then read using a spear tip piercing pH electrode (Sensorex, California, USA), and a 1:3 dilution with ultra-pure water was carried out on the neutralised sample. Phytic acid content was then measured using the total phytate Megazyme K-Phyt™ assay described above starting from section B. Enzymatic Dephosphorylation reaction. Susceptible phytate content was calculated by:

$$\frac{\text{Mean phytic acid content of the sample from the susceptible phytate assay (g/100g)}}{\text{Mean total phytic acid content of sample from total phytic acid assay (g/100g)}}$$

2.5.17. Preparation and Analysis of Samples For 16S rRNA Sequencing- Study 1

2.5.17.1 DNA Extraction

For DNA extraction from cecal contents, one caecum was defrosted overnight in the fridge. Prior to commencement of extraction all surfaces, pipettes, weighing balance were disinfected with freshly prepared virkon. In addition, the pipettes, pipette tips, racks etc were treated with UV in the UV hood. DNA was extracted at the Poultry Research Unit lab using the QIAGEN DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol with some modifications described below. Briefly, 0.25 – 0.26 g of cecal contents were weighed into the power bead tubes and the weight of each sample was

recorded. After addition of 750 µl of power bead solution and 60 µl of solution C1, the tubes were vortexed till the sample, beads and solutions were uniformly mixed. The samples were homogenized using Precellys 24 homogenizer (Bertin Technologies, France) with bead beating at 6500 rpm for 40s (x3) with 30 s interval between two cycles. Then steps 5 to 18 of the manufacturer's Quick-Start Protocol (August 2016) was followed except that in step 17, 60µl of solution C6 was added to the centre of the white filter membrane instead of 100 µl.

The DNA concentration and purity of extracted DNA was determined using a NanoPhotometer NP80 (Implen, Germany). The spectrophotometer lens was cleaned with ethanol and lens tissue prior to use. 1-2 µl of DNA solution was pipetted at the centre of the lens and the concentration values displayed were recorded. This was done twice for each sample. Samples with DNA concentration above 30 ng/µl, 260:280 ratio of approx. 1.8, 260:230 ratio between 2.0 – 2.2 were accepted for 16S rRNA analysis. Extraction was repeated with samples where they were found to be contaminated with RNA or protein based on the 260:280 and 260:230 ratio values. The extracted DNA was then stored at -80^{o}C. Usually, caecal contents were collected using aseptic techniques into RNase free containers and the working area was maintained as RNase free using a commercial product (RNaseZap, Sigma Aldrich, UK).

2.5.17.2. DNA Normalization

Prior to sequencing the extracted DNA was defrosted at 4-6^{o}C and was diluted to 5 ng/µl using 10 mM Tris-Cl, pH 8.5, quantified using Quant-iTTM 1x dsDNA HS assay kit (Life technologies Corp., Oregon, USA) according to manufacturer's protocol and fluorescence was read using QubitTM4 fluorometer (Life Technologies Holdings Pte Ltd, Singapore).

2.5.17.3. 16S rRNA Sequencing

The normalized concentration (5 ng/µl) of purified genomic DNA was used as a template to analyse the microbial communities. 16S rRNA metagenomic sequencing library was prepared following the Illumina 16S Metagenomic Sequencing library preparation's instructions (Part No 15044223 Rev. B) which involves amplification of the V3 - V4 region of the gene encoding 16S rRNA using a two-step PCR protocol.

The following 16S, V3 - V4 specific primers were used (containing both 16S specific primers as well as adapter tails for adding indices and Illumina flow cell adapters) and were selected from Klindworth *et al.* (2014)

Forward Primer = 5'

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse Primer=

5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

The first step was PCR amplifications (Amplicon PCR), which were carried out using 25 µl reaction mixtures of 2.5 µl microbial DNA (5 ng/µl); 5 µl Amplicon PCR Forward Primer (1 µM); 5 µl Amplicon PCR Reverse Primer (1 µM) and 12.5 µl 2x KAPA HiFi HotStart ReadyMix (KAPA-Germany) in a 96 well plate. The plate was sealed using microseal before PCR was performed

in a thermal cycler (Techne, TC-512, UK) using the following program: 95°C for 3 minutes; 25 cycles of: 95°C for 30 seconds; 55°C for 30 seconds; 72°C for 30 seconds; 72°C for 5 minutes. Then PCR amplicons were cleaned up as follows: 20 µl of AMPure XP beads was added to each well of the PCR plate, and mixed by gently pipetted up and down and incubated at room temperature for 5 min. Then the plate was placed on a magnetic stand for 2 min until the supernatant cleared. The clear supernatant was discarded without disturbing the beads. The beads were washed with 200 µl freshly prepared 80% ethanol twice. Then excess ethanol was carefully removed, the beads were air-dried and then the plate was taken off the magnetic stand. Next, 53 µl of 10 mM Tris-Cl, pH 8.5 was added to each well of the PCR plate and the beads were fully resuspended by pipetting up and down and then incubated at room temperature for 2 minutes. The plate was again placed on the magnetic stand till a clear supernatant was obtained. Fifty µl of the supernatant was transferred to a new 96 well plate and the plate with magnetic beads was discarded. The purified amplicons of 15 random samples were diluted 1:5 in molecular grade water before mixing with 2 µl sample buffer and run on Agilent High Sensitivity D1000 screen tape (Agilent Technologies, INC, CA, USA) according to manufacturer's instructions to verify amplicon size.

The next step was the Index PCR to attach the dual indices and the Illumina sequencing adapters which was performed as follows: A fresh 96-well PCR plate placed in the TruSeq Index Plate Fixture (Illumina, USA) and the Index 1 and Index 2 primers were arranged on the same fixture (figure 2.2). The following reactions were set up: 5 µl amplicon DNA, 5 µl Nextera XT Index Primer 1 (N71-12) horizontally, 5 µl Nextera XT Index Primer 2 (S51-8) vertically, 25 µl of 2x KAPA HiFi HotStart ReadyMix, 10 µl molecular grade water.

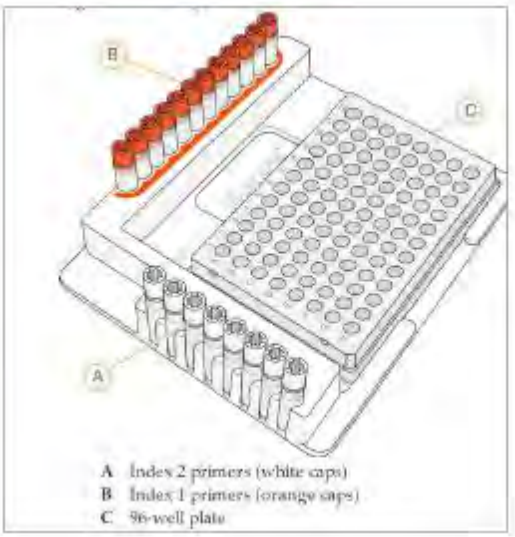


Figure 2.2. Illumina TrueSeq plate fixture showing arrangement of indices and 96-well plate

This mixture was gently mixed, and the plate was sealed with a microseal. PCR was performed on a thermal cycler using this programme: 95°C for 3 min, 8 cycles of: 95°C for 30 sec 55°C for 30 sec 72°C for 30 sec, 72°C for 5 min, then hold at 4°C. The Index PCR products were cleaned up using AMPure XP beads in the same manner as described for Amplicon PCR above except that after the ethanol washes the beads were resuspended in 27.5 µl of 10 mM Tris-Cl, pH 8.5 and 25 µl of the supernatant free of beads was transferred to a new plate. Size of the indexed amplicon was verified using Agilent High Sensitivity D1000 screen tape as described above.

The last step was library quantification, normalization and pooling. The libraries were quantified using QubitTM dsDNA HS assay kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions and fluorescence read using QubitTM 4 fluorometer (Life Technologies Holdings Pte Ltd, Singapore). DNA concentration was calculated in nM, based on the size of DNA amplicons based on the average size of DNA amplicons as determined by on Agilent High Sensitivity D1000 screen tape using the formula,

$Concentration\ in\ nM$ = {$Concentration\ in\ (ng/\mu l)$÷660 (g/mol)×$Average\ library\ size$}×106

$where, Average\ library\ size$ = $Average\ base\ pair\ from\ Agilent\ Screen\ Tape$

660 (g/mol) = $molecular\ weight\ of\ double\ stranded\ DNA$

The DNA was diluted to a 4 nM concentration and 5 µl aliquot of the diluted DNA from each library was pooled as follow; first the MiSeq reagent cartridge was removed from -15°C storage and thawed at room temperature. Then DNA was denatured by combining the following volumes in a microcentrifuge tube: 4 nM pooled library (5 µl) and 0.2 N NaOH (5 µl), vortexed briefly then centrifuged at 280 × g at 20°C for 1 minute before incubating for 5 min at room temperature. Then 990 µl of pre-chilled Hybridization Buffer HT1 was added to the tube containing denatured DNA (10 µl) Library resulting in a 20 pM denatured library in 1 mM NaOH and placed on ice until use. PhiX as a sequence control was denatured and diluted to 4 nM by combining 10 nM PhiX library (2 µl) and 10 mM Tris pH 8.5 (3 µl). Then 4 nM PhiX library (5 µl) and 0.2 N NaOH (5 µl) was combined in a microcentrifuge tube and vortexed briefly before incubating for 5 minutes at room temperature to denature the PhiX library into single strands. Then 990 µl pre-chilled HT1 was added to the tube containing 10 µl denatured PhiX library to result in a 20 pM PhiX library. This was then diluted to the same loading concentration as the Amplicon library to get 8pM by mixing 240 µl of 20 pM denatured library and pre-chilled HT1 (360 µl). The Amplicon Library and PhiX Control were combined in volume of 570 µl and 30 µl respectively. This was then set aside on ice until it was time to heat denature the mixture immediately before loading it onto the MiSeq v3 reagent cartridge at which point the mixture was incubated at 96°C for 2 minutes by using a heat block. Afterward

the tube was mixed and placed in the ice-water bath. Finally, the template allocations of samples wsere set up in the Illumina sheet, then the combined sample library and PhiX was loaded into the well in the Miseq cartridge then loaded in the machine using version 3 (300 × 2) on the MiSeq instrument (Illumina Inc., USA) according to manufacturer's instructions.

2.5.17.4. Bioinformatic Analyses

Raw reads were trimmed for Illumina Nextera XT adapters and read through using Trimmomatic version 0.38 (Bolger *et al*., 2014). Adapter trimmed reads were checked for quality through FastQC version 0.11.7 (ref: https://github.com/s-andrews/FastQC) for adapter sequences and average sequencing quality drop off below Phred 20.

Dada2 was used to estimate error rates within the fastq files, and further trim the sequences PhiX reads were filtered, and reads were discarded if they contained any Ns. Reads were merged and denoised, and chimeric reads removed. All unique reads from the positive control (mock reference) were extracted and compared against a FASTA of 16S sequences from the strains in the ATCC mock. Taxonomy was assigned to sequences by comparison to the Silva database v132.

At this point, data was transferred to Phyloseq v1.30.0. Phyloseq requires a count for each OTU (or unique read), a taxa table (obtained through the comparison with the Silva database) and metadata. OTU names, were changed from nucleotide sequence and given an amplicon sequence variant (ASV) number. Samples were pruned, to remove negative and positive

control as well as samples that failed PCR or sequencing reaction. Any ASV that was present in less than two samples was also removed from the dataset.

2.5.17.5. Ecological and Statistical analyses of sequencing data

Further downstream analyses and graphical outputs were generated using Marker Data Profiling module of Microbiome Analyst a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data.

Marker Data Profiling module was used as according to Chong *et al*. (2020). Under data filtering, the low count and low variance (based on inter-quantile range) were both selected to be 10%. Data was neither rarefied nor transformed and total sum scaling (TSS) was selected for normalization. Both alpha and beta diversity metrics were used to estimate microbial community diversity. Species richness (Observed ASVs and Chao1) and evenness (Shannon and Simpson index) were selected for alpha diversity estimations. To compare alpha diversity metrics among groups, non-parametric Kruskal-Wallis test was selected. For beta diversity analysis, dissimilarity matrix between samples Bray Curtis method was selected, and was further visualized with a Non-Metric Multidimensional Scaling (NMDS) ordination technique. Bray Curtis dissimilarity was also used to perform hierarchical clustering (dendrogram) with average as the clustering algorithm.

2.6 Data Analysis

Outliers were removed from each data set if they fell above or below 2x the standard deviation of the mean. All data was analysed using SPSS software, version 25 for Windows (IBM Statistics, 2018). KS testing was applied to determine normality, then either ANOVA or

Kruskal Wallis was applied to determine significance. Univariate analysis was used to determine interactions between analysed factors. Significant differences were elucidated by post hoc test (Tukey HSD) of the statistical software package.

Correlations were analysed by bivariate correlation using Pearson correlation, chosen because it computes based on true values and depicts linear relationships. Multiple linear regressions were used to determine the unique contribution and relatedness of factors. Interpretations of the strength between relationships was based on those of Cohen (1988): small r = 0.1-0.29, medium r = 0.30-0.39 and large r = 0.50 to 1.0. Statistical significance was declared at $p<0.05$.

CHAPTER 3: The effect of standard (500 FTU) and super-dosing (3000 FTU) of phytase on performance and whole body phosphorus content of broilers

3.1. Introduction

Phytic acid is considered an anti-nutritional factor in monogastric animals not only due to its low phosphorus digestibility in the gastrointestinal tract, but also as it reduces bioavailability of minerals (Plumstead *et al*., 2008), protein (Liu *et al*., 2009) and starch (Dr Sonia Yun Liu, 2022) through complex formation in the gastro-intestinal tract (Schlemmer *et al*., 2009). Phytic acid also increases mucus production (Cowieson *et al*., 2004) and reduces broiler performance (Linares *et al*., 2007). To reduce the adverse effects of this anti-nutritional factor, several methods have been practiced among which, use of exogenous phytase has gained much interest. Phytase improves efficiency of utilization of amino acids and energy by reducing anti-nutritional factors and improves growth in broilers (Cowieson *et al*., 2009). Scientists have also recently suggested the generation of myo-inositol through a phytase-initiated enzymatic cascade is responsible for a part of the beneficial effect of microbial phytase in poultry (Walk *et al*., 2014). Phytates also reduce protein digestibility by making complex with them which results in increased intact protein and influences the secretion of more HCL and pepsin (Cowieson *et al*., 2008). When the acidity increases more bicarbonate ions are released and increased intestinal pH, results in reducing the solubility of phytate-protein complex and some of these undigested proteins find their way to large intestine where they are fermented by intestinal bacteria which may lead to poor performance and risk of intestinal disease. These changes in intestinal pH is very important as it can shift the endogenous microbiota profiles and their activity and a lower pH is preferable in the small

intestine for intestinal integrity and microecology by reducing pathogenic and increasing beneficial bacterial population (Tamim and Angel, 2003).

Traditionally, the supplementation of a lower dose (500 FTU/kg feed) of phytase has been practiced as an effort to achieve better utilization of plant P in the diet and thereby reduce inorganic P inclusion, increase animal performance and limit environmental P pollution. The economic use of exogenous phytase as a source of phosphorus often limits the inclusion in broiler diets to approximately 500 FTU/kg, however superdosing of phytase (> 1500 FTU/kg) has become a growing opportunity for poultry producers due to rising ingredient costs and lower enzyme prices. Superdosing of phytase in broiler diets has shown to further improve broiler performance by increasing phytate hydrolysis, improving nutrient availability and perhaps generating myo-inositol with vitamin like effects. The hydrolysis of IP6 is evident to be dose dependant as addition of 500 FTU/kg phytase in a maize-soya based diet catalysed the IP6 hydrolysis around 67% and 78% in jejunum and ileum, while inclusion of 12,500 FTU/kg phytase improved the hydrolysis of IP6 by 91-92% (Zeller *et al*., 2015). The superdosing of phytase is also considered to generate extra-phosphoric effects such as improving amino acid digestibility (Pieniazek *et al*., 2017) and enhancing digestibility of other nutrients (Cowieson *et al*., 2006), and improving antioxidant status of the animals (Karadas *et al*., 2010) which can translate to improved body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) beyond that seen when using traditional doses.

Supplementation of phytase improves the bone mineralisation in broilers (Walters *et al*., 2019). Bone mineralisation especially the tibia phosphorous contents are still widely used as

an easy estimate to determine the whole body phosphorous retention in broiler which is laborious to estimate. More studies explored the relationship between bone phosphorous content and phosphorous availability, but few studies evaluated the relatedness between bone phosphorous content and whole body phosphorous content. Some studies (Hurwitz 1964) reported tibia P is a reliable indicator of whole body phosphorous as the WBP:TP ratio (19.6) was constant throughout the trial while others (Shastak *et al*., 2012c) reported different WBP:TP ratio at varying ages (21.3 and 19.8 on day 21 and 35). Sanni (2017) also reported more phosphorous retained in the tibia compared to whole body phosphorous with the advancement of broiler age. So, it is important to evaluate while superdosing, tibia phosphorous content can be used as an alternative indicator to whole body phosphorous content for accurate evaluation of phosphorous retention in broiler.

The objective of the present study was to investigate the benefits associated with phytate hydrolysis with the inclusion of a super-dose of phytase in a diet not severely limited in available P. The overarching hypothesis for the study is that superdosing of phytase might improve broiler performance and whole body mineralisation by further increasing the hydrolysis of IP6 and producing myoinositol while ensuring better gut health (gut microbiota and mucosa). A secondary hypothesis is that phytase dosage will impede the use of tibia phosphorus levels to predict whole body phosphorus requirements of growing broiler chicks.

The aims of the study were-

1. Examine the effect of super-dosing phytase supplementation on performance and whole body phosphorus content of broilers and relative concentration of differing inositol esters inositol esters (IP6 through to myo-inositol) within the broiler GIT
2. Investigate the relationship between traditional measures of phytase efficacy such as overall growth performance and degree of bone mineralisation with the whole body phosphorus
3. Examine the effect of exogenous phytase supplementation on mucin content in the broiler gastro-intestinal tract and gut microbiota of broilers

3.2. Materials and Method

The broiler feeding trial was undertaken in old Poultry Research Unit at Nottingham Trent University. Ethical approval to conduct the trial was sought from the Nottingham Trent University local ethical review group and permission obtained with allocated code (ARE488) to conduct the trial according to NC3R Arrive guidelines (Kilkenny *et al.,* 2010).

3.2.1. Bird Husbandry

Day old male and female Ross 308 birds (250 males and 250 females) were ordered by NTU from PD Hook and collected from the Cote (Oxford) hatchery. Birds were from a flock aged approximately 40 weeks to optimize bird quality and had a target weight range of approximately 37-43g. The poultry room was heated to 31°C by infra-red heat lamps and wall heaters prior to the birds arrival on day 0. Pens were labelled with treatment, bird sex, diet

number and pen number. Water was provided in the drinkers and trial diet in the feed troughs. Upon arrival at the Poultry Research Unit (PRU), day old male and female Ross 308 were weighed before random allocation to 48 mesh sided pens bedded on wood shavings (3 cm thick). Additional shavings were added as required throughout the trial. Feed and water were available ad libitum, with care taken to ensure the birds ate and drank as soon as possible. Temperature was set at 32°C on day of hatch and reduced by approximately 1°C per day to 21°C by day 21. The room was then maintained at 21°C for the remainder of each trial. Birds were reared as described in chapter 2 section 2.2.

3.2.2. Experimental diets

Four diets were manufactured as a pellet for starter phase by a commercial collaborator and crushed to crumb on-site by PRU staff. Another four diets were also manufactured as a pellet for grower phase by the commercial collaborator. Starter diets were fed from d 0 to 21 and grower diets were fed from d 21 to 35. Each diet had 12 replicate pens (6 male and 6 female) with 10 broilers per replicate.

The phytase Quantum Blue was sourced from AB Vista, UK. Diet specification and calculated nutritional content are presented in the table 3.1. One kg samples of each diet treatment were reserved for proximate analysis and stored at -20°C.

Table 3.1. Experimental diets with rates of inclusion (%)

	Starter (0-21)		Grower (21-35)	
Ingredient (%)	PC	NC	PC	NC
Maize	56.79	56.79	67.11	67.11
Soybean meal	36.56	36.56	27.57	27.57
Soya oil	2.30	2.30	2.15	2.15
Salt	0.24	0.24	0.23	0.23
Limestone	1.42	1.23	0.96	0.78
Monocalcium Phosphate	1.46	0.94	0.94	0.44
Sodium Bicarbonate	0.25	0.25	0.20	0.20
Lysine HCl	0.18	0.18	0.18	0.18
DL-Methionine	0.31	0.31	0.23	0.23
Threonine	0.09	0.09	0.06	0.06
Vitamin & Mineral premix	0.4	0.4	0.4	0.4
Inert filler (sand)	0	0.71	0	0.65
Calculated nutrient values				
ME, MJ/kg	12.55	12.55	12.97	12.97
Protein, %	23.00	23.00	19.42	19.41
DM, %	87.79	87.08	87.57	86.89
Lysine, %	1.28	1.28	1.05	1.05
Methionine, %	0.62	0.62	0.50	0.50
M+C, %	0.95	0.95	0.79	0.79
Tryptophan, %	0.24	0.24	0.19	0.19
Threonine, %	0.86	0.86	0.70	0.70
Arginine, %	1.42	1.42	1.16	1.16
Isoleucine, %	0.88	0.88	0.72	0.72
Valine, %	0.95	0.95	0.80	0.80
Calcium, %	0.96	0.81	0.68	0.53
Avail P, %	0.48	0.35	0.34	0.21
Sodium, %	0.18	0.18	0.16	0.16
Chloride, %	0.22	0.22	0.22	0.22

The four diets are-

A) Positive Control (0.48 & 0.34% non-phytate phosphorous (nPP) in starter & grower diet)
B) Negative Control (0.35 % 0.21% nPP in starter and grower)
C) NC + 500 FTU/kg Quantum Blue phytase
D) NC + 3000 FTU/kg Quantum Blue phytase

The nutrient composition of the experimental diets are shown in table 3.2.

Table 3.2: Analysed nutrient composition of the experimental diet

Phase	**Starter**				**Grower**			
Diet	A	B	C	D	A	B	C	D
Dry matter (%)	89.92	90.43	90.38	89.72	89.18	90.00	89.20	89.48
Gross energy (MJ/kg DM)	16.53	16.73	16.82	16.79	16.63	16.61	16.71	16.75
Ash (g/kg)	5.38	5.45	5.49	5.48	4.30	4.39	4.39	4.57
Crude protein (g/kg DM)	23.44	23.00	23.06	22.94	19.81	19.00	19.81	19.75
Fat (g/kg)	4.89	4.82	4.84	4.74	4.79	4.78	4.68	5.33

NB: Diet A= Positive Control (PC), Diet B= Negative Control (NC), Diet C= NC + 500 FTU/kg, Diet D= NC + 3000 FTU/kg

3.2.3. Treatment schedule or randomization plan

A replicate consisted of a pen containing 10 birds. The weight of each pen (10 birds) was recorded on day 0 and treatments were randomly allotted to pens around the trial room using an online randomizer allocated (by an individual not involved in the study to prevent bias), to reduce any possible effects of ventilation and room placement. There were 12 replicates per

treatment consisting of 6 replicates of male and another 6 of female broilers. Di*et al*location and pen and room layout is shown in figure 3.1 and 3.2 respectively.

pen	di*et al*location		pen	di*et al*location
pen 1	A (M)		pen 25	A (M)
pen 2	A (F)		pen 26	A (F)
pen 3	B (M)		pen 27	B (M)
pen 4	B (F)		pen 28	B (F)
pen 5	C (M)		pen 29	C (M)
pen 6	C (F)		pen 30	C (F)
pen 7	D (M)		pen 31	D (M)
pen 8	D (F)		pen 32	D (F)
pen 9	A (M)		pen 33	A (M)
pen 10	A (F)		pen 34	A (F)
pen 11	B (M)		pen 35	B (M)
pen 12	B (F)		pen 36	B (F)
pen 13	C (M)		pen 37	C (M)
pen 14	C (F)		pen 38	C (F)
pen 15	D (M)		pen 39	D (M)
pen 16	D (F)		pen 40	D (F)
pen 17	A (M)		pen 41	A (M)
pen 18	A (F)		pen 42	A (F)
pen 19	B (M)		pen 43	B (M)
pen 20	B (F)		pen 44	B (F)
pen 21	C (M)		pen 45	C (M)
pen 22	C (F)		pen 46	C (F)
pen 23	D (M)		pen 47	D (M)
pen 24	D (F)		pen 48	D (F)

Figure 3.1: Allocation of treatments to pens and plots

19		20	41		42
18		21	40		43
17		22	39		44
16		23	38		45
15		24	37		46
14		25	36		47
13		26	35		48
12		27	34		
11		28	33		
10		29	32		1
9		30	31		2
8					
Spare	7	6	5	4	3

Figure 3.2: Pen and room layout

3.2.4. Bird preparation

Upon removal from the pen, each bird was individually euthanised by cervical dislocation and weighed. The birds were then dissected, and content in the crop, proventriculus, intestine, and caeca, emptied and flushed with ultra-pure water. The birds including the emptied organs were re-weighed to determine whole body weight after which both right and left bones (tibia, femur, and feet, according to the bone type studied) were removed as described in Chapter 2, Section 2.4.5. Whole body (WB) as used in this study is defined as the whole bird including feather, cleaned viscera and bones but emptied of digestive tract content. Briefly, flesh from the tibia and femur was carefully removed with a scalpel whilst ensuring cartilage caps were kept intact. Care was taken to ensure the flesh removed from the bones was added back to the remaining carcasses. The feet were not defleshed. The whole bird including feathers and viscera but excluding the tibia, femur or feet were then individually cut into small pieces and stored at -20^{o}C until further processing. Feathers were included in the measure for whole body phosphorus content to ensure all bodily requirements for phosphorus (however small) were accounted for in order to ensure maximum accuracy. Their associated bones (tibia, femur, or feet) were also individually stored at -20^{o}C until further processing.

At processing, whole body (excluding sampled bones) were individually ground from slightly frozen in an electric mincer as described in Chapter 2, Section 2.4.6. Briefly, the cut pieces of the whole body were fed through the mincer and kneaded by hand before re-mincing. This process was repeated 4 times until the sample was thoroughly ground and homogenised. Between each bird minced, the electric mincer was fully cleaned and rinsed with ultra-pure water in order to prevent carryover of tissue. A 200g representative sample of each ground bird was then collected and stored at -20^{o}C until further analysis.

3.2.5. Determined parameters

The weight of the birds and feed per pen were recorded at the onset and then weekly to calculate average bird weight (BW), Body weight gain (BWG), Feed intake and feed conversion ratio are described in Chapter 2, Sections 2.4.1.

One bird was sampled on days 0, 7, 14 21, 28 and 35 to determine the mucin layer thickness, ash %, tibia mineral content and whole body phosphorous content. Prior to sampling, birds were humanely euthanized via cervical dislocation by trained operatives in a separate room in line with the guidelines of the Scientific procedures Act (ASPCA, 1986).

Bones (tibia & femur) were defleshed and bone (tibia, femur & feet) weight and ash weight and ash percentage were determined as previously described in Chapter 2, Sections 2.5.4.2.

Whole body samples were processed as described in previous section 3.2.4 and in Chapter 2, Section 2.4.6. Briefly, ground whole body samples (excluding bones) were oven dried at 105^{o}C for approximately 5 days until a constant weight was achieved to determine moisture content and then extracted of fat using the Soxtherm hot fat extraction method for 2h as previously described in Chapter 2, Section 2.5.3. The dry fat extracted samples were then ground, and 10g subsamples weighed to determine ash content. The 10g subsamples were gradually ashed in a furnace for 9h at 450^{o}C, and then for a further 15h at 650^{o}C. Both bone and whole body ash were analysed for total calcium and total phosphorus content using the ICP-OES assay as previously described in Chapter 2, Section 2.5.6.

A 1cm^2 piece of ileal tissue was collected from 1 bird per pen to measure the mucin layer thickness by a modification of Corne's method (Corne *et al.,* 1974) which was described briefly in chapter 2 section 2.5.11. Ileal, jejunal, crop and gizzard weight and content were also collected into labelled pot per pen. Digesta weight and digestive tract weight were measured according to Chapter 2, section 2.4.3. All digesta samples were stored at -20°C until required. Samples were later freeze dried (LTE Scientific, Oldham, UK) for 7 days before being re-weighed and then ground manually with a pestle and mortar to a homogenous fine powder. One set of ground digesta samples were sent to University of East Anglia to determine inositol esters and the method is described in chapter 2, section 2.5.14. Both caeca from each bird were excised post-mortem and collected separately in clean labelled bags. The caeca were weighed before being snap frozen on dry ice. Caeca were then stored at -20°C and used later to determine gut microbiome diversity using 16s rRNA sequencing method described in Chapter 2, Section 2.5.17.

3.2.6. Experimental design and data analysis

The experimental design of trial 1 was randomised block design where the experimental units are grouped into blocks (in this trial experimental units are grouped into male vs female, so sex is block here)

Data of trial 1 of each phase (weekly) were analysed separately. The following model was used to analyse the data

$$Y_{(ij)} = M + Diet_{(i)} + Sex_{(j)} + Diet*Sex_{(ij)} + E_{(ij)}$$

Where, Y is the dependant variable, M is the overall mean, Diet is the effect of the experimental diet, Sex is the effect of bird sex and RE is the residual effect. Diet*Sex is the interaction between the diet and the sex.

Mean values of all right and left bone measurements were used for statistical analysis. WBP and WBCa content for each bird were calculated as the sum of calcium and phosphorus content determined in the whole bird plus that determined in tibia and femur. All data was analysed using SPSS software, version 25 for Windows (IBM Statistics, 2018). The Two-way ANOVA model was used to evaluate the main effect of either diet, bird sex (Male or Female) or age (days) on performance, mucin layer thickness, bone parameters and WBP content for the whole dataset. Univariate analysis was used to determine interactions between analysed factors. Significant differences between means were elucidated by post hoc test (Tukey HSD) of the statistical software package. The performance data was also analysed using polynomial model to better explained the data but no significant effect was found (Appendix-C). The strength of the relationship between the calcium and phosphorus content and ash % in tibia, ash % in femur & feet and whole body; and with all other bone measurements were examined using the Pearson correlation procedure. Interpretations of the strength of relationships were based on those of Cohen (1988): small r = 0.1 - 0.29, medium r = 0.30 - 0.49 and large r = 0.50 - 1.0. Regression analysis was performed to examine the relatedness between the phosphorus content of the tibia, and their respective WBP content. Statistically significant difference was declared at $P < 0.05$.

3.3. Results

3.3.1. Environment

No environmental abnormalities occurred during this trial. The temperature, humidity, lighting and ventilation all were in standard condition.

3.3.2. Health and Condition

The mortality was 3.75% during the 35 days feeding trial which is considered standard for trials conducted at poultry research unit at NTU. Table 3.3 represents the weekly mortality of four dietary treatments and there was no apparent effect of diet on mortality.

Table 3.3: Weekly mortality of broilers

Diet	d0-7	d7-14	d14-21	d21-28	d28-35	total
Positive Control	4	0	1	0	0	5
Negative Control	2	1	0	1	0	4
Negative Control +500 FTU Phytase	3	1	0	1	0	5
Negative Control +3000 FTU Phytase	2	0	1	0	1	4

3.3.4. Broiler Uniformity

The mean start weights of the broilers during the trial are shown in table 3.4. The start weight of the broilers were similar among the treatments at the onset of the trial period.

Table 3.4: Average start weights for broilers (SE)

Diet	D0 bird weight (g)
Positive Control	43.13 (0.36)
Negative Control	43.09(0.36)
Negative Control +500 FTU Phytase	43.12(0.29)
Negative Control +3000 FTU Phytase	42.95(0.29)
p-value	0.974

3.3.5. Broiler Performance

To evaluate the effect of external phytase on broiler performance, weight gain and feed intake were measured. FCR was calculated from BWG and FI. The mean weight gain and feed intake and FCR were shown in table 3.5.

Table 3.5: Performance of broilers (±SE) fed diets with or without supplementation of external phytase

Day	Treatment	Body Weight Gain (g)		Feed Intake (g)		Feed Conversion Ratio	
		male	female	male	female	male	female
0-21d	**Positive Control (PC)**	1089.1 (16.9)	1001.7 (11.3)	1420.2 (25.4)	1333.6 (14.2)	1.31 (0.01)	1.33 (0.01)
	Negative Control (NC)	1103.7 (15.9)	975.4 (12.5)	1470.4 (30.3)	1317.9 (19.4)	1.33 (0.02)	1.35 (0.01)
	NC+500 FTU/kg	1103.2 (9.6)	988.6 (16.9)	1459.7 (32.2)	1334.4 (21.7)	1.32 (0.02)	1.35 (0.02)
	NC+3000 FTU/kg	1120.8 (37.4)	991.5 (6.9)	1447.9 (42.4)	1310.8 (13.2)	1.29 (0.01)	1.32 (0.01)
21-35d	**Positive Control (PC)**	1634.7 (39.5)	1418.2 (30.7)	2480.2 (53.9)	2235.4 (29.4)	1.52 (0.03)	1.58 (0.02)
	Negative Control (NC)	1708.5 (38.1)	1395.9 (27.3)	2611.4 (35.9)	2229.0 (33.2)	1.53 (0.02)	1.60 (0.02)
	NC+500 FTU/kg	1719.8 (24.4)	1415.2 (22.3)	2564.8 (28.7)	2262.6 (34.9)	1.49 (0.03)	1.60 (0.02)
	NC+3000 FTU/kg	1638.5 (99.2)	1374.1 (38.2)	2402.7 (114.8)	2189.8 (80.4)	1.48 (0.07)	1.59 (0.04)
0-35d	**Positive Control (PC)**	2723.8 (38.5)	2419.8 (38.4)	3900.4 (70.0)	3569.1 (39.6)	1.43 (0.02)	1.48 (0.01)
	Negative Control (NC)	2812.8 (43.6)	2371.4 (35.7)	4081.8 (47.4)	3546.9 (47.9)	1.45 (0.01)	1.50 (0.01)
	NC+500 FTU/kg	2822.9 (23.1)	2403.8 (33.3)	4024.5 (31.7)	3597.0 (36.1)	1.42 (0.02)	1.50 (0.01)
	NC+3000 FTU/kg	2759.4 (133.6)	2365.5 (42.7)	3850.6 (146.3)	3500.5 (89.3)	1.40 (0.04)	1.48 (0.03)

Table 3.6: Effect of different diets and sex on Body Weight Gain (BWG), Feed Intake (FI) and Feed Conversion Ratio (FCR) of broiler

P value	BWG			FI			FCR		
Phase	0-21	21-35	0-35	0-21	21-35	0-35	0-21	21-35	0-35
Sex	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.010	0.001	<0.001
Diet	0.838	0.564	0.826	0.828	0.142	0.191	0.070	0.877	0.475
Diet*Sex	0.630	0.717	0.666	0.642	0.504	0.500	0.973	0.830	0.763

No significant effect of phytase supplementation was observed in the body weight gain, feed intake or feed conversion ratio of broilers during different time periods of the trial (Table 3.6) ($P>0.05$) but better feed conversion ratio was recorded for male broilers compared to that of female (Table 3.6) ($P< 0.05$). No significant interaction of diet and sex was observed throughout the trial.

3.3.6. Mucin layer thickness of broiler

Addition of phytase in the diet had no effect on altering mucin layer thickness in broilers in this study. It has been recorded that mucin layer thickness altered with the age of the broilers (Table 3.7)**.** At day 7 mucin layer thickness was higher which reduced at day 14, followed by increasing significantly at day 21. At day 28 it was similar to day 21 but decreased again at day 35.

Table 3.7: Effect of diet on mucin layer thickness (µg/cm^2) at different age of broiler

Day	Treatment	N	Mucin layer thickness	SE
0-7	A	12	65.16	5.78
	B	12	70.37	9.47
	C	12	65.89	7.22
	D	12	68.83	4.36
8-14	A	11	27.47	2.37
	B	12	31.76	4.09
	C	12	28.96	2.31
	D	12	28.49	3.12
14-21	A	12	38.89	1.69
	B	12	41.49	3.54
	C	12	44.13	3.12
	D	12	44.81	2.23
22-28	A	12	38.08	2.84
	B	12	41.71	2.55
	C	12	35.05	2.25
	D	11	38.21	4.60
29-35	A	12	30.85	2.09
	B	12	27.34	1.54
	C	12	30.38	2.12
	D	12	26.81	1.20
	Age	Diet	Diet*Age	
P value	<0.001	0.802	0.973	

3.3.7. Whole Body Mineralisation

The effect of different dietary treatments and sex of broiler on Whole Body Ca (WBCa) and Whole Body Phosphorous (WBP) content in different ages has been illustrated in Table 3.8.

Table 3.8: Effect of diet and sex on Whole body mineralisation of broiler at different ages

	WBCa (g/kg)	WBP (g/kg)	WBCa:P	WBP:Tibia P
Diet				
Positive Control	11.77	12.67	0.96	79.42
Negative Control	11.35	12.28	0.95	77.67
Negative Control +500 FTU Phytase	10.78	12.12	0.92	76.94
Negative Control+3000 FTU Phytase	11.42	12.43	0.95	79.11
SEM	0.41	0.25	0.02	1.80
Sex				
Male	11.13	12.28	0.95	76.66
Female	11.53	12.46	0.95	79.91
SEM	0.29	0.18	0.01	1.27
Age				
d0	12.10^{b}	9.79^{c}	1.24^{a}	57.68^{d}
d7	8.09^{d}	11.61^{b}	0.66^{c}	66.86^{c}
d14	13.98^{a}	12.61^{b}	1.09^{b}	141.21^{a}
d21	13.63^{ab}	12.41^{b}	1.09^{b}	65.7^{c}
d28	10.33^{c}	18.53^{a}	0.55^{d}	88.44^{b}
d35	9.86^{c}	9.29^{c}	1.07^{b}	49.91^{e}
SEM	0.50	0.31	0.02	2.19
P-values				
Diet	0.40	0.49	0.20	0.73
Sex	0.33	0.47	0.90	0.07
Age	<0.05	<0.05	<0.05	<0.05
Diet*Sex	0.88	0.99	0.19	0.85
Diet*Age	0.84	0.60	0.94	0.73
Sex*Age	<0.05	<0.05	<0.05	<0.05
Diet*Sex*Age	0.86	0.76	0.63	0.93

Superscript letters denote significance at P<0.05

The WBCa and WBP contents of broiler were similar among different diets and there was no significant difference of those minerals between male and female broilers. However, WBCa

and WBP content did differ significantly in different ages of birds. The WBCa content was reduced in day 7 following by an increase on day 14 and 21 and then reduced again at day 28 and 35. On the contrary, WBP content was increased with increasing age and reduced at day 35 ($p<0.05$). The WBCa: P was reduced in day 7 birds with an increase on day 14 and 21 and reduced again at day 28 following an increase on day 35 ($p<0.05$). The highest WBCa: P was observed at day 0. The WBP:TibiaP did not follow any pattern, it was highest at day 14 and then reduced again at day 21 following an increase at day 28 and decrease again at day 35. There were interaction effects between sex and age. At day 14 WBCa, WBP, WBCa:P and WBP: tibia P were significantly higher in female broilers compared to male. On the contrary Male broilers had higher WBCa, WBP and WBCa:P at day 35. (Appendix A)

3.3.8. Tibia Bone Mineralisation

Diet or sex had no effect on tibia Ca and P content of the broilers but when diet interacts with sex it affected the TibiaCa:P significantly ($p=0.05$) (Table 3.9). However, Tibia mineral content varied significantly among different ages of broilers where TibiaCa:P was increased with increasing age, reduced at day 21 following by an increase at day 28 until day 35. The highest TibiaCa:P was observed at day 35 ($p<0.05$). There was no effect of diet and sex on weekly TibiaCa, TibiaP and TibiaCa:P content of the broilers ($P<0.05$).

Table 3.9: Effect of diet and sex on Tibia bone mineralisation of broiler at different ages

Diet	TibiaCa (mg)	TibiaP (mg)	TibiaCa:P
Positive Control	305.98	173.36	1.81
Negative Control	305.06	169.08	1.83
Negative Control +500 FTU Phytase	305.56	168.46	1.83
Negative Control +3000 FTU Phytase	304.58	169.46	1.82
SEM	1.48	1.81	0.01
Sex			
Male	304.69	170.76	1.82
Female	306.20	169.40	1.83
SEM	1.05	1.28	0.01
Age			
d0	310.62^{d}	169.72^{c}	1.83^{d}
d7	348.23^{c}	174.12^{c}	2.0^{c}
d14	182.70^{f}	89.44^{d}	2.00a^{b}
d21	189.43^{e}	189.44^{b}	1.00^{e}
d28	419.10^{a}	211.56^{a}	2.01^{bc}
d35	382.60^{b}	186.21^{b}	2.05^{a}
SEM	1.81	2.22	0.01
P-values			
Diet	0.92	0.23	0.206
Sex	0.31	0.45	0.20
Age	<0.05	<0.05	<0.05
Diet*Sex	0.60	0.40	<0.05
Diet*Age	0.74	0.41	0.63
Sex*Age	0.71	0.29	0.40
Diet*Sex*Age	0.73	0.46	0.65

Superscript letters denote significance at P<0.05

3.3.9. Bone Ash %

Diet had a significant effect on ash % tibia, femur and feet and the dry matter (DM) content tibia. Diets supplemented with a super dose of phytase had the highest ash % irrespective of the bone.

Table 3.10: Weekly Bone parameter (DM % & Ash % dry bone weight) with or without supplementation of dietary Phytase

	Feet		Tibia		Femur	
Diet	**DM**	**Ash**	**DM**	**Ash**	**DM**	**Ash**
Positive Control	28.42	15.63^{a}	35.46^{ab}	35.61^{a}	35.33	34.16^{ab}
Negative Control	28.55	14.88^{b}	35.17^{b}	34.34^{b}	35.34	33.14^{b}
Negative Control +500 FTU Phytase	28.93	15.34^{a}	35.47^{ab}	35.50^{a}	35.23	35.01^{a}
Negative Control +3000 FTU Phytase	29.14	15.40^{a}	36.12^{a}	36.09^{a}	35.37	35.22^{a}
SEM	0.40	0.13	0.34	0.30	0.27	0.40
Sex						
Male	28.32	15.19	35.02^{b}	34.89^{b}	35.05^{b}	33.86^{b}
Female	29.20	15.43	36.09^{a}	35.88^{a}	35.59^{a}	34.91^{a}
SEM	0.59	0.09	0.52	0.21	0.41	0.28
Age						
d0	21.54^{c}	13.42^{c}	26.91^{f}	23.52^{d}	29.05^{f}	22.10^{d}
d7	31.66^{b}	16.25^{a}	31.29^{e}	38.64^{ab}	31.95^{e}	38.48^{a}
d14	30.82^{b}	15.88^{ab}	34.68^{d}	37.67^{b}	34.38^{d}	36.72^{a}
d21	34.16^{a}	15.69^{b}	37.31^{c}	39.49^{a}	36.56^{c}	38.19^{a}
d28			40.89^{b}	35.07^{c}	38.42^{b}	34.92^{b}
D35			42.25^{a}	35.95^{c}	41.54^{a}	33.86^{b}
SEM	0.40	0.13	0.34	0.37	0.27	0.49
P-values						
Diet	0.92	<0.05	0.02	<0.05	0.98	<0.05
Sex	0.14	0.06	<0.05	<0.05	<0.05	<0.05
Age	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Diet*Sex	0.99	0.86	0.41	0.67	0.93	0.72
Diet*Age	0.99	0.09	0.27	0.41	0.34	0.47
Sex*Age	0.95	<0.05	<0.05	0.11	0.92	<0.05
Diet*Sex*Age	0.99	0.86	0.252	0.67	0.75	0.79

Superscript letters denote significance at P<0.05

Ash was expressed as a percentage of dry bone weight. Female broilers had higher Tibia and Femur DM and Ash % compared to male broilers (p<0.05) (Table 3.10). Tibia and femur ash %

were increased with the advancement of age until day 21 and reduced again at day 28 to 35 ($p<0.05$). Tibia ash% was reduced in the NC diet compared to the PC diet and with superdosing of phytase it was improved in NC diet at day 21 and 28 (Table 3.11). The lowest femur ash % also observed with NC diet and it was highest with Superdosing of phytase at day 7.

Table 3.11: Weekly Bone Ash % (% dry bone weight) with or without supplementation of dietary Phytase

Weekly bone ash %	PC	NC	NC+ 500 FTU/kg	NC+ 3000 FTU/kg	P-value
D0 Tibia ash %	23.41	22.50	24.82	23.34	0.584
D7 Tibia ash %	37.88	37.67	39.45	38.63	0.114
D14 Tibia ash %	38.06	37.02	37.72	39.29	0.166
D21 Tibia ash %	40.02^{a}	37.79^{b}	39.66^{ab}	40.07^{a}	0.015
D28 Tibia ash %	36.32^{a}	33.17^{b}	34.36^{ab}	36.45^{a}	0.045
D35 Tibia ash %	36.44	35.52	35.14	36.50	0.423
D0 Femur ash %	21.52	21.95	22.92	22.02	0.854
D7 Femur ash %	38.21^{ab}	37.41^{b}	38.94^{ab}	40.23^{a}	0.047
D14 Femur ash %	37.41	36.32	36.97	37.71	0.164
D21 Femur ash %	37.33	37.63	39.00	38.75	0.508
D28 Femur ash %	34.46	31.79	37.70	36.80	0.172
D35 Femur ash %	35.10	32.72	33.46	34.35	0.267
D0 Feet ash %	13.51	13.49	13.47	13.32	0.905
D7 Feet ash %	16.32	15.91	16.48	16.45	0.279
D14 Feet ash %	16.12	15.19	15.96	15.88	0.171
D21 Feet ash %	16.55^{a}	14.72^{b}	15.85^{ab}	15.95^{ab}	0.016

Superscript letters denote significance at $P<0.05$

Female broilers had higher tibia (day 21 & 28), femur (day 7) and feet ash % (day 0) compared to male broilers, while male broilers had higher feet ash % only at day 7 (Appendix B).

3.3.10. Gastrointestinal tract

Dietary treatment had a significant effect on the overall Gastrointestinal tract weight ($p<0.05$) which was similar and higher in all diets except the diet supplemented with superdose of phytase at day 21 and 28. The digesta and ceca weight of the broilers were not affected by diet (Table 3.12). Male broilers had higher GI tract weight compared to that of females at both day 7 and 21 (Table 3.13).

Table 3.12: Different parameters of broiler gastrointestinal tract (g/kg of BW) with or without supplementation of Phytase in broiler diet.

Days	Diets	GI Tract Weight		Digesta Weight		Caeca Weight	
		Mean	SEM	Mean	SEM	Mean	SEM
7	**Positive Control**	121.63	4.26	79.79	5.50		
	Negative Control	118.77	2.08	84.82	4.52		
	Negative Control +500 FTU Phytase	119.13	1.79	80.60	3.47		
	Negative Control +3000 FTU Phytase	115.40	1.97	81.75	3.57		
14	**Positive Control**	89.45	2.02	52.90	2.78	5.69	0.31
	Negative Control	87.83	2.14	51.82	5.32	4.63	0.79
	Negative Control +500 FTU Phytase	93.61	3.33	48.86	2.87	5.20	0.47
	Negative Control +3000 FTU Phytase	85.96	2.27	52.38	1.75	5.98	0.56
21	**Positive Control**	69.59^{ab}	2.29	39.44	1.76	5.17	0.34
	Negative Control	71.13^{a}	2.14	39.00	2.14	4.64	0.50
	Negative Control +500 FTU Phytase	69.79^{ab}	1.40	42.86	3.07	5.31	0.44
	Negative Control +3000 FTU Phytase	63.78^{b}	1.24	35.91	2.23	5.22	0.32
28	**Positive Control**	58.67^{ab}	1.53	45.25	2.25	5.74	0.48
	Negative Control	60.00^{ab}	1.26	37.99	1.44	5.34	0.44
	Negative Control +500 FTU Phytase	60.61^{a}	1.62	39.01	2.76	5.52	0.58
	Negative Control +3000 FTU Phytase	55.38^{b}	0.89	39.58	2.45	5.41	0.24
35	**Positive Control**	57.95	1.96	38.88	4.10	4.91	0.51
	Negative Control	55.43	1.79	38.29	2.97	4.26	0.40
	Negative Control +500 FTU Phytase	53.19	1.73	34.87	2.53	4.11	0.39
	Negative Control +3000 FTU Phytase	53.05	1.98	32.58	2.39	5.19	0.88

Superscript letters denote significance at $P<0.05$

Table 3.13: Effect of diet and sex on different parameters of broiler gastrointestinal tract with or without supplementation of Phytase in broiler diet

P value	GI Tract Weight					Digesta Weight					Ceca Weight			
Days	7	14	21	28	35	7	14	21	28	35	14	21	28	35
Diet	0.371	0.154	0.019	0.036	0.220	0.866	0.852	0.270	0.137	0.410	0.390	0.652	0.930	0.462
Sex	0.007	0.081	0.008	0.055	0.134	0.426	0.542	0.761	0.230	0.665	0.761	0.474	0.760	0.660
Diet*Sex	0.172	0.240	0.419	0.526	0.570	0.844	0.704	0.908	0.918	0.120	0.901	0.244	0.513	0.051

3.3.11. Concentration of different inositol esters

Diet had a significant effect on the concentration of different inositol esters at different ages. At day 7, crop InsP4 and InsP4 concentration were greater in the NC+500 FTU phytase diet compared to NC+ 3000 FTU phytase diets. Crop InsP0 concentration were significantly higher with the superdose of phytase compared to the standard dose. At day 14, crop InsP5 and InsP6 concentration were higher with the standard dose compared to the superdose of phytase.

At day 21 gizzard InsP3, InsP4, InsP5 and InsP6 concentrations were greater with the standard dose of phytase compared to that of superdosed. On the other hand, at day 21 ileal concentration of InsP0, InsP3, InsP4 were greater with the superdose of phytase and, InsP5 & InsP6 were higher in the standard dose of phytase. The concentration of different inositol esters at jejunum followed a similar trend to ileum except InsP4 which concentration were similar in both standard and superdose phytase containing diets at day 21.

At day 35 gizzard InsP3 & InsP4 concentrations were greater with the standard dose of phytase compared to the superdose of phytase. InsP0 concentrations were greater with the superdose of phytase and InsP5 and InsP6 were higher in the standard dose of phytase in the ileum which is similar to that seen at day 21. At jejunum the concentration followed the same trend as ileum at day 35 except InsP4 which was also higher with the standard dose.

The significance of these varying level of inositol phosphates in different GIT amongst diets were discussed is discussion section.

Table 3.14: Concentration of different inositol phosphate (InsP) isomers (nmol/g DM) in digesta sample

Day	Place	Conc.	Diet		SEM	P-Value		
			NC+500 FTU/kg	NC+3000 FTU/kg		Diet	Sex	Diet*Sex
7	**Crop**	**InsP0**	2452.59[b]	3175.10[a]	124.19	<0.05	0.34	0.73
		InsP3	1305.29[a]	669.71[b]	120.58	0.01	0.18	0.43
		InsP4	3346.49[a]	1735.33[b]	379.57	0.03	0.16	0.52
		InsP5	726.39	452.36	114.04	0.25	0.46	0.97
		InsP6	3869.10	2451.81	666.73	0.32	0.63	0.91
14	**Crop**	**InsP0**	3111.72	2591.38	388.58	0.53	0.30	0.44
		InsP3	886.15	959.78	56.23	0.45	0.78	0.07
		InsP4	2851.15	2937.87	235.09	0.79	0.85	0.12
		InsP5	2085.93[a]	1288.17[b]	139.38	<0.05	0.11	0.41
		InsP6	13094.43[a]	8402.07[b]	986.81	0.01	0.08	0.65
21	**Gizzard**	**InsP0**	1677.57	1994.64	156.04	0.33	0.35	0.86
		InsP3	941.30[a]	296.32[b]	82.00	<0.05	0.59	0.88
		InsP4	2381.60[a]	280.60[b]	282.31	<0.05	0.26	0.44
		InsP5	488.25[a]	47.04[b]	74.43	<0.05	0.32	0.35
		InsP6	1215.47[a]	193.58[b]	150.18	<0.05	0.45	0.55
	Ileum	**InsP0**	15890.87[b]	26038.59[a]	1395.88	<0.05	0.48	0.15
		InsP3	1632.37[b]	3404.40[a]	281.48	<0.05	0.74	0.20
		InsP4	5231.70[b]	7039.57[a]	456.71	0.05	0.91	0.34
		InsP5	8413.86[a]	3720.75[b]	574.27	<0.05	0.08	0.92
		InsP6	34077.30[a]	8545.59[b]	618.01	<0.05	0.01	0.05
	Jejunum	**InsP0**	16602.54[b]	26431.65[a]	1250.17	<0.05	0.66	0.47
		InsP3	873.80[b]	1847.96[a]	181.06	0.01	0.91	0.79
		InsP4	2396.74	3355.24	310.60	0.14	0.83	0.58
		InsP5	3538.13[a]	1590.12[b]	357.81	0.01	0.85	0.73
		InsP6	16592.38[a]	3934.21[b]	1713.00	<0.05	0.30	0.28
35	**Gizzard**	**InsP0**	4549.26	3276.51	782.73	0.40	0.10	0.15
		InsP3	718.63[a]	370.62[b]	64.58	0.01	0.52	0.60
		InsP4	1094.61[a]	282.98[b]	153.89	0.01	0.44	0.78
		InsP5	622.26	12.88	256.21	0.24	0.25	0.26
		InsP6	2622.35	130.34	1073.47	0.25	0.27	0.28
	Ileum	**InsP0**	22100.08[b]	29041.13[a]	1584.54	0.02	0.13	0.12
		InsP3	1075.34	1285.46	124.62	0.40	0.11	0.88
		InsP4	2851.10	1955.10	317.32	0.15	0.08	0.40
		InsP5	3380.77[a]	574.26[b]	443.72	<0.05	0.02	0.06
		InsP6	14658.20[a]	2000.26[b]	1796.78	<0.05	0.04	0.09
	Jejunum	**InsP0**	17471.69[b]	21433.17[a]	971.79	0.04	0.38	0.61
		InsP3	727.62	696.69	45.41	0.72	0.04	0.22
		InsP4	1168.19[a]	723.53[b]	95.81	0.02	0.39	0.82
		InsP5	947.70[a]	250.64[b]	124.94	<0.05	0.67	0.46
		InsP6	5118.86[a]	1045.52[b]	710.80	<0.05	0.94	0.77

Superscript letters denote significance at P<0.05

3.3.12. Microbial diversity of broiler based on 16S r RNA gene data

Sequencing of the 16S r RNA genes in the caecal samples produced a total of 536144 reads after quality filtering, with a mean sequence number of 10722 ± 16241 reads per sample.

3.3.12.1. Alpha diversity

An increase in species richness was observed as the chickens fed the PC diet was compared to that of the NC, as indicated by Observed operational taxonomic units (OTUs) and Chao1 (Table 3.15, Fig 3.3) but it was not significant. Both estimators showed similar patterns reassuring that the sequencing depth obtained was sufficient. The Kruskal-Wallis tests of Richness, Shannon and Simpson indicated that bacterial diversity in chickens did not differ significantly among diets. The mean number of observed OTUs identified was 120.58 for the positive control diet and 119.27 for the negative control diet. When the negative control diet supplemented with standard dose of Phytase the observed OTUs increased whereas with the superdose of phytase it was similar to the NC samples.

Table 3.15: Effect of diet on alpha diversity of broiler microbiome

Diversity index	PC	NC	NC+ 500 FTU/kg	NC+ 3000 FTU/kg	P-value
Shannon	4.06	4.04	4.06	4.03	0.63
Observed OTUs	120.58	119.27	121.41	119.25	0.98
Chao1	127.44	126.53	128.08	125.75	0.98
Simpson	0.97	0.97	0.97	0.97	0.89

Table 3.16: Effect of sex on alpha diversity of broiler microbiome

Diversity index	Male	Female	P-value
Shannon	4.05	4.04	0.48
Observed OTUs	121.08	119.17	0.62
Chao1	127.71	126.18	0.62
Simpson	0.97	0.97	0.78

An increase in species richness was observed in male broilers as indicated by observed OTUs and Chao1 but that was not significant, which means gender (Table 3.16) had no effect on the species richness in broilers.

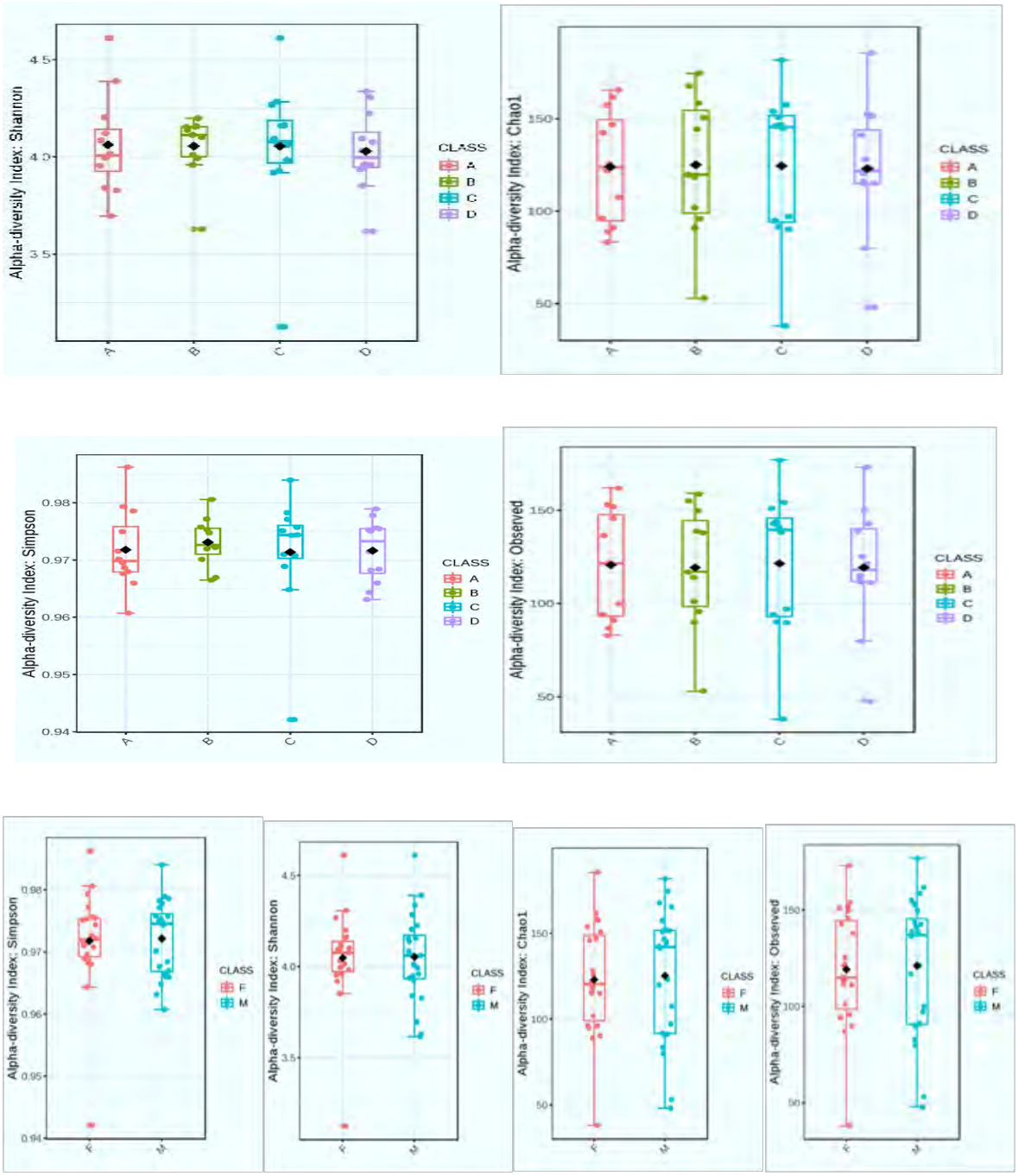
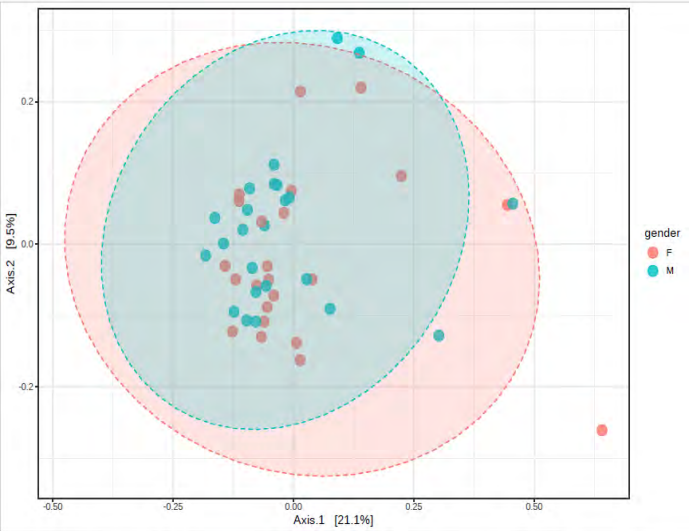
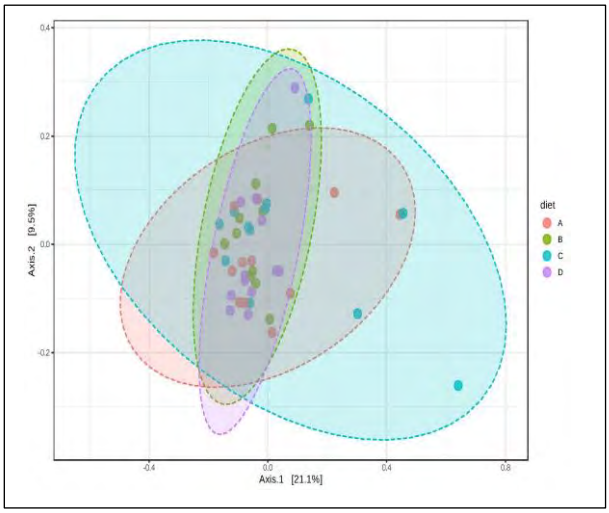


Fig 3.3: Boxplots representing alpha diversity metrics of richness (Observed OTUs and Chao1) and evenness (Shannon and Simpson) grouped according to diet type and sex. Non-parametric Kruskal-Wallis test for multiple comparisons was conducted. Each point represents the diversity score for a sample and points are color-coded according to age diet type (A =PC, B=NC, C= NC+ 500 FTU/kg, D = NC+ 3000 FTU/kg) and sex (M=Male, F=Female). The box represents the first (Q1) and third (Q3) quartiles of the distribution and the line within the box marks the median. The whiskers extend from Q1 to Q3 and values beyond these whiskers are considered as outliers.

3.3.12.2. Beta diversity

The overall microbial community structure exhibited no significant shift either by variation in gender (F=0.75, R2=0.02, p<0.79) or diet (F= 0.86, R2=0.06, p<0.76) based on PERMANOVA. Beta diversity revealed that individual variation in the community structure was greater in the diet with the standard level of Phytase in the NC diet but not significant. The PCoA plot based on the Bray-Curtis dissimilarity matrix showed no diet related clustering (Figure 3.4). The PCoA plot also visually confirmed both male and female broilers shared a similarity in community structure (Figure 3.4). These results indicate that the chickens shared a core set of microbiota in the cecum regardless of the dietary supplementation or gender.





[PERMANOVA] F-value: 0.75345; R-squared: 0.016468; p-value < 0.798 gender

[PERMANOVA] F-value: 0.86284; R-squared: 0.05678; p-value < 0.764 diet

Fig 3.4: Principal coordinates analysis (PCoA) plots based on the Bray-Curtis dissimilarity matrix on relative abundance data. Colours indicate sex or diet type. Ellipses indicate 95% confidence intervals of multivariate t-distribution around centroids of the groupings with sex or diet as factor. Diet type (A =PC, B=NC, C= NC+ 500 FTU/kg, D = NC+ 3000 FTU/kg) and sex (M=Male, F=Female).

3.3.12.3. Taxonomic classification of the bacteria using 16S r RNA genes

3.3.12.3.1. Relative abundance of microbes at phylum, family and genus level

Comparisons of the relative abundances of the gut microbiota compositions at the phylum, family and genus levels are shown in Fig. 3.5, 3.6 and 3.7.

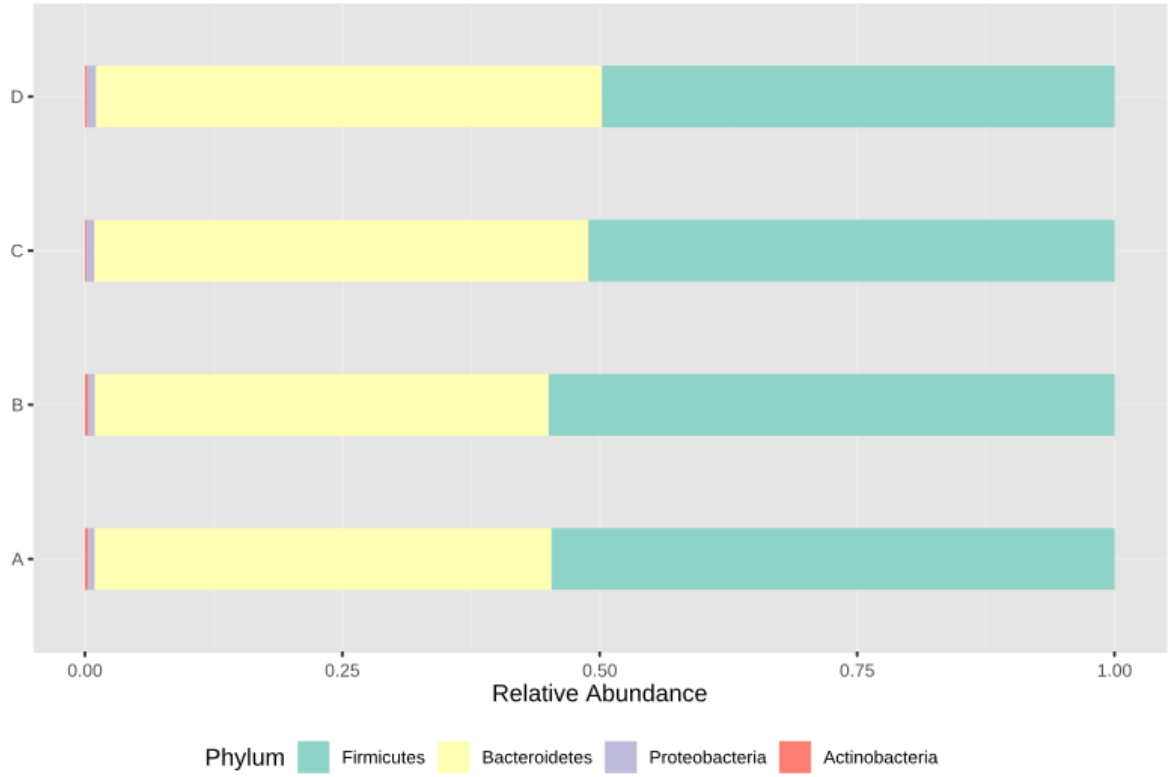


Fig 3.5: Microbial community composition of chicken caecal content. Stacked bar plots representing relative abundances of the different phyla in all samples according to diet type (A =PC , B=NC , C= NC+ 500 FTU/kg , D = NC+ 3000 FTU/kg)

There was no significant difference in the relative proportions of microbial communities according to diet or gender at the phylum level when comparing the whole data set (Table 3.17). At phylum level, Firmicutes (>50%) formed more than half of the caecal microbiome in all treatment groups. The second predominant phyla was Bacteroidetes (46%), other minor

phyla are Proteobacteria (0.63%) and Actinobacteria (0.25%). There was no significant difference in the proportion of either Firmicutes, Proteobacteria or Actinobacteria due to supplementation of phytase, either standard or in superdoses of phytase (Table 3.17).

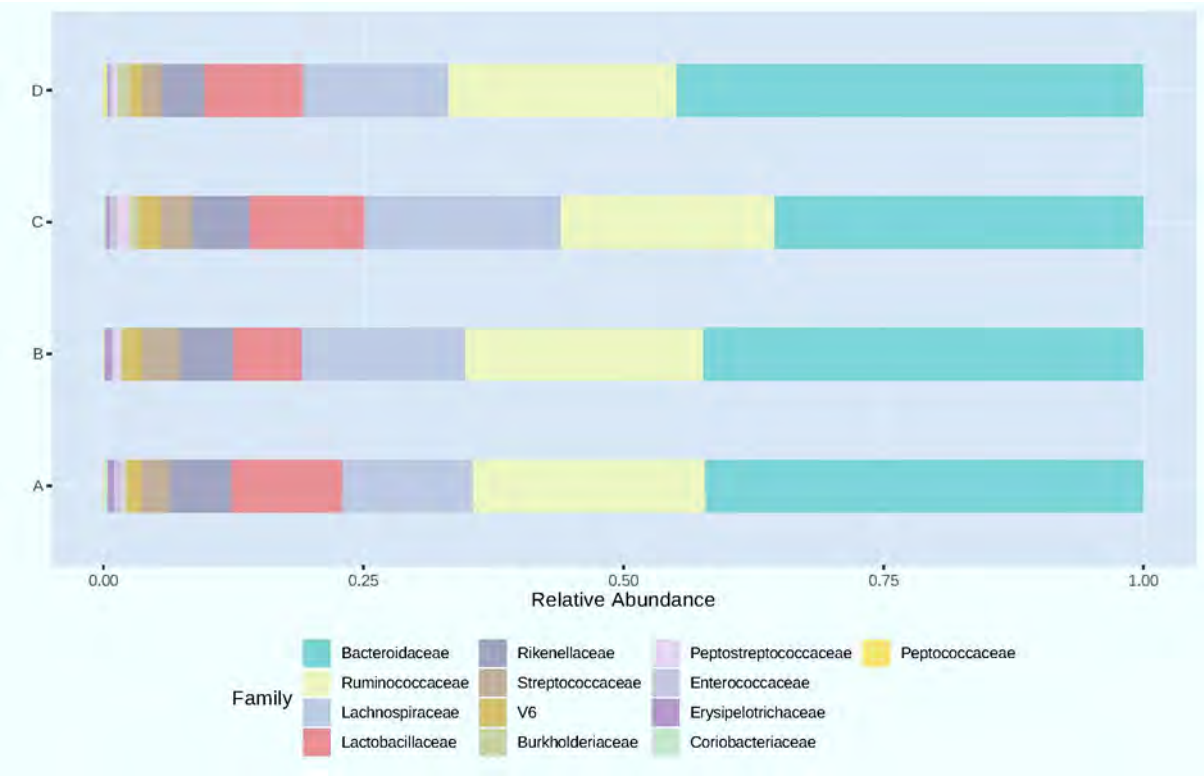


Fig 3.6: Microbial community composition of chicken caecal content. Stacked bar plots representing relative abundances of the different family in all samples according to diet type (A =PC, B=NC, C= NC+ 500 FTU/kg, D = NC+ 3000 FTU/kg)

At the family level, the four most abundant bacterial families in broiler caecal microbiota primarily consisted of Bacteriodaceae (41%), Ruminococcaceae (21.38%), Lachnospiraceae (15.31%) and Lactobacillaceae (9.79%). The remaining minor families were Rikenellaceae,

Streptococcaceae, V6, Burkholderiaceae, Peptostreptococcaceae, Enterococcaceae, Erysipelotrichaceae, Coriobacteriaceae and Peptococcaceae.

The most predominant family was Bacteriodaceae whose relative abundance was numerically decreased with supplementation of the standard dose of Phytase (34.48%) compared to the other dietary treatments (43%) but there was no statistical difference among the diets.

Firmicutes was dominated by families within the order Clostridiales (Ruminococcaceae and Lachnospiraceae) whose relative abundance was similar in every treatment group (Figure 3.6 and table 3.17). In fact, most of the genera identified in every treatment group, belonged to the order Clostridiales although there was no significant difference ($p > 0.05$) in the relative abundance of Clostridiales due to the dietary supplementations. The relative abundance of Lachnospiraceae was numerically higher in the negative control group (15.47%) with the standard (18.86%) and superdose (14.20%) of phytase compared to that of the positive control diet (12.74%). *Blautia* (4.77%) was the top genera within the Lachnospiraceae family and was present at all sampling points followed by the genera Faecalibacterium (3.86%). Sporobacter (2.36%), Subdoligranulum (2.26%), Ruminococcus torques group, Ruminococcus_2 were also observed but in few sampling points.

Lactobacilliales was the next dominant order after Clostridiales in the phylum Firmicutes. The relative abundance of Lactobacilliaceae family was statistically similar among the dietary treatments, although it was numerically higher in the diet supplemented with the standard (11.38%) and super dose (10%) of phytase and positive control diet (11.02%) compared to that of the negative control diet (6.47%). *Lactobacillus* genus was present as the core microbiome and the relative abundance was 9.79%.

Streptococcaceae (another family of Lactobacilliales) was also a part of the core microbiome accounting for nearly 3% of bacterial family whose relative abundance was similar among the dietary treatments. *Streptococcus genus was present in most of the samples whose relative abundance was 3%*.

Rikenellaceae, another family whose relative abundance was 5.02% similar among most of the dietary treatment but slightly lower with the superdose of Phytase (3.84%).

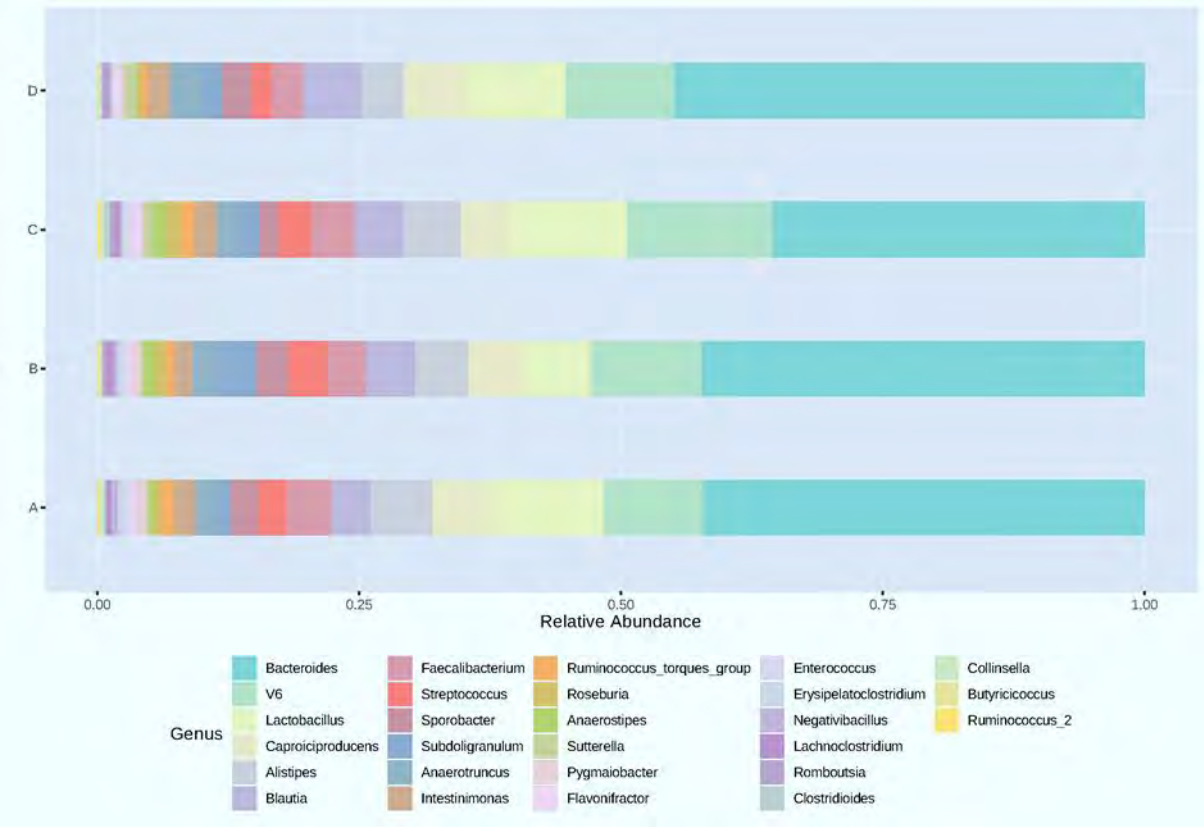


Fig 3.7: Microbial community composition of chicken caecal content. Stacked bar plots representing relative abundances of the different genus in all samples according to diet type (A =PC, B=NC, C= NC+ 500 FTU/kg, D = NC+ 3000 FTU/kg)

At the genus level, Bacteroides (40%) was the topmost enriched genera in the broiler caecal samples in each dietary treatment which was followed by genera Var.1 (11%), Lactobacillus (9.79%), Caproiciproducens (5.25%), Alistipes (5.02%), Blautia (4.77%) and Faecalibacterium (3.86%). These genera were observed in all samples, but none were significantly different when comparing the effect of treatment (Figure 3.7 and Table 3.17). At the genus level, Anaerostipes and pygmiobacter differ significantly among the diets. Anaerostioes usually belongs to the clostridium cluster and its relative abundance was reduced with the superdose of phytase compared to that of the standard dose (3.9). Pygmiobacter was another genus for which relative abundance was reduced with superdosing of phytase compared to that of the positive and negative control diets (Figure 3.8).

Table 3.17: Effect of diet and sex on microbial community at different taxonomic level

	P-values		FDR		Statistics	
phylum	diet	gender	diet	gender	diet	gender
Proteobacteria	0.12	0.47	0.49	0.97	5.79	250.50
Firmicutes	0.39	0.92	0.54	0.97	2.98	271.00
Bacteroidetes	0.40	0.94	0.54	0.97	2.92	280.00
Actinobacteria	0.73	0.97	0.73	0.97	1.30	278.00
family						
Burkholderiaceae	0.12	0.97	0.57	0.97	5.79	278.00
Lachnospiraceae	0.14	0.46	0.57	0.68	5.49	311.50
Lactobacillaceae	0.15	0.20	0.57	0.55	5.26	337.00
Erysipelotrichaceae	0.21	0.36	0.57	0.67	4.53	317.00
Rikenellaceae	0.22	0.18	0.57	0.55	4.42	340.00
Peptococcaceae	0.48	0.65	0.83	0.83	2.50	259.00
Bacteroidaceae	0.50	0.84	0.83	0.91	2.39	266.00
Peptostreptococcaceae	0.59	0.34	0.83	0.67	1.92	233.00
Enterococcaceae	0.59	0.17	0.83	0.55	1.90	219.00
Coriobacteriaceae	0.73	0.47	0.83	0.68	1.30	250.50
Streptococcaceae	0.76	0.56	0.83	0.18	1.15	159.50
Ruminococcaceae	0.94	0.70	0.94	0.83	0.39	257.50
genus						
Pygmaiobacter	0.02	0.90	0.48	1.00	9.40	270.00
Anaerostipes	0.04	0.95	0.48	1.00	8.56	279.50
Sutterella	0.12	0.97	0.71	1.00	5.79	278.00
Lactobacillus	0.15	0.20	0.71	0.68	5.26	337.00
Roseburia	0.16	0.46	0.71	0.85	5.20	311.00
Erysipelatoclostridium	0.21	0.36	0.71	0.82	4.53	317.00
Alistipes	0.22	0.18	0.71	0.68	4.42	340.00
Subdoligranulum	0.22	0.20	0.71	0.68	4.37	336.50
Butyricicoccus	0.24	0.04	0.71	0.52	4.25	206.00
Romboutsia	0.40	0.83	0.82	1.00	2.92	267.00
Ruminococcus_2	0.48	0.39	0.82	0.82	2.46	315.00
Blautia	0.49	0.73	0.82	0.98	2.41	293.00
Bacteroides	0.50	0.84	0.82	1.00	2.39	266.00
Caproiciproducens	0.51	0.63	0.82	0.94	2.31	253.00
Ruminococcus_torques_group	0.53	1.00	0.82	1.00	2.23	276.00
Lachnoclostridium	0.56	0.66	0.82	0.94	2.08	293.50
Anaerotruncus	0.59	0.29	0.82	0.78	1.93	320.00
Enterococcus	0.59	0.17	0.82	0.68	1.90	219.00
Faecalibacterium	0.68	0.66	0.82	0.94	1.50	255.00
Flavonifractor	0.69	0.55	0.82	0.93	1.48	302.50
Negativibacillus	0.72	0.20	0.82	0.68	1.33	228.00
Collinsella	0.73	0.47	0.82	0.85	1.30	250.50
Streptococcus	0.76	0.52	0.82	0.36	1.15	159.50
Clostridioides	0.79	0.10	0.82	0.68	1.05	217.00
Intestinimonas	0.79	0.39	0.82	0.82	1.04	235.00
Sporobacter	0.86	0.26	0.86	0.77	0.76	222.50

The FDR (False discovery rate) is the ratio of false positive results to the total positive results. The FDR value is very useful in multiple testing system associated with RNAseq because a FDR value of 0.1 means the test result or P- value is 90% real. FDR and statistics both are unitless.

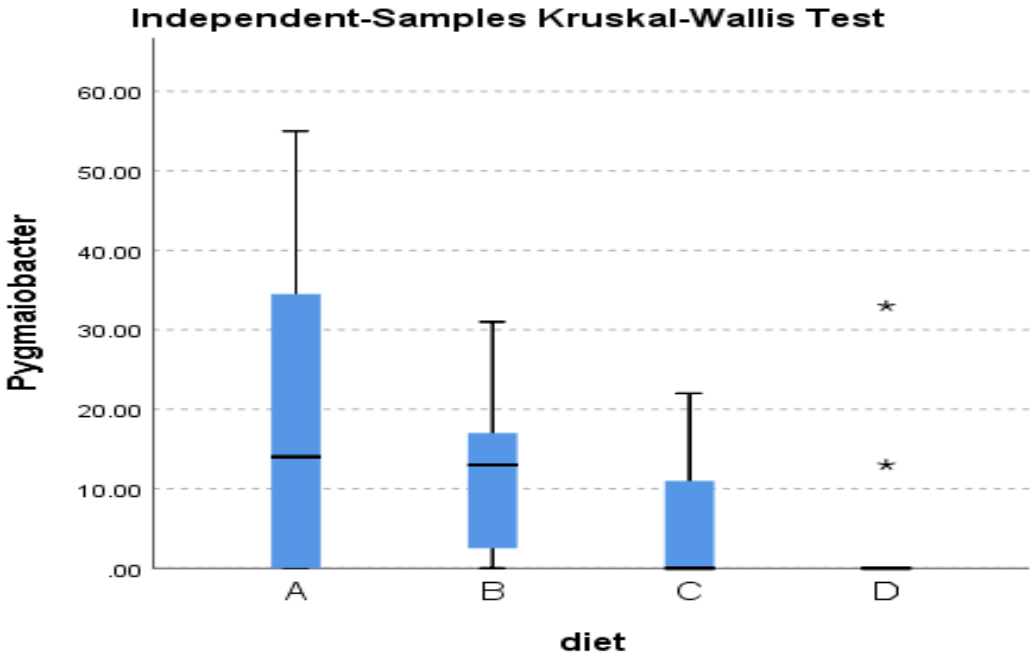


Figure 3.8: Effect of diet on relative proportions of pygmaiobacter in broiler caecum

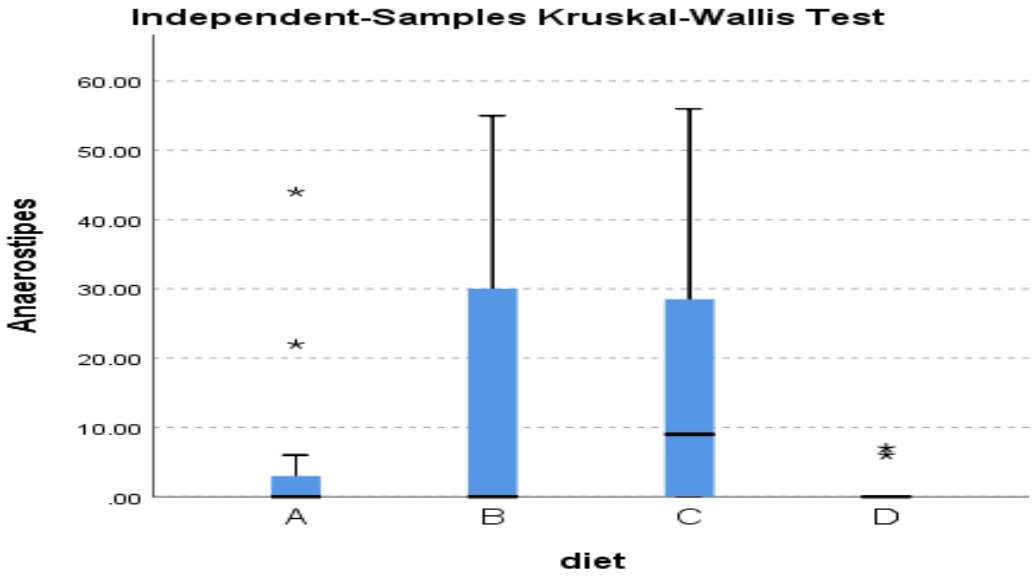


Figure 3.9: Effect of diet on relative proportions of Anaerostipes in broiler caecum

3.4. Discussion

3.4.1. Broiler Performance

There was no effect on broiler body weight gain, feed intake and feed conversion ratio of either the inclusion or level of inclusion of phytase. However, the male broilers had improved FCR compared to that of female broilers. This appears to be due to higher body weight gain in male broilers, which leads to improved FCR throughout the time period. Though the FCR from day 0 to 35 was not statistically significant, the small improvements of 1.48 with phytase superdosing compared to 1.50 with the NC diet may represent financial gains – particularly on a commercial scale where margins are tight and tiny savings per bird are meaningful. Very small changes in FCR have major economic impact on poultry production (Waller, A. 2007) but often a level that is economically relevant such as 0.02 units is beyond the sensitivity that can be assessed with replicates that are viable in a research pen facility. The result of the current study contradicts the findings of other studies as most reported a response to Phytase inclusion and positive control diets would therefore be expected to show an improvement over negative control diets (Walters *et al*., 2019, Santor *et al*., 2014, Powell *et al*., 2011, Singh 2008, Ravindran *et al*., 2008, Selle and Ravindran 2007, Onyango *et al*., 2004).

The difference in the performance data of the present study compared to others may be due to differences in husbandry practices, environmental conditions, dietary requirements, or other factors such as digestibility of the ingredients used in the basal diet, degree of P deficiency in the diet, and the type and level of the phytase used.

A possible explanation to justify the difference in performance during present study may be due to feed spillage as the feed system (open troughs) and mash feed (PC and NC starter diets) encourages the chicks to climb in the feeders, spill feed and dust bathe. The initial body weight of broilers among the diets were similar and there was no dispute in the environmental factors like temperature, humidity or ventilation. The average daily weight gain (analysed from the trial data) was in very close comparison with the ROS 308 performance objectives (Aviagen, 2019; Figure 3.10). The apparent deviation from the ROSS 308 performance objectives is an artefact of the thinning process, where the largest birds are removed for slaughter and the rest left to grow a bit longer.

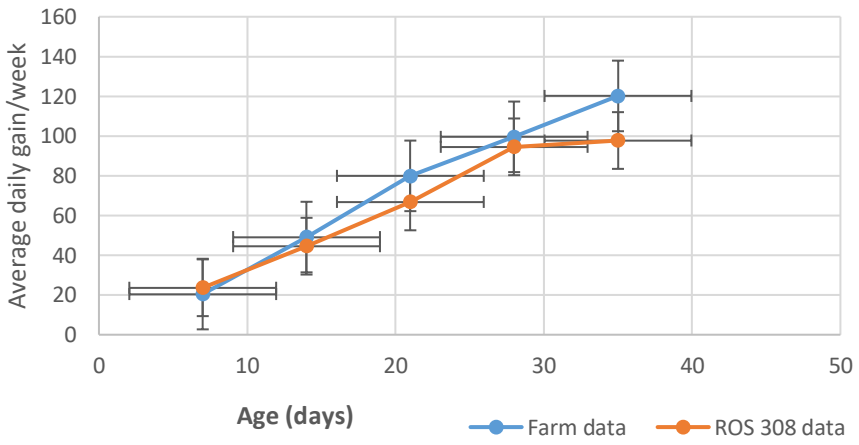


Figure 3.10: Growth performance of trial bird in comparison with ROSS 308 performance objectives (Aviagen, 2019)

However, when compared with the Ross 308 performance objectives (Aviagen, 2020), the FCR of the present study were relatively higher at day 7 and slightly higher on other weeks which is an indication of lower feed efficiency of broilers in the present study, though they were not huge (Table 3.18). Unfortunately, this result is possibly due to feed spillage as the feed system (open troughs) encourages the chicks to climb in the feeders, spill feed and dust bathe.

Table 3.18: Comparison of Ross 308 Broiler objectives FCR with the mean results of the present trial

Age (days)	ROS 308 objectives for FCR	Current study FCR
7	0.821	1.269
14	1.057	1.294
21	1.201	1.361
28	1.336	1.512
35	1.473	1.596

The phytase activity of the diets has also been measured and there was no substantial difference in the calculated value and the analysed value (Table 3.19)

Table 3.19: Phytase activity of test diet samples (Quantum Blue, ELISA method)

Phase	Diet	Phytase Activity (QB ELISA FTU/kg)
Starter	Positive Contol	<50
	Negative Control	<50
	Negative Control +500 FTU Phytase	394
	Negative Control +3000 FTU Phytase	2830
Grower	Positive Contol	<50
	Negative Control	<50
	Negative Control +500 FTU Phytase	419
	Negative Control +3000 FTU Phytase	2570

Walk *et al*. (2013) also reported superdosing of 1500 FTU/kg phytase in the NC diet did not change the FI at day 21 and day 49 or BWG at day 49 which is similar to the present study as there was no effect of diet on FI and BWG. Walk *et al*. (2013) suggested that the P was not

particularly limiting, even in the NC diet. But they reported superdosing of phytase improved FCR compared to that of the NC diet at both day 21 and 49. The difference in performance may be explained by the optimal nutrient composition of the diets, specifically the P content, was not limiting so the phytase did not have scope to work, as it has been evident that addition of phytase to diets containing low amounts of available P (Cowieson *et al*., 2006; Zhang *et al*., 1999), or low amounts of available P and ME (Olukosi *et al*., 2008), improved broiler performance to levels comparable to that of birds fed diets containing sufficient amounts of available P and ME.

3.4.2. Mucin layer thickness

In the present study mucin layer thickness was not affected by the diets, but it was higher on day 7 and reduced after a further week. A possible explanation of that is pathogenic bacteria start to enter into the digestive system just after hatch and may cause inflammation of intestinal cells as the broiler's digestive system is still developing. As an innate immune response, the goblet cells begin to produce mucin to protect the cells from enteric bacterial inflammation so that at a young stage the mucin thickness is higher. After that the thickness gradually reduces, which is an indication that the digestive system has properly developed, and that the pathogenic bacterial inflammation has reduced. However, it has been reported that, phytase inclusion influences mucin excretion through the destruction of phytate (Onyango *et al.*, 2008), as phytate is an antinutritional factor, and like other antinutritional factors eg. Non starch polysaccharides (Ayoola *et al*., 2015), phytate may affect digesta viscosity and influence the secretion of mucin. However, there are limited studies of the effect of phytase supplementation on mucin layer thickness of broilers. For a better understanding

of mucin dynamics in the present study, a correlation between feed intake or body weight gain and mucin thickness has been investigated and very weak or small linear relation found between mucin thickness and BWG but no linear relation found between feed intake and mucin thickness (Figure 3.11 & 3.12).

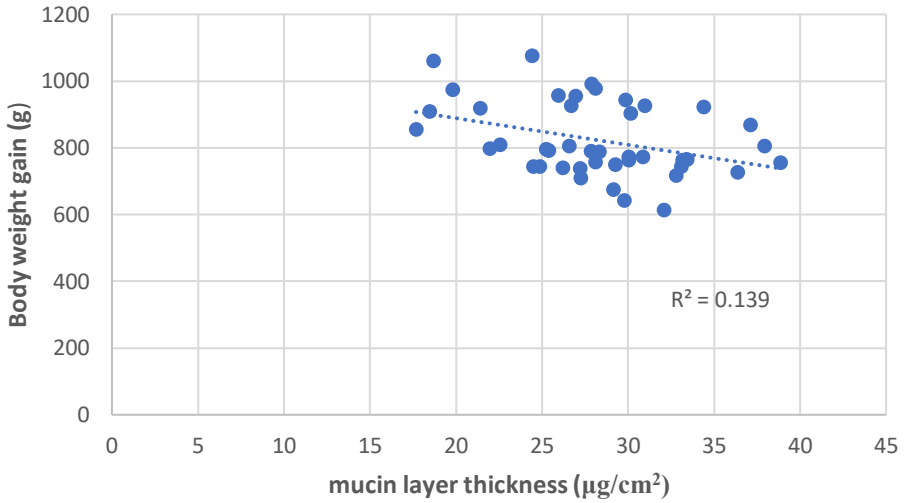


Figure 3.11: Correlation between mucin layer thickness and body weight gain at day 35

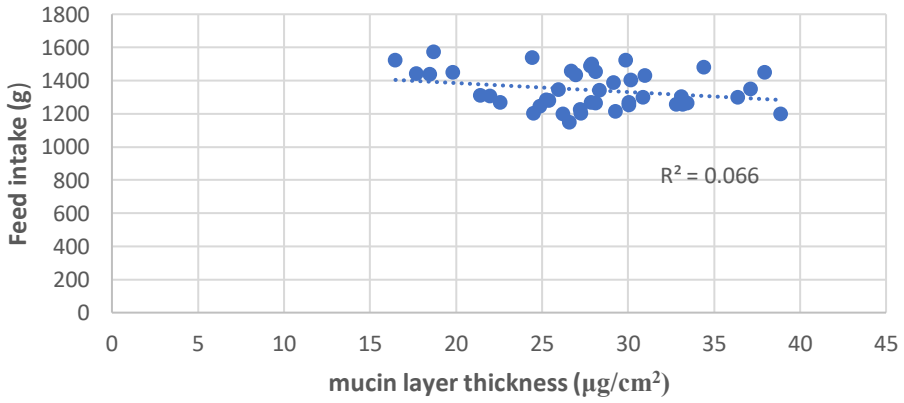


Figure 3.12: Correlation between mucin layer thickness and feed intake at day 35

3.4.3. Bone mineralization

For the evaluation of bone mineralization, typically two criteria have been used which are bone ash weight and bone ash percentage, as 80% of the total body phosphorous is retained in the bones (De Groote and Huyghebaert, 1997). The bone ash percentage data in different literature vary widely as there are major differences in methodology which makes it difficult to compare data between studies. Despite the difficulties in comparing data patterns between studies, changes within a study may still be discussed usefully.

In the present study the bone ash percentage has been observed weekly throughout the rearing period to evaluate the changes over time, which is very important as in most of the literature the bone ash percentage was discussed at only one time point. As with the advancement of age the tibia bone ash percentage was increased up to 21 days and then reduced at day 28 and day 35. When the broilers are small, they are cartilaginous so that bone ash content is lower, but with the advancement of age the skeleton mineralises and the ash percentage increases. However, when the broilers reach maturity the flesh proportion is higher and skeletal proportion is lower so after day 21 the ash percentage tends to be lower as well. Barreiro *et al*. (2009) reported bone ash percentage was 43%, 47.7% and 43.7% on days 8, 22 and 43 respectively; and concluded that the higher bone ash percentage determined on day 22 was due to a greater demand for minerals in response to rapid growth at that age which also supports the data of the present study although the data were lower compared to their study. The mean tibia bone ash percentage at different sampling ages determined in this study were 23.52%, 38.64%, 37.67%, 39.49%, 35.07% and 35.95% on day 0, 7, 14, 21, 28 and 35 respectively. In contrast, Skinner and Waldroup (1995) reported a

numerical increase in bone ash percentage with age in floor-reared male and female broilers (39.3% and 40.4% at day 14; and 42.8% and 43.8% at day 42 respectively).

In the present study the addition of the standard dose of phytase in the diet improved the tibia ash percentage at day 21 and day 28 compared to that of the NC diet and with super-dosing of phytase tibia ash was highest at day 21 and 28. Walters *et al.*, 2019 also reported phytase supplementation in broiler diet improved tibia bone mineralisation expressed as bone ash % and with superdosing of 2000 FTU/kg and 3000/1000 FTU/kg phytase in the NC diet, the tibia ash % was highest compared to that of the NC diet (nPP 0.25 & 0.23% for starter and grower) and the NC plus other phytase containing diets at day 14 and day 28. These authors have also reported superdosing of phytase in the NC diet yielded tibia ash % similar to that of the PC diet (nPP 0.43 & 0.39% for starter and grower). Khodambashi *et al*. (2013) investigated the effect of phytase supplementation and also reported an increased bone ash percentage with 500 FTU phytase supplementation at day 28. They have reported an increase in ash percentage of 34.16 with the NC diet to 40.37 % with 500 FTU phytase supplementation where in the present study it was 33.17% with NC diet and 34.36% with supplementation of 500 FTU phytase at day 28.

Woyengo *et al*. (2010) also reported improved tibia ash content at day 21 ($P<0.05$) from 38.3% of the NC diet (0.46% nonphytate P; 1.1% Ca) to 42.4% with the addition of phytase (600 FTU/kg) in the NC diet, which is similar to the findings of present study. They have reported increased tibia ash content as a result of improved ileal digestibility of P from 29.5 to 43% with the addition of phytase in the broiler NC diet. Olukosi *et al*., 2007 also reported supplementation of phytase 1000 FTU/kg improves ileal P digestibility ($P<0.01$) at day 21

compared to the NC (marginal in P and ME) diet. So superdosing of phytase in broiler diet improves tibia bone mineralisation which is also in agreement with other studies.

3.4.4. Tibia Ca and P content

There was no effect of inclusion of phytase and sex on tibia Ca and P content of the broilers at any time point, but age significantly affected the tibia P and Ca content. Both tibia P and Ca content were lower and increased with the advancement of age until they reached day 14, at day 14 both mineral contents reduced and increased again until day 28 following by lower levels by day 35. Dersjant-Li, Evans and Kumar, (2018) also reported no effect of phytase inclusion on tibia ash and tibia Ca and P content of broilers at day 27 and 41, however they reported an increase in tibia P content at day 10 with 1000 FTU/kg phytase diet (Ca 0.73% and P 0.31%) compared to that of 500 FTU/kg phytase diet (Ca 0.83% and P 0.33%) but both values did not differ from the control diet (0.96% Ca and 0.48% P). The sampling time point and the diet in that study slightly differ from the present study. On the contrary, Walters *et al*., 2019 reported addition of phytase regardless of dose, improved tibia Ca and P content when compared with the NC diet (nPP 0.25 & 0.23% for starter and grower) throughout the study. They have also reported, inclusion of phytase at 2000 FTU/kg yielded P percent values similar to that of PC (nPP 0.43 & 0.39% for starter and grower) on day 14 and 28 while addition of the highest level of phytase (3000/1000 FTU/kg) increased P content beyond that of PC on day 14. Also at day 28, the inclusion of phytase at 250,750 and 3000/1000 FTU/kg increased tibia Ca content beyond that of the NC diet and comparable to that of the PC diet.

No effect of dietary P reduction with or without supplementation of phytase on tibia bone density was seen in this study, suggesting that the balance of Ca:P in these treatments were

sufficient despite the reduced level of P in the diet. The dietary Ca percentage needs to be take into consideration in any future study as addition of phytase in diets with reduced Ca levels increases the potential to improve bone mineral density in diets deficient in dietary P (Kim *et al*., 2017). When applying phytase matrix values, currently some feed manufacturers practice matching Ca to digestible or available P reduction ratio around 1:1. So, Superdosing of phytase in broiler diet has no effect on tibia P and Ca content when compared to the control diet.

3.4.5. Whole body Ca and P content

In the present study no significant difference was observed in WBP or WBCa content when analysed by diet, or sex (Table 3.8), however the WBP content improved with the advancement of age up to day 28 while WBCa content was recorded highest at day 14 and 21 and reduced again at day 28. There was no effect of phytase supplementation on WBP and WBCa content in the present study and no literature is available to compare the findings related to phytase inclusion.

The overall mean WBP and WBCa content recorded in this study were 12.35 and 11.09 g/kg respectively which are higher than reported in other studies (Table 3.20) used for comparison.

The mean WBP and WBCa content determined in present study were not in close agreement with other studies, however the trend of WBCa content increasing with age until day 21 and reducing at day 28 were similar to the trend reported by Van Krimpen *et al*. (2016) in cockerels. The trend in WBP content changing with age were also in close agreement with Van Krimpen

et al. (2016), however the WBP content tends to reduce after 28 days in the present study whereas in the aforementioned paper it began reducing after 21 days.

Table 3.20: WBP (g/kg) and WBCa (g/kg) from previous and present study reports

Age (days)	Cockerels		Pullets		Unsexed		Source
	WBP	WBCa	WBP	WBCa	WBP	WBCa	
0					3.4	4.3	WPSA 1985
21					4.9	6.8	
42					4.8	6.7	
21-42	5.1	6.9					Nieß *et al*., 2005
21					2.2-3.9	2.4-5.5	Shastak *et al*., 2012b
35					3.0-4.5	3.4-6.8	Van Krimpen *et al*., 2013
35 & 43	4.7		4.5				Van Krimpen *et al*., 2016
10	4.5	5.3-6.1					
21	4.75	5.7-6.8					
30	4.48	6.0					
38	4.19	5.6					
14					5.01	6.73	Sanni 2017
28	5.20	7.38	5.11	7.05			
36	4.98	6.81	5.07	6.98			
0					9.79	12.10	Sumaiya 2023
7					11.61	8.09	
14					12.61	13.98	
21					12.41	13.63	
28					18.53	10.33	
35					9.29	9.86	

Shastak (2012c) also reported the amount of WBP as well as Tibia Phosphorous (TP) content increased with the age of broilers and irrespective of the difference in data, the trend is similar to the present study. They also reported the WBP/TP ratio in day 21 ROSS 308 broilers was 21.3 and 19.8 in day 35 broilers, while in the present study it was 65.7 and 49.5 at day 21 and day 35 respectively.

In the Shastak (2012c) experiment, a basal diet of 0.35% total P was used, and another 6 treatments were included where P concentrations were balanced on an incremental basis by 0.08%, 0.16% and 0.24% from two different P sources but all the diets were calculated to be

below the P requirement of the broilers. While in the present study the study design was different as the P level was adequate or marginally adequate to the requirements of the broilers.

Harwitz (1964) also reported a Carcass P/TP ratio of 19.6 at day 24 when fed P at different levels and found that the ratio was unaffected by nutritional treatments. The present study also supports the findings of Harwitz (1964) as the diet has no effect on the WBP:TP ratio of the broilers irrespective of age. So, Superdosing has no effect on WBP and WBCa content of broiler when compared to the standard dose of phytase.

3.4.6. Relatedness between bone ash percentage with tibia P content and/or WBP content

Tibia ash % was negatively correlated with tibia P and Ca content and WBCa content in this study. However, tibia ash % was positively correlated with WBP content but the correlation was small (r≥ 0.173) (Table 3.21). Other bone ash % (feet and femur) were also positively correlated with WBP content and the correlation is small (r≥ 0.206) with femur ash% and medium (r≥ 0.380) with feet ash % (Table 3.22). Both feet and femur ash % were negatively correlated with WBCa (Figure 3.22).

Table 3.21: Pearson correlation between tibia ash, tibia Ca & P and whole body Ca & P content

	Body Weight (g)	Tibia P content (mg)	Tibia Ca content (mg)	WBP (mg)	WBCa (mg)	Tibia ash %
Body Weight (g)	1	0.409**	0.436**	0.128*	-0.101	0.212**
Tibia P content (mg)	0.409**	1	0.621**	0.234**	-0.227**	-0.069
Tibia Ca content (mg)	0.436**	0.621**	1	0.198**	-0.436**	-0.203**
WBP (mg)	0.128*	0.234**	0.198**	1	0.439**	0.173**
WBCa (mg)	-0.101	-0.227**	-0.436**	0.439**	1	-0.006
Tibia ash %	0.212**	-0.069	-0.203**	0.173**	-0.006	1

**Correlation is significant at the 0.01 level (2-tailed)

*Correlation is significant at the 0.05 level (2-tailed)

Table 3.22: Pearson correlation between Feet ash, Femur ash, and whole body Ca & P content

	Body Weight (g)	WBP (mg)	WBCa (mg)	Femur ash %	Feet ash %
Body Weight (g)	1	0.128*	-0.101	0.149*	0.333**
WBP (mg)	0.128*	1	0.439**	0.206**	0.380**
WBCa (mg)	-0.101	0.439**	1	-0.003	-0.054
Femur ash %	0.149*	0.206**	-0.003	1	-
Feet ash %	0.333**	0.380**	-0.054	-	1

**Correlation is significant at the 0.01 level (2-tailed)

*Correlation is significant at the 0.05 level (2-tailed)

Bone ash % is routinely used to evaluate bone mineralization in poultry, however the small to medium correlation with WBP and negative correlation with WBCa content found in this study suggests that it is not a sensitive measure of WBP and WBCa content. Sanni (2017) reported tibia ash % was weakly correlated with tibia phosphorous content and WBP content and in case of feet ash% it has medium strength correlation with WBP content, which is similar to the findings of this study. The use of bone ash % to assess phosphorus availability or bone

mineralization has been questioned by other investigators (Hall *et al*., 2003; Li *et al*., 2015) due to poor reliability. Others also suggested bone ash weight or bone breaking force are more sensitive indicators to evaluate the relative biological availability of a P source compared to that of bone ash % (Coon *et al*. 2007).

3.4.7. Relatedness between tibia P with WBP content

Positive correlations were found between the tibia P content and WBP content of broiler in the present study, however the correlation was very small or weak (r≥ 0.234). In contrast strong correlations (r≥ 0.978) were reported by Sanni (2017) between tibia P and WBP content of broilers, who suggested tibia P content is a reliable indicator to measure WBP content. Like Sanni (2017), lots of other investigators also suggested tibia P as an indicator for WBP measurement, because it is very difficult and time consuming to measure the WBP content in broilers (Hurwitz, 1964; Huyghebaert *et al.,* 1980; Shastak *et al.*, 2012c). Hurwitz (1964) observed a constant WBP:tibia P ratio of 19.6 irrespective of the dietary treatments and suggested tibia P content might be a reliable estimate of WBP. Shastak *et al*. (2012c) also reported a WBP:tibia P ratio of 21.3 and 19.8 at day 21 and 35 respectively while fed the broilers incremental level of dietary P from different sources. Recently, Sanni (2017) observed a mean WBP:tibia P ratio of 29.33 in broilers. The WBP:tibia P ratio obtained in the present study were very high compared to the values reported by other investigators (Hurwitz, 1964; Huyghebaert *et al.,* 1980; Shastak *et al*., 2012c, Sanni 2017). The higher WBP content is due to the difference in the methodology used the calculate the WBP content where both tibia and femur P were considered whereas in other studies only tibia P content was considered.

Moreover, Shastak *et al*. (2012c) also observed WBP content in broiler increased linearly with the tibia P content and reported that each mg of P retained in the tibia the whole body P content increased by 17.7 mg at both day 21 and day 35 (r^2=0.97 & 0.92 at day 21 and 35). In the present study, the increase in WBP when related to the increase in TP was linear and the slopes were 20.97 and 34.17 at day 28 and day 35 respectively which indicates with each mg of P retained in tibia the WBP retained increased by 20.97 mg and 34.17mg at day 28 and day 35 respectively but the strength of the relationship is very weak (r^2=0.03 and 0.01 at day 28 and 35) (Figure 3.13 and 3.14). Sanni (2017) also reported a linear increase of 25.85 mg retention in WBP for each mg increase of tibia P retention in broiler at day 35 and the relationship is very strong (r^2=0.97).

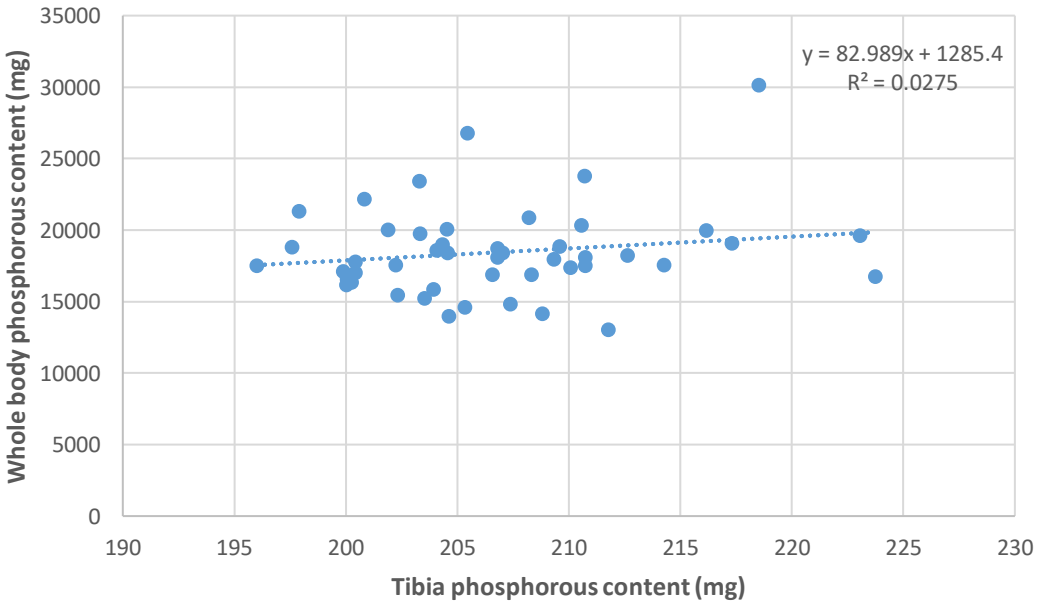


Figure 3.13: Linear relationship between tibia P and whole body P examined in 28 day-old broilers

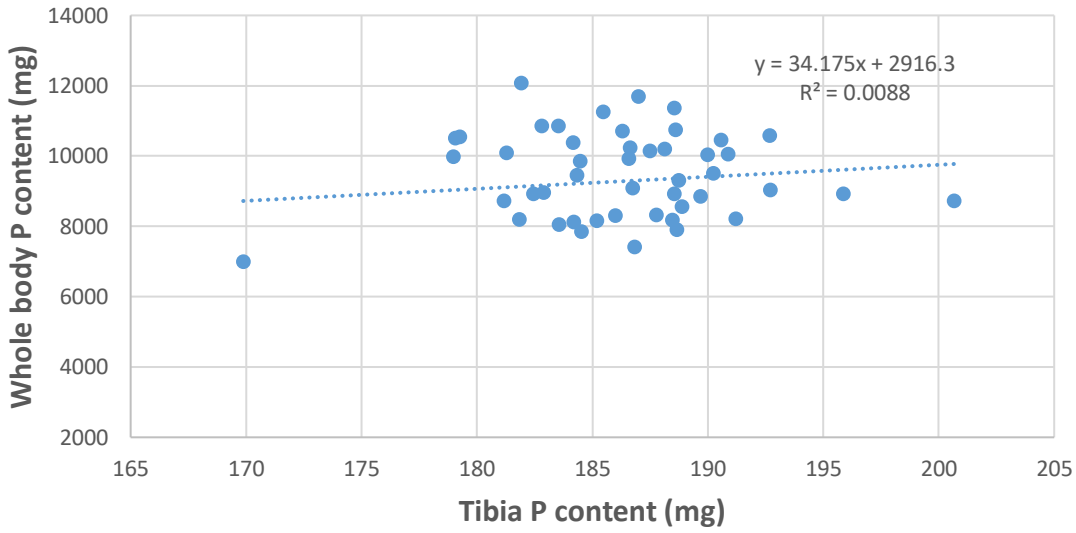


Figure 3.14: Linear relationship between tibia P and whole body P examined in 35 day old broilers.

Data from the present study suggested the WBP:Tibia P ratio may not be as constant as previously assumed which also reported by Sanni (2017), who found WBP:Tibia P ratio changed with the age of broiler. The differences in these data might be related to lots of factors such as length of experimental period, differences in dietary P content, age of the broilers at sampling and changes in bird composition due to use of different breed and these factors were also noted by Shastak *et al.* (2012c). The differences might also happen due to environmental or husbandry effects such as lighting, litter quality, health management, humidity and temperature which could change the bone quality (Hester, 1994) and warrants further investigation. Moreover, the differences in the results may be due to adequate P content of the diet or the broilers were not in any physiological stress which did not allow the phytase to show it's effect. Through support for improved nutrient digestibility, phytase reduced the physiological stress experience by fast growing broilers (Walk et al., 2013).

The findings from this study do not support that for each age assessed the tibia P content will certainly indicate the true WBP value of broilers, rather than where possible the Whole body p content should be assessed to measure the whole mineral status of broilers.

3.4.8. Effect of phytase superdosing on digestive tract weight

Caeca and digesta weights were unaffected by treatment, although birds fed 3000 FTU/kg phytase had lower GIT weights compared to those fed the negative control diet on days 21 and NC+ 500 FTU/kg diet respectively at day 28.

Phytase supplementations reduce digesta viscosity and increase intestinal mobility and the resultant reductions in pathogenic microbial activity which reduce intestinal tissue growth (Brenes *et al*., 2003). It is also possible that microbial phytase may act in a similar manner to that of exogenous xylanase in disrupting the cell wall matrix of wheat (Ravindran *et al.,* 1999), thus degrading the NSP and lowering digesta viscosity and reducing the microbial activity of intestinal digesta (Choct *et al*., 1999, Sinlae and Choct, 2000). Wu *et al*., 2004 reported phytase reduced the relative weight and length of small intestine by 11.4 and 14.1% compared to the basal diet. They fed the broiler a basal diet of adequate P content (4.5 g/kg nPP) and supplemented phytase (500 FTU/kg) and reported a small intestinal weight of 29.7 g/kg body weight and 26.3 g/kg of body weight for basal and phytase supplemented diets respectively. They also reported phytase supplementation had no effect on ceca weight (3.4 and 3.7 g/kg BW for basal and phytase supplemented diet), which is consistent to the results

of the present study. This may suggest that there were no substantial differences in the bacterial make-up of caecal content.

Reduced intestinal weight is considered to be beneficial for broiler as it increases the activity of digestive enzymes (Dibner *et al*., 2007)and may also spare energy from tissue growth which can be utilized by host for growth or improve the digestibility of nutrients (Miles *et al*., 2006). Other authors also observed improvement in nutrient retention because of the thinning of intestinal wall (Huyghebaert *et al*., 2011, Sharifi *et al*. 2012). So superdosing of phytase reduce GIT weight which may improve nutrient digestibility in broiler.

3.4.9. Effect of phytase superdosing on concentration of esters

Superdosing of phytase has demonstrated further improvement to weight gain and feed conversion ratio (FCR) compared with standard doses and scientists have suggested that the further improvement in performance may be related to the phytate destruction rather than P provision (Cowieson *et al*., 2011). Walk *et al*., 2013 also reported the further improvement in FCR was an 'extra-phosphoric' effect of superdosing as they observed no additional benefit of phytase on bone P level when fed diet with high P level, but still showed positive FCR response. There is a possibility that the production of myoinositol through the breakdown of phytic acid plays important part in the benefit of phytase superdosing. The importance of myoinositol is very evident for cell survival and growth (Eagle *et al*., 1956, Holub, 1986)as it is absorbed and utilized in a number of biological functions within the animal (Lee and Bedford, 2016).

In the present study, the concentration of myoinositol (IP0) in crop on day 7 and in ileum and jejunum on day 21 and 35 were significantly higher, with superdosing of phytase compared to that of the standard dose. Lee *et al.*, (2017) suggested that superdosing of phytase generates myoinositol which is responsible for positive FCR response in broilers. Improvement in body weight gain and FCR have already been demonstrated with myoinositol supplementation of low P diets in broiler from day 0 to 21 (Zyla *et al.*, 2004). Though there was no effect of superdosing on body weight gain and FCR of broiler in the present study irrespective of improving myoinositol concentration in the digesta, which might suggest a deviation of energy expenditure from growth towards absorption and utilisation of excess myoinositol being delivered to the animal. As the rephosphorylation of absorbed myoinositol required for functionality which might suggest a greater supply of myoinositol will increase the P requirement of the animal.

In the present study, superdosing also reduced the concentration of IP6 and lower inositol esters (IP5, IP4 and IP3) compared to that of the standard dose of phytase except on day 21 the lower esters (IP4 & IP3) were higher with superdosing, which indicates that more IP6 and lower esters were degraded due to superdosing. Not only IP6 but also the lower esters have an anti-nutritional effect in the animal and superdosing may reduce the potential for chelation through degradation of these esters. Lower molecular IP esters (mono & diphosphates) have limited capacity to form chelates and to inhibit digestibility of protein (Harland and Narula, 1999) and P from those lower ester (IP4 or IP3) has a high availability for poultry (Angel *et al.*, 2002). Superdosing may also be beneficial for reducing endogenous nutrient loss such as amino acids (methionine, cysteine, threonine & Serine) and minerals (P,

K, Na & Ca) as it reduces the concentration of IP6 (Selle *et al*., 2000 & Cowieson, Acamovic and Bedford, 2006).

3.4.10. Effect of phytase superdosing on cecal microbiome diversity

Dietary treatments had no effect on species richness and relative abundance of microbial communities at phylum, family or genus level. The number of OTUs were relatively higher 524, 584 and 500 with 0, 500 & 12500 FTU/kg phytase supplemented diet in other studies (Witzig *et al*., 2015) compared to the present study on day 25. Though the Shanon diversity observed (4.43, 4.65 & 4.48) was similar to the present study. Borda-Molina *et al*., 2016 also reported a Shanon diversity of 4.15 in caecal digesta, which is similar to our study though they reported an observed OTU of 24 which is less than the present study. The remarkably similar Simpson score indicated that bacterial diversity in chickens did not differ at all among diets and sex. Montso *et al.* 2022 also observed Simpson score which followed similar pattern to the present study.

Analysing the effect of phytase superdosing on microbial diversity is very important. (Zanu *et al*., 2020) reported superdosing of phytase (1500 FTU/kg) reduced *Bacillus* spp. counts in cecal digesta on day 16 compared to that of standard dose when the broilers were challenged with necrotic enteritis (NE). They suggested high phytase decreased the count of *Bacillus* spp. By perhaps promoting the outgrowth of *C. perfringens* through the nutrient supply (competitive exclusion) and the impact of NE on the reduction of beneficial bacteria in the gut is well evident (Yang *et al*., 2018).

Akyurek *et al*., 2011 Reported caecal microbiota Lactobacillus spp and E coli count increase with 500 FTU phytase supplementation compared to that of the negative control diet (0.20% Npp) and the positive control diet (0.45% Npp) at day 21. Though in the present study, the bacterial count has not been observed at species level, but at genus level the lactobacillus was numerically higher in the diet supplemented with the standard and super dose of phytase and the positive control diet compared to that of the negative control diet on day 35 (Figure 3.7). Lactobacillus produce lactic acid which reduce gut pH and inhibit the development of infection by pathogenic bacteria such as C. difficile and C. perfringens, Campylobacter jejuni etc. They also promote mucus production and secretion of SCFAs whose beneficial effect is highly evident in broiler (Dempsey & Corr, 2022).

Firmicutes was dominated by families within the order Clostridiales in caeca which is also observed by other investigators (Witzig *et al*., 2015, Stanley *et al*., 2013a; Deusch *et al*., 2015) on day 25 and similar to the present study. Stanley *et al*, 2014 reported at family level caecal digesta was colonized mainly by Bacteriodaceae, Ruminococcaceae, Lachnospiraceae, Lactobacillaceace which is also similar to present study. The relative abundance of Bacteriodaceae was numerically reduced with the standard phytase dose compared to the other diets 22.70% (0 phytase), 21.86% (500 phytase) & 24.42% (12,500 phytase) which is also similar to the present study.

Borda-Molina *et al*, 2016 reported a high proportion of microbes belonging to the order clostridiales were detected in caecal digesta was similar to the result of the present study. The microbes in this group are associated with SCFA metabolism (Choietal.,2015) and hence

the presence of this group in the digesta is an indication of healthy chicken. The beneficial effects of SCFA are well evident as they increase the growth of epithelial cells, stimulate mineral absorption, inhibit the growth and adherence of pathogenic microbes by decreasing P^H (Walugembeetal.,2015). Borda-Molina *et al*, 2016 reported a relative abundance of Lachnospiraceae was 12 to 22% of diet supplemented with P (6.9%) with or without the superdosing of phytase (1500 FTU), which is similar to the observation of the present study where the relative abundance was ranged 12 to 19 % in control diets and control diets with supplementation of standard and superdose of phytase. Ruminococcaceae is also a common family detected in caeca (Bjerrum *et al.,* 2005; MohdShaufi *et al.,* 2015) and the relative abundance observed in the present study was higher (21.38%) compared to other (4.8%) studies (Borda-Molina *et al*, 2016). Both families play an important role for the maintenance of gut health and they have the capability to produce SCFA by breaking cellulose and hemicellulose (Biddle *et al*., 2013). Erysipelotrichaceae was also observed as a minor family in caeca which is also reported by (Borda-Molina *et al*, 2016) who observed it in 2% with diet supplemented with P (6.9%) with or without superdosing of phytase (1500 FTU). Eeckhaut *et al*., 2011; De Maesschalck *et al*., 2014 reported protein sequences related to the production of butyryl-CoA have been detected on this family.

At the genus level, Anaerostipes and pygmiobacter differ significantly among the diets & it's relative abundance reduced with the superdose of phytase compared to that of the standard dose. A novel species belonging to the Anaerostipes genus (Anaerostipes butyraticus sp. nov.) was previously identified in 4 weeks old chicken caecum and described as butyrate producing bacterium under clostridium cluster by (Eeckhaut *et al*., 2010). (Bui *et al*., 2021) suggested the beneficial effect of Anaerostipes spp on promoting host heath by anaerobic conversion of inositol stereoisomers into propionate and acetate and also phytate derived inositol into

propionate by the abundant intestinal genus Anaerostipes. They have also reported reduced fasting glucose level in mice when myoinositol was co-administerded with live A. rhamnosivorans in western diet compared to heat killed A. rhamnosivorans after 6 weeks treatment.

Pygmiobacter was another genera which relative abundance reduced with the superdosing of phytase compared to that of the positive and negative control diet. This genus was first described by (Bilen *et al.*, 2017) who isolated a new species *Pygmaiobacter massiliensis*' from a stool sample of a healthy 47-year-old Pygmy woman. Sun *et al.*, 2020 also isolated pygmaiobacter in septic rat feaces and described its proportion was reduced when septic rat were treated with adipose tissue mesenchymal stem cells. Peng *et al.*, 2020 also described the presence of pygmaiobacter in chicken gut and its relative abundance was 1.72, 3.42, 0.59 & 0.75% in the control, bicalin fed group, Avian Escherichia coli induced colibacillosis (APEC) group and the Bicalin treated APEC group.

3.5. Conclusion

Though superdosing of phytase in broiler diet increased the generation of myoinositol content by improving phytate degradation compared to that of standard dose of phytase this did not translate to any further benefit on performance of broilers compared to other diets. Moreover, improved tibia ash percentage has been observed, but the tibia and whole body Ca and P content were not affected by superdosing of phytase compared to the standard dose. The finding from this study do not support the hypothesis that superdosing effects dependant on yield of myoinositol by further breakdown of phytate (IP6). So, to observe the effect of

superdosing the ability of the animal to convert the myo-inositol-mediated signals to lean gain is also important.

The WBP: Tibia P ratio of broiler varied for each age assessed which does not support the hypothesis that WBP: Tibial P will be constant in broiler which indicates that tibia P might not be a reliable estimate of WBP content. Though WBP is very laborious to measure, future studies should measure the WBP content and P digestibility to estimate the exact P retention in broilers.

There was no effect of phytase superdosing on caecal microbial diversity compared to other diets, this indicates there is no adverse effect of superdosing on gut health and the negative control diet was not actually limiting on P level. These findings do not support the hypothesis that the effect of superdosing of phytase could have been related to a shift in intestinal P^H with subsequent effect on microbiota profiles. Though Superdosing reduced the relative abundance of genus Anaerostipes and Pygmaiobacter, there was lack of information on the effect of phytase on this two genus and future studies should investigate further. Also, in future studies measuring the viscosity of digesta without Xylanase addition in the diet and measuring volatile fatty acid production in caecal digesta may help further to determine the beneficial effect of phytase superdosing in broiler diet. Xylanase already improves the degradation of the NSP and reduce the antinutritional effect of NSP and makes all the diet more palatable and digestible (Choct *et al*., 1999, Sinlae and Choct, 2000). So, to see the actual effect of phytase a more challenged diet without Xylanase can be introduced in future.

There was no effect of phytase superdosing on mucin layer thickness, caeca and digesta weight but superdosing reduced the digestive tract weight in broilers at a certain age point which could be considered beneficial. The improvement of myoinositol generation due to superdosing didn't reflect in broiler performance is a possible indication of adequate P level in the diets which was not actually limiting. So, focussing on the P level of the diet in future studies could be beneficial to measure the effect of superdosing on performance.

CHAPTER 4: Comparison of amino acid digestion and physiological response of broilers fed diets containing graded levels of a standard wheat or a novel strain of HIGHPHY wheat containing high levels of phytase

4.1. Introduction

While the majority of studies focus on exogenous addition of phytase enzymes to broiler diets, an alternative method of increasing the overall P digestibility of poultry diets is to identify strains of wheat naturally expressing high levels of phytase such as purple acid phosphatase. Mature grain phytase activity (MGPA) of cereal grains could play an important role in hydrolysis of phytate to make the phytate P available to birds, so scientists have attempted to increase the MGPA through in planta expression of microbial phytase in transgenic crops (Brinch-Pedersen *et al.*, 2002). Microbial HAP (Histidine Acid Phosphatase) phytases are considered the main group favoured for increasing MGPA through transgene overexpression (Lei *et al.*, 2007). However, scientists have recently developed a wheat mutant (HIGHPHY) holding extra copies of purple acid phosphatase phytase (PAPhy) through which increased MGPA (to ~6200 FTU/kg) has been achieved (Brinch-Pedersen *et al.*, 2012; Holme *et al.*, 2012). In the HIGHPHY mutant PAPhy's are considered to attribute to the bulk of MGPA rather than conventional HAP phytases (Dionisio *et al.*, 2007 and 2011).

The HIGHPHY wheat strain has been recently shown in a broiler study to have comparable efficacy in phosphorus release to an exogenous enzyme (Scholey *et al.,* 2017). However, before such a strain of wheat can be considered for inclusion in commercial poultry diets, a broader investigation into the nutritional profile of the wheat and subsequent response of

the bird is required. Therefore, the primary aim of this study was to determine the amino acid digestibility of HIGHPHY wheat compared to a standard wheat, and the secondary aim was to investigate the physiological response of the bird. The physiological responses investigated were digesta pH in the crop, gizzard and ileum, mucin layer thickness in the ileum, and rate of inositol hexaphosphate (InsP6) degradation in key regions of the gastrointestinal tract (GIT). In addition to determination of amino acid digestibility of each wheat, the digestibility of calcium and phosphorus was also determined to verify previous findings (Scholey *et al.,* 2017) and to provide contextual data for the inositol ester ratios in key regions of the GIT.

The hypothesis of this study was that HIGHPHY wheat may improve broiler performance by improving digestibility of different nutrients especially amino acids and hydrolysis of inositol esters and can be used as an alternative to external phytase.

The aims of the study were

1. Examine the effect of wheat type on pH and mucin content in the broiler gastro-intestinal tract;
2. Examine the effect of HIGHPHY wheat on relative concentration of differing inositol esters (InsP6 through to myoinositol) in the broiler GI tract;
3. Evaluate the effect of HIGHPHY wheat on amino acid digestibility and growth performance of broilers;
4. Investigate the effect of HIGHPHY wheat on ileal Ca and P digestibility of broilers.

4.2. Materials and Method

Wheat grains used in the feeding trial were standard field grown wheat, *T. aestivum L. cv Skagen* with a phytase activity of 1060 FTU/kg and HIGHPHY *T. aestivum L.* with a phytase activity of 6196 FTU/kg.

A linear regression approach to determining nutrient digestibility was adopted where birds were fed one of three levels of test material in diets containing a fixed base level of key ingredients and an inert marker, allowing determining of coefficient of amino acid digestibility (Rodehutscord *et al.,* 2004). The three rates of test material inclusion and two test wheats (standard and HIGHPHY) created a 2*3 factorial design. Ethical approval to conduct the trial was sought from the Nottingham Trent University local ethical review group and permission obtained with allocated code (ARE460) to conduct the trial according to NC3R Arrive guidelines (Kilkenny *et al.,* 2010).

4.2.1. Bird Husbandry

Male Ross 308 birds were sourced one-day post hatch from PD Hook Cote hatchery, Oxford, from a parent flock aged 43 weeks. All birds were group housed and fed a commercial starter crumb sourced from GLW Feed Ltd (Shropshire, UK) until day 14-post hatch. The gross composition of commercial starter diet is shown in table 4.1. Birds were reared as described in chapter 2 section 2.2. On day 15, 384 birds were weighed into groups of 8 birds before random allocation to one of 48 mesh sided pens bedded on wood shavings. Each diet was fed to 8 replicate pens, with one pen of 8 birds as one experimental unit.

Table 4.1: Ingredient composition and calculated nutrient profile of commercial starter diet

Ingredients (%)	%
Wheat	40.77
Soyabean meal	29.00
Oats	15.00
Maize	10.00
Limestone flour	0.88
Dicalcium phosphate (18%)	1.17
Soya oil	0.80
Salt	0.23
Sodium bicarbonate	0.10
Liquid lysine 50(T)	0.61
Methionine H-A liquid	0.51
Soya oil spray	0.40
Vitamin Premix[1]	0.35
L-Threonine	0.15
Ronozyme liquid 35.7%[2]	0.03
Total	**100**
Metabolisable Energy MJ/Kg	12.56
Dry Matter	87.72
Moisture	12.28
Crude protein	21.81
Crude Ash	5.12
Crude Fat	3.74
Crude fibre	3.92
Calcium (g/kg)	0.78
Phosphorus (g/kg)	0.58

[1]*Vitamin/mineral premix supplied per kg diet: Selenium: 0.25mg, Iron: 50mg, Manganese: 120mg, Molybdenum: 1mg, Vitamin A: 12,000iu, Vitamin D: 2500iu, HyD: 2500iu, Vitamin E: 100iu, Vitamin K: 5mg, Vitamin B_1: 3mg, Vitamin B_2: 8mg, Vitamin B_6: 6mg, Vitamin B_{12}: 30µg, Iodine: 2mg, Folic: 2mg, Nicotinic: 70mg, Cal-D-Pant: 18mg, Biotin: 0.3mg, Choline: 250mg, Copper: 20mg, Zinc: 100mg,*

[2]*Ronozyme WX: 100mg, Ronozyme HiPhos: 100mg, Ronozyme ProAct: 200mg, Maxiban: 625mg, CRINA poultry plus: 300mg, Aresto: 25mg.*

4.2.2. Experimental diets

Six test diets were manufactured (table 4.2), where diets A, B, C contained HIGHPHY wheat at an inclusion rate of 60, 40 and 20% respectively and diets D, E, F contained Standard wheat at 60, 40 and 20% inclusion rate. All the diets contained identical volumes of rapeseed meal and soya bean meal as a source of phytate, with a 50:50 starch-dextrose mix used to supply energy and balance diet volumes and titanium dioxide added at 0.5% as an inert digestibility marker. All the test diets were made as 35 kg batches with 4 kg of the allocated diet weighed out into individual bags (one bag per pen) to allow feed intake to be recorded. The six dietary treatments were-

A= Diet containing 60% HIGHPHY wheat

B= Diet containing 40% HIGHPHY wheat

C= Diet containing 20% HIGHPHY wheat

D= Diet containing 60% Standard wheat

E= Diet containing 40% Standard wheat

F= Diet containing 20% Standard wheat

The analysed nutrient composition of the experimental diets are shown in table 4.3. All the diets were designed to meet the nutritional requirement of the birds but, as per the experimental design, the protein content of the diet gradually decreased as the inclusion level of wheats were gradually decreased from 60% to 20% (Table 4.3)

Table 4.2: Dietary formulation (%)

Diet	A	B	C	D	E	F
HIGHPHY Wheat	60.00	40.00	20.00	0.00	0.00	0.00
STANDARD Wheat	0.00	0.00	0.00	60.00	40.00	20.00
Low glucosinolate extruded Rapeseed	9.00	9.00	9.00	9.00	9.00	9.00
Extruded Soyabean meal[1]	21.12	21.12	21.12	21.12	21.12	21.12
Soya oil	1.65	1.65	1.65	1.65	1.65	1.65
Limestone	1.16	1.16	1.16	1.16	1.16	1.16
Salt	0.18	0.18	0.18	0.18	0.18	0.18
Sodium bicarbonate	0.25	0.25	0.25	0.25	0.25	0.25
Lysine HCl	0.14	0.14	0.14	0.14	0.14	0.14
Methionine	0.18	0.18	0.18	0.18	0.18	0.18
Xylanase[2]	0.01	0.01	0.01	0.01	0.01	0.01
Vitamin / mineral premix	0.40	0.40	0.40	0.40	0.40	0.40
Corn starch	2.68	12.70	22.70	2.68	12.70	22.70
Dextrose	2.68	12.70	22.70	2.68	12.70	22.70
Titanium (IV) dioxide	0.50	0.50	0.50	0.50	0.50	0.50

[1]Crude protein content 48%

[2]Econase XT liquid 0.01%

[3]Vitamin and Mineral Premix content added at 4kg/tonne, providing (per kg diet): Mn 100 mg, Zn 88 mg, Fe 20 mg, Cu 10 mg, I 1 mg, Mb 0.48 mg, Se 0.2 mg, Retinol 13.5 mg, Cholecalciferol 3 mg, Tocopherol 25 mg, Menadione 5.0 mg, Thiamine 3 mg, Riboflavin 10.0 mg, Pantothenic acid 15 mg, Pyroxidine 3.0 mg, Niacin 60 mg, Cobalamin 30 µg, Folic acid 1.5 mg, Biotin 125 µg.

Table 4.3: Analysed nutrient content of diets

Diet	Diet with 60% HIGHPHY	Diet with 40% HIGHPHY	Diet with 20% HIGHPHY	Diet with 60% Standard	Diet with 40% Standard	Diet with 20% Standard	Standard Wheat	HIGHPHY Wheat
DM (g/kg)	886	883	862	867	870	882	892	857
Ash (g/kg DM)	50.23	44.73	61.25	53.17	45.98	40.02	12.78	16.34
Fat (g/kg DM)	28.10	31.82	28.19	26.99	36.90	32.65	9.19	12.84
Protein (g/kg DM)	246.39	221.97	190.26	236.22	195.52	178.34	122.65	172.00
GE (MJ/kg DM)	17.07	17.07	17.06	16.15	16.15	16.15	18.10	19.91
Ca (g/kg DM)	9.73	8.6	8.11	10.76	8.73	8.68	0.61	0.6
P (g/kg DM)	9.87	8.27	6.48	9.9	7.6	5.54	3.72	3.85
Analysed amino acid content (g/kg DM)								
Cystine	13.692	11.118	10.513	12.059	10.604	9.324	7.629	9.181
Aspartic acid	27.808	23.688	21.818	24.964	21.268	19.716	4.307	6.002
Methionine	7.229	5.616	5.013	6.351	5.366	5.85	1.264	1.841
Threonine	12.288	10.087	9.101	10.406	8.989	8.067	2.670	3.662
Serine	17.743	15.274	13.165	15.623	13.484	11.136	4.530	6.743
Glutamic acid	88.98	72.951	61.434	75.69	61.743	47.715	25.654	40.814
Glycine	14.494	12.352	11.106	13.139	11.262	9.378	3.667	4.950
Alanine	12.564	10.655	9.526	11.258	9.657	8.309	3.206	4.356
Valine	13.972	9.745	9.228	9.891	8.409	7.341	3.211	4.510
Isoleucine	12.012	8.001	7.912	8.144	6.887	6.216	2.206	3.081
Leucine	30.618	24.663	21.807	25.705	21.119	18.46	5.463	8.294
Tyrosine	8.145	5.521	4.299	6.554	4.808	4.16	0.350	0.833
Phenylalanine	16.555	12.897	11.334	13.79	11.082	9.489	3.104	4.920
Lysine	17.909	14.133	12.049	13.938	12.776	11.471	2.497	3.168
Histidine	8.427	6.716	5.915	6.697	5.973	5.176	1.874	2.896
Arginine	19.292	15.494	13.961	16.497	13.443	12.104	4.056	5.699
Proline	13.456	10.321	8.998	10.839	9.055	6.515	7.132	10.750

4.2.3. Treatment schedule / randomisation plan

A replicate consisted of a pen containing 8 birds, with only birds weighing between 500 g and 580 g placed. The weight of each pen was recorded on day 15 and treatments randomly allotted to pens around the trial room using an online randomiser allocated (by an individual not involved in the study to prevent bias), to reduce any possible effects of ventilation and room placement. Pen layout and di*et al*location are shown in figure 4.1.

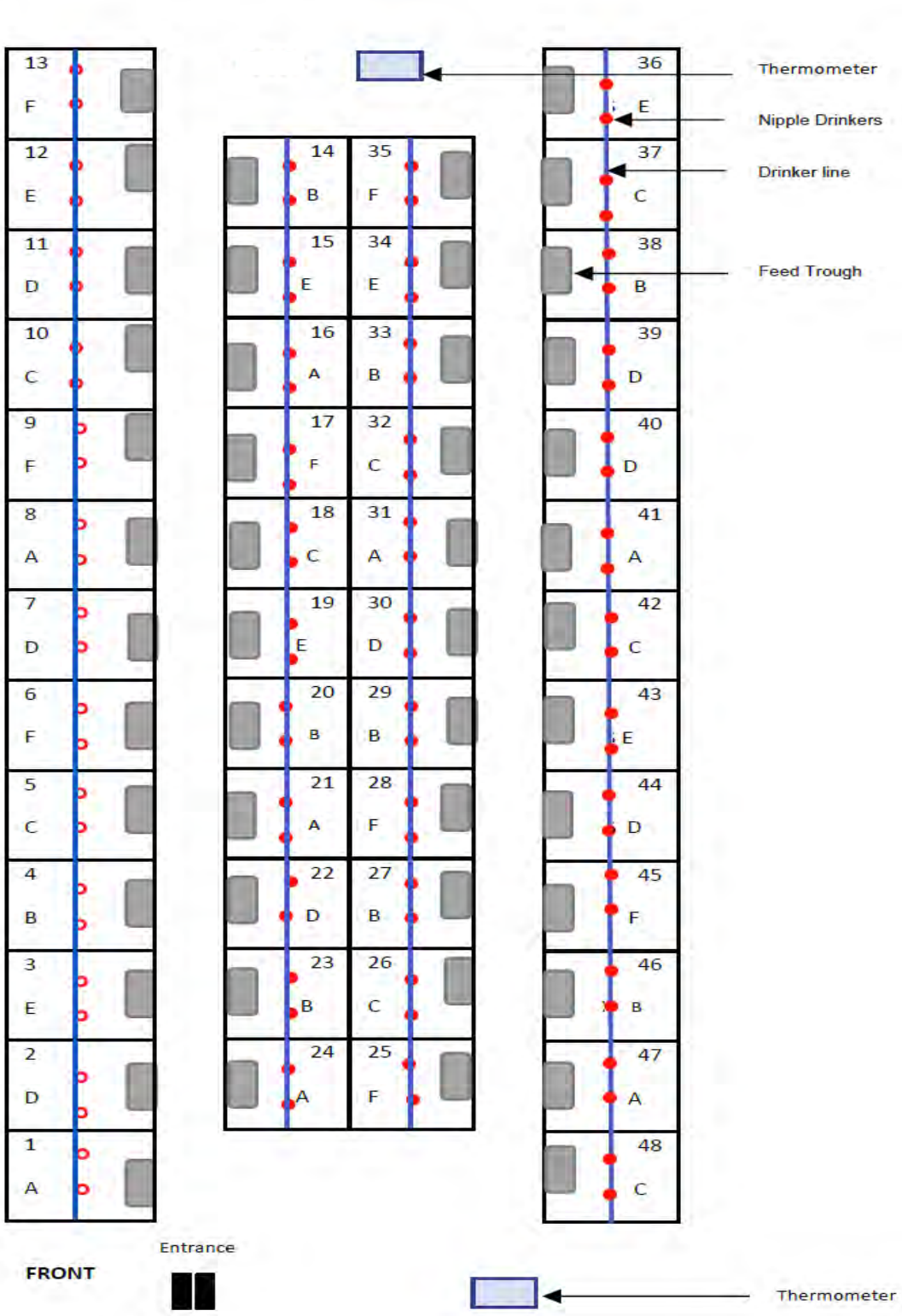


Figure 4.1.: Pen/Room layout with di*et al*location

4.2.4. Determined Parameters

Weight of the birds and feed per pen were recorded at the onset and end of the trial period at 15 and 20 days respectively to calculate the bodyweight gain (BWG) of the birds. Pens of birds were fed from their designated treatment diet from d15-20 and leftover feed was weighed back to measure feed intake.

On d20, all 8 birds from each pen were humanly euthanized by cervical dislocation. Immediately post euthanasia, from 2 birds per pen, pH was recorded inside the gizzard, ileum and crop described in chapter 2 section 2.5.10. A $1cm^2$ piece of intestinal tissue was collected from 1 bird per pen to measure the mucin layer thickness by a modification of Corne's method (Corne *et al.,* 1974) which was described briefly in chapter 2 section 2.5.11. Ileal digesta was collected from 10cm below Meckel's Diverticulum to the ileo-caecal-colonic junction from all birds per pen and pooled into one pot per pen and stored at -20°C.

All digesta samples were freeze dried and ground before splitting into 2 pots. One set of ileal digesta and crop digesta pots were sent to University of Hohenheim for inositol phosphate ester analysis as described in chapter 2 section 2.5.15. The remaining ileal digesta was analysed at NTU for amino acid content, Ca and P content and Ti as described in chapter 2 sections 2.5.8, (amino acids) 2.5.6 (Ca and P) and 2.5.5 (Titanium dioxide).

4.2.5. experimental Design and Statistical Analysis of data

The experiment was designed following factorial experimental design where the effect of multiple factors or independent variables were examined. In this experiment it was 2x3 factorial experiment where 2 wheat types (HIGHPHY wheat and Standard wheat) and 3 inclusion levels (20%, 40% and 60%) were investigated.

Statistical analyses of data were performed in SPSS (2016. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp). Data were analysed using the following model

$$Y_{(ij)} = M + WT_{(i)} + lL_{(j)} + WT*IL_{(ij)} + E_{(ij)}$$

Where, Y is the dependant variable, M is the overall mean, E is the residual effect, WT is the effect of wheat type and IL is the effect of inclusion level and WT*IL is the interaction between the effect of wheat type and inclusion level on BWG, FI, pH, mucin layer thickness of the ileum. The same model was used to compare phytate hydrolysis and the coefficients of digestibility of both amino acids and minerals (Ca and P). All these data were subjected to standard ANOVA procedures using SPSS. Data were considered statistically significant when $P < 0.05$.

The partial digestibility of each amino acid (AA) from the HIGHPHY Wheat and Standard Wheat were obtained by calculating the linear regression coefficient between the quantitative AA intake and the amount of AA digested up to the terminal ileum as described by Rodehutscord *et al*., (2004). Linear regressions were calculated using SPSS. Each pen was considered as one unit in the regression analysis. Both parameters for the goodness of fit and r^2 will be presented. The 95% confidence interval and P-value were both taken into account to evaluate the effect of wheat type on the slopes. When regression analysis is applied to quantitative data for both AA intake and AA flow at the terminal ileum, the slopes describe

the proportions of incremental intake (in this case from HIGHPHY Wheat and Standard Wheat) which did not reach the terminal ileum. The difference in slope from 1.0 contains both the unabsorbed part of the AA from those wheats and the specific endogenous loss caused by wheats. Thus, the slopes can be interpreted without corrections as ileal digestibility of the AAs from wheat. Using this approach, the basal endogenous loss is contained in the intercept and hence does not need to be further accounted for. By regression analysis, the partial digestibility of the AAs from wheats were separated from the digestibility determined for the entire diet, where the wheats contributed only part of the total protein. A separation into unabsorbed AAs and endogenous AAs secretion is not necessary with this type of regression analysis as the slope itself is applied as the coefficient of AA digestibility. The amino acid digestibility data of two wheat type were regressed separately, which generated two slopes. Prececal digestibility usually referred to partial digestibility because the digesta sample was collected from ileum not from total digestive tract.

The partial Ca and P digestibility of the test wheats were also obtained by calculating the linear regression coefficient between the quantitative Ca and P intake and the amount of Ca and P digested up to the terminal ileum as per the procedure followed to analyse the amino acid digestibility.

The strength of relationship between mucin layer thickness and amino acid digestibility; dietary Ca level and inositol hydrolysis were examined using the Pearson correlation procedure. Interpretations of the strength of relationships were based on those of Cohen (1988): small r = 0.1 - 0.29, medium r = 0.30 - 0.49 and large r = 0.50 - 1.0.

4.3. Results

4.3.1. Environment

No environmental abnormalities occurred during this trial.

4.3.2. Health and Condition

Mortality data shown in the table 4.4 demonstrates that there were no significant concerns regarding the health of the broilers. There were no significant differences in mortality between treatments during the 5 days trial period.

Table 4.4: Bird mortality during the trial period (per bird)

Treatment	Mortality
Diet with 60% HIGHPHY	1
Diet with 40% HIGHPHY	0
Diet with 20% HIGHPHY	1
Diet with 60% Standard	0
Diet with 40% Standard	0
Diet with 20% Standard	1

4.3.3. Broiler Uniformity

The mean start weights of the broilers during the trial are shown in table 4.5. The start weight of the broilers were similar among the treatments at the onset of the trial period.

Table 4.5: Average start weights for broilers (±SE).

Treatment	D15 BW/bird (g)
Diet with 60% HIGHPHY	556 (15.8)
Diet with 40% HIGHPHY	541(17.7)
Diet with 20% HIGHPHY	540 (2.0)
Diet with 60% Standard	547 (11.9)
Diet with 40% Standard	537 (25.1)
Diet with 20% Standard	545 (17.1)
P-Value	0.452

4.3.4. Performance of broilers fed graded levels of two feed wheats

There were no effects of feed type on BGW, and FI respectively, but there were significant effects of inclusion level on BGW and FCR recorded (Table 4.6). Table 4.6 shows no interaction effects of feed type and inclusion level were present.

Table 4.6: The Effect of the Inclusion of HIGHPHY Wheat and Standard Wheat on broiler performance

Feed Type (FT)	**HIGHPHY Wheat**			**Standard Wheat**			**P (ANOVA)**		
Inclusion Level (IL)	60%	40%	20%	60%	40%	20%	FT	IL	FT X IL
Daily Weight gain	43.11[a]	38.46[b]	33.41[c]	43.74[a]	38.47[b]	29.17[c]	0.316	<0.001	0.196
Daily Feed Intake	76.06	79.43	78.87	79.23	82.13	77.91	0.388	0.368	0.618

Superscript letters denote significance at P<0.05

4.3.5. Gastrointestinal P[H] of broilers fed graded levels of two feed wheats

Effect of diet on pH of crop, gizzard and ileum are shown in figures 4.2, 4.3 and 4.4 respectively. There were no effects of wheat type or inclusion level on the pH of different parts of broiler gastrointestinal tract.

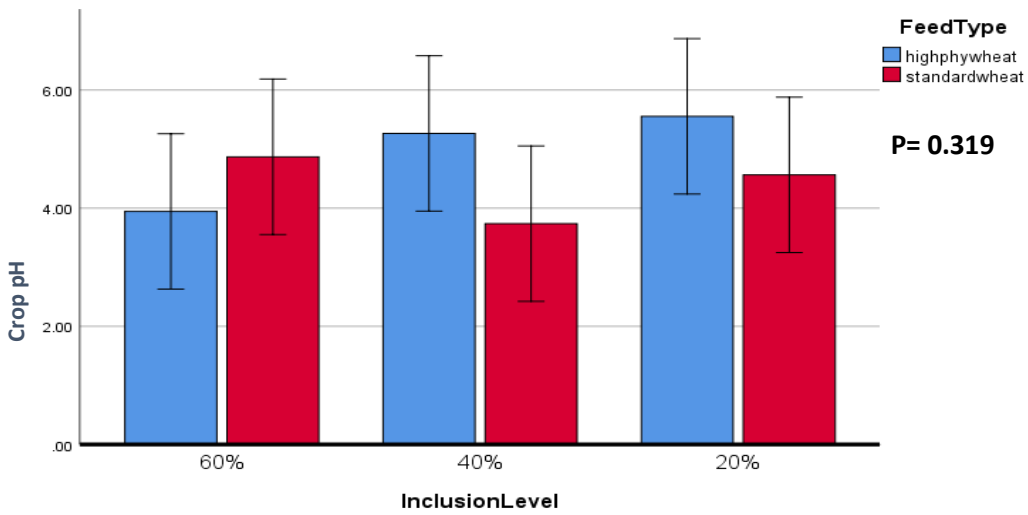


Figure 4.2: pH of the crop in birds fed graded levels of two wheats

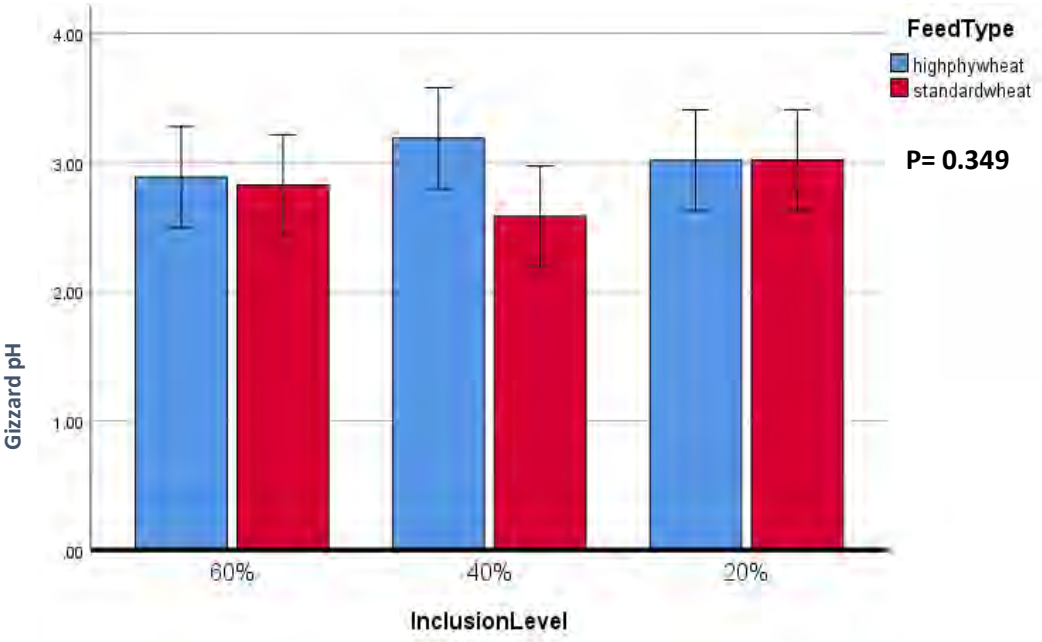


Figure 4.3: pH of the gizzard in birds fed graded levels of two wheats

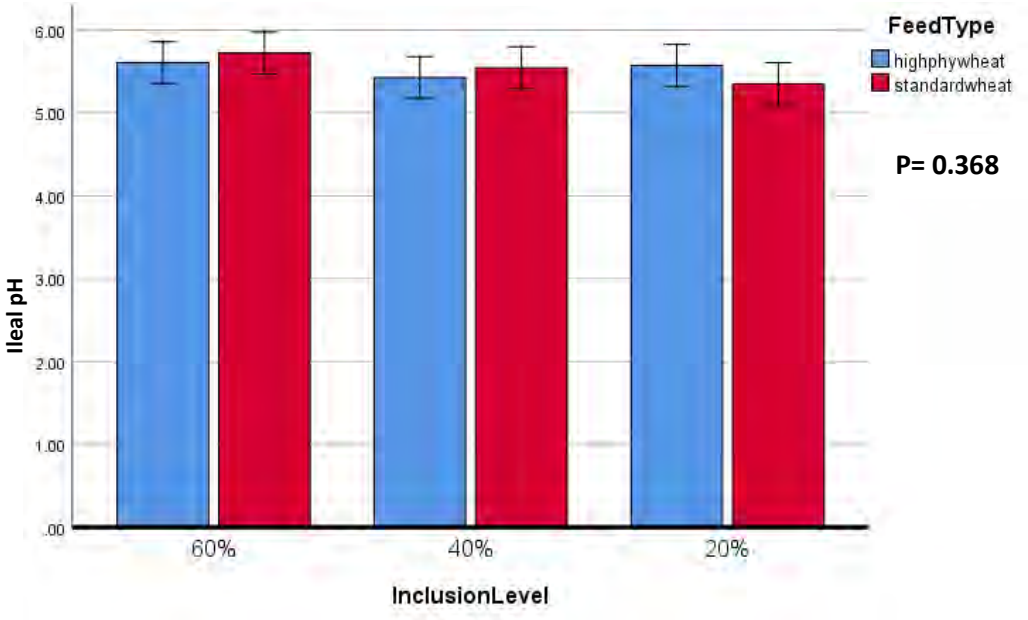


Figure 4.4: pH of the ileum in birds fed graded levels of two wheats

4.3.6. Ileal Mucin layer thickness of broilers fed graded levels of two feed wheats

There was no significant effect of diet on mucin layer thickness of broilers though the thickness were numerically variable among the diets (Table 4.7). Also, there was no effect of inclusion level and no interaction between wheat type and inclusion level for mucin layer thickness of broilers in this study.

Table 4.7: Effect of diet, feed type (FT) and inclusion level (IL) on ileal mucin layer thickness (±SE)

Diet	HIGHPHY Wheat			Standard Wheat			P (ANOVA)		
	60%	40%	20%	60%	40%	20%	FT	IL	FT x IL
Mucin layer thickness (µg/cm^2)	93.98 (9.67)	97.92 (10.40)	124.40 (19.26)	108.61 (14.14)	120.89 (17.86)	90.80 (10.03)	0.908	0.834	0.108

4.3.7. Amino acid digestibility of broiler fed graded levels of two feed wheats

The ileal digestibility coefficients of the individual AAs from the diets ranged between 0.547 and 0.879, with the lowest and highest values being determined for cysteine and methionine, respectively (Table 4.8). Among the essential AAs, methionine was most highly digested (0.879). The effects of HIGHPHY Wheat and Standard Wheat on digestibility coefficients were compared for all AAs. Wheat type significantly affected the digestibility of the AAs in the total diet, apart from cysteine and methionine, though the latter was approaching significance (P=0.054). The coefficients of digestibility were significantly higher in HIGHPHY Wheat diets compared to that of Standard Wheat Diets for all amino acids. There was no significant effect of inclusion level on AA digestibility, apart from for glutamic acid, tyrosine and proline, which were improved with higher inclusion levels. No significant interactions of wheat type and inclusion level were detected for most of the of the AAs, although the data indicated an improvement in methionine, valine, lysine, histidine and isoleucine digestibility because of

interaction. This improvement could be due to bird-to-bird variation, particularly in terms of feed intake in high and low inclusion diet.

Table 4.8: Ileal digestibility co-efficient of amino acids determined for the diets with different concentrations of HIGHPHY Wheat and Standard Wheat

Feed Type (FT)	HIGHPHY Wheat			Standard Wheat			P (ANOVA)		
Inclusion Level (IL)	60%	40%	20%	60%	40%	20%	FT	IL	FT X IL
Cysteine	0.604	0.547	0.606	0.578	0.562	0.583	0.628	0.303	0.733
Aspartic acid	0.734	0.696	0.708	0.640	0.642	0.673	<0.001	0.350	0.179
Methionine	0.879	0.844	0.847	0.824	0.822	0.861	0.054	0.209	0.035
Threonine	0.689	0.632	0.644	0.564	0.576	0.602	<0.001	0.486	0.076
Serine	0.744	0.716	0.713	0.656	0.662	0.654	<0.001	0.594	0.524
Glutamic acid	0.874	0.851	0.844	0.842	0.815	0.794	0.002	0.030	0.791
Glycine	0.729	0.706	0.713	0.652	0.675	0.667	<0.001	1.000	0.297
Alanine	0.747	0.718	0.726	0.656	0.680	0.681	<0.001	0.966	0.277
Valine	0.742	0.659	0.664	0.564	0.591	0.598	<0.001	0.356	0.009
Isoleucine	0.818	0.744	0.753	0.669	0.684	0.698	<0.001	0.333	0.039
Leucine	0.832	0.795	0.790	0.744	0.748	0.760	<0.001	0.498	0.138
Tyrosine	0.848	0.803	0.752	0.799	0.721	0.697	0.001	0.003	0.738
Phenylalanine	0.830	0.793	0.783	0.750	0.740	0.746	<0.001	0.162	0.345
Lysine	0.815	0.771	0.755	0.687	0.726	0.736	<0.001	0.951	0.012
Histidine	0.809	0.773	0.776	0.712	0.744	0.754	<0.001	0.898	0.024
Arginine	0.838	0.804	0.809	0.763	0.761	0.785	<0.001	0.341	0.146
Proline	0.794	0.748	0.717	0.694	0.688	0.591	<0.001	0.002	0.408

Table 4.9: Amount of amino acid (mg) digested per day in ileum depending on the respective daily intake, described by a linear regression (parameter estimate and SE of estimate)

	Slope (coefficient of digestibility)		R^2		Confidence Interval		P-value
	HIGHPHY Wheat	Standard Wheat	HIGHPHY Wheat	Standard Wheat	HIGHPHY Wheat	Standard Wheat	
Cysteine	0.537± 0.130	0.509± 0.115	0.437	0.469	0.267-0.807	0.269-0.748	0.873
Aspartic acid	0.684±0.089	0.563±0.059	0.730	0.803	0.500-0.868	0.439-0.686	0.263
Methionine	0.912±0.048	0.776 ±0.065	0.943	0.868	0.813-1.011	0.642-0.910	0.099
Threonine	0.710± 0.094	0.475 ±0.068	0.722	0.690	0.515-0.905	0.334-0.615	0.049
Serine	0.718 ±0.076	0.631 ±0.053	0.804	0.864	0.561-0.875	0.520-.0741	0.353
Glutamic acid	0.895± 0.040	0.878 ±0.053	0.958	0.927	0.812-0.977	0.6769-0.987	0.799
Glycine	0.673± 0.075	0.623 ±0.050	0.788	0.877	0.519-0.828	0.520-0.726	0.582
Alanine	0.696± 0.084	0.608± 0.062	0.756	0.816	0.521-0.871	0.480-0.735	0.404
Valine	0.859± 0.065	0.501 ±0.073	0.889	0.682	0.724-0.993	0.349-0.652	0.001
Isoleucine	0.916 ±0.058	0.612 ±0.075	0.919	0.750	0.796-1.036	0.455-0.768	0.003
Leucine	0.879± 0.059	0.710 ±0.051	0.910	0.899	0.757-1.000	0.605-0.815	0.036
Tyrosine	0.942± 0.034	0.914± 0.070	0.972	0.886	0.872-1.013	0.769-1.058	0.721
Phenylalanine	0.881 ±0.053	0.742 ±0.046	0.926	0.922	0.771-0.991	0.647-0.838	0.054
Lysine	0.887± 0.061	0.600 ±0.085	0.905	0.695	0.759-1.014	0.424-0.775	0.009
Histidine	0.828 ±0.057	0.640 ±0.058	0.906	0.847	0.710-0.945	0.520-0.760	0.026
Arginine	0.852 ±0.056	0.715± 0.044	0.913	0.924	0.735-0.968	0.628-0.806	0.061
Proline	0.866± 0.084	0.786 ±0.066	0.829	0.865	0.692-1.040	0.648-0.923	0.458

By regression analysis, the partial digestibility of the AAs from wheats were separated from the digestibility determined for the entire diet, where the slopes indicate the amino acid digestibility coefficient of HIGHPHY and standard wheat (Table 4.9).

The slopes of the regression lines shown in Table 4.9 ranged from 0.537 (cysteine) to 0.942 (tyrosine) for HIGHPHY Wheat, and for standard wheat from 0.475 (threonine) to 0.914 (tyrosine). The 95% confidence intervals estimated for the slopes within one AA overlap widely, apart for threonine, valine, isoleucine, lysine, methionine and histidine (Table 4.10). The pre-caecal digestibility of most of the essential amino acids such as threonine, valine, leucine, isoleucine, lysine, histidine were significantly higher in HIGHPHY Wheat compared to that of standard wheat (Table 4.8). Hence, it was concluded that Wheat type had significant effect on the slopes or digestibility for those amino acids. Therefore, HIGHPHY wheat is more digestible and has greater nutritional value compared to standard wheat.

4.3.8 Hydrolysis of phytate esters in broilers of broilers fed graded levels of two feed wheats

Feed type had no significant effect on InsP6 hydrolysis in the ileum, but inclusion level did have a significant effect as shown in Table 4.10. InsP6 hydrolysis was highest in diets with 20% wheat inclusion and lowest in 60% inclusion of either wheat type. There were no significant differences in the concentration of inositol phosphate isomers in the ileum between HIGHPHY Wheat or Standard wheat containing diets in broilers (Table 4.11).

Table 4.10: Percentage of dietary InsP6 (dihydrogen phosphate) hydrolysed in ileum of broiler chickens (Mean values and P value for ANOVA)

Feed Type (FT)	**HIGHPHY Wheat**			**Standard Wheat**			**P (ANOVA)**		
Inclusion Level (IL)	**60%**	**40%**	**20%**	**60%**	**40%**	**20%**	**FT**	**IL**	**FT X IL**
$InsP_6$ Hydrolysis	70.39^{b}	75.91^{ab}	87.30^{a}	76.86^{b}	85.39^{ab}	86.65^{a}	0.106	0.004	0.402

Superscript letters denote significance at P<0.05

Table 4.11: Concentration of different inositol phosphate (InsP) isomers (µmol) in ileal digesta (Mean values and P value for ANOVA)

	HIGHPHY Wheat	**Standard Wheat**	**P (ANOVA)**
Ins(1,2,6;1,4,5;2,4,5)*P_3	0.45	0.36	0.179
Ins(1,5,6)P_3	0.48	ND	0.607
Proportion of $InsP_3$ in $\sum InsP_{3\text{-}5}$	0.06	0.03	0.513
Ins(1,2,3,4)P_4	0.32	0.30	0.741
Ins(1,2,5,6)P_4	0.44	0.40	0.564
Proportion of $InsP_4$ in $\sum InsP_{3\text{-}5}$	0.08	0.20	0.207
Ins(1,2,3,4,6)P_5	0.61	0.58	0.274
Ins(1,2,3,4,5)P_5	1.50	1.50	0.959
Ins(1,2,4,5,6)P_5	0.69	0.70	0.876
Proportion of $InsP_5$ in $\sum InsP_{3\text{-}5}$	0.88	0.77	0.262
$InsP_6$	23.77	23.61	0.917

4.3.9. Ca and P digestibility of broilers fed graded levels of two feed wheats

The coefficients of digestibility for Ca were similar for both HIGHPHY and standard wheat containing diets (Table 4.12). There were no significant effects of either feed type or inclusion level on Ca digestibility coefficients of broilers at day 20. However, P digestibility coefficients were significantly improved by inclusion of HIGHPHY wheat compared to standard wheat. Coefficients of apparent ileal digestibility of P were different with two different wheat containing diets. In HIGHPHY wheat containing diet 60% diet had the highest coefficient of P digestibility whereas in standard wheat it was highest in 40% wheat containing diet. In standard wheat it improved with 40% diet compared to 20%, but in HIGHPHY wheat the values are almost similar with different inclusion level.

Table 4.12: Coefficient of apparent ileal digestibility of Ca and P in broilers fed varying levels of HIGHPHY wheat and standard wheat at day 20

Feed Type (FT)	**HIGHPHY Wheat**			**Standard Wheat**			**SEM**	**ANOVA**		
Inclusion Level (IL)	**60%**	**40%**	**20%**	**60%**	**40%**	**20%**		**FT**	**IL**	**FT*IL**
Ca	0.561	0.561	0.561	0.566	0.571	0.556	0.008	0.838	0.929	0.924
P	0.663^{a}	0.647^{ab}	0.647^{ab}	0.626^{ab}	0.664^{a}	0.577^{b}	0.008	0.040	0.041	0.051

Superscript letters denote significance at P<0.05

The co-efficients of digestibility of Ca and P for the two wheats eg. HIGHPHY and standard have been illustrated (Table 4.13) according to the method reported by Rodehutscord *et al*. (2004), where the digestibility co-efficients of amino acids were illustrated rather than Ca or

P. In this study the same method has been used to illustrate the Ca and P digestibility of the wheats however, this study was specifically designed to estimate the amino acid digestibility of the wheats, so it is not ideal to estimate the Ca and P digestibility. The slopes of the regression line expressed the coefficient of digestibility, and the basal endogenous loss lies within the intercept.

Table 4.13: Slopes estimated for P & Ca digested up to the terminal ileum depending on P & Ca intake from diets containing different levels of HIGHPHY Wheat and Standard Wheat (described by linear regression)

	Intercept		**Slope (coefficient of digestibility)**		**R^2**		**p value**
	HIGHPHY Wheat	Standard Wheat	HIGHPHY Wheat	Standard Wheat	HIGHPHY Wheat	Standard Wheat	
P	44.415±32.160	57.881±28.66	0.722 ±0.048	0.726± 0.046	0.912	0.917	0.952
Ca	58.254±59.506	67.833±57.403	0.644± 0.083	0.658 ±0.078	0.732	0.765	0.903

4.4. Discussion

4.4.1. Broiler performance

There were no effects of wheat type on the body weight gain (BWG) and feed intake of broilers over the five-day test feeding period. While this feeding period is too short to fully evaluate the effect of wheat type on FI and BWG, these data indicate that broilers ate well and gained weight which ultimately suggests that there was no detrimental effect of HIGHPHY wheat on diet palatability. The reason for short feeding period was the semisynthetic diets which were fed for a short acclimatization period (5 days) to minimise the negative effects of poor diet structure. There are only a few research papers that have been published on HIGHPHY Wheat; in one study, it was reported that broiler performance was unaffected between HIGHPHY wheat and standard wheat groups, which is similar to our results (Scholey *et al.,* 2017).

To further understand the possible mechanisms underpinning the growth performance assessed by (Scholey *et al.*, (2017) *in vitro* viscosity analysis of the HIGHPHY wheat and standard wheat was performed. This showed the viscosity of the HIGHPHY Wheat was significantly lower (0.46cps) compared to that of standard wheat (0.56cps), which may be an indication of better nutrient utilization (Table 4.14).

Table 4.14: In vitro viscosity of HIGHPHY Wheat and standard wheat

Measurements	HIGHPHY wheat	Standard wheat	SEM
pH	6.19	6.39	
Viscosity (cps)	0.46^{b}	0.56^{a}	0.07
Viscosity (cps) + Enzyme A	0.35^{b}	0.45^{a}	<0.01
Viscosity (cps) + Enzyme B	0.48^{b}	0.60^{a}	0.02

Superscript letters denote significance at P<0.05

Table 4.15: In vitro viscosity of diets containing graded level of HIGHPHY Wheat and standard wheat

Feed Type (FT)	HIGHPHY Wheat			Standard Wheat			SEM
Inclusion Level (IL)	**60%**	**40%**	**20%**	**60%**	**40%**	**20%**	
Viscosity (cps)	0.86	0.79	0.79	0.78	0.73	0.73	0.02

However, in this study the viscosity of the diets themselves were similar (Table 4.15), which may be due to the effect of the xylanase enzyme (Econase XT, AB Vista, UK) added to all diets to reduce both the anti-nutritional effect as well as the viscosity as industry standard practice (Smulikowska *et al*., 2002). The wheat type may be shown to be impactful over longer feeding periods as in this study the feeding period was short (14 -20 days) and the HIGHPHY wheat contains more protein and phosphorous in addition to the higher phytase activity compared to that of standard wheat (Scholey *et al.,* 2017).

4.4.2. Gastrointestinal P^{H}

Phytase activity is affected by gut pH and the optimum pH of the enzyme. For most of the 6 phytases and 3 phytases the optimum pH range from 4.5-5 and 2.5 -5 respectively, the only exception is Bacillus phytases effective at neutral pH (Simon and Igbasan 2002). Therefore, phytate is most susceptible to attack by 6 phytases in the crop where the pH is 4-5 and proventriculus and gizzard where pH is 0.5-4 (Wyatt *et al*., 2004). Conversely, the pH optimum for wheat grain PAPhy is 5.5. The distal gut pH in the present study was not affected by the wheat type but gizzard pH was lower than the pH optimum for wheat grain PAPhy. The higher alkalinity in the ileum better meets the pH optimum for wheat grain PAPhy of 5.5, irrespective of the wheat type. It has been suggested that 60% of phytate remains after the gizzard and may be hydrolysed further along the gastrointestinal tract (Morgan *et al*., 2015), and a higher

pH optimum may facilitate this phytate breakdown in the small intestine where the pH tends to be higher and in the present study it ranged from 5.35 to 5.7. Mineral bioavailability tends to be reduced in the small intestine when the pH is neutral which facilitates the formation of insoluble phytate-mineral complexes (Tamim and Angel, 2003), therefore wheat grain PAPhy has the potential to breakdown more phytate in the ileum and reduce formation of phytate-mineral complexes and increase the bioavailability of mineral in small intestine.

4.4.3. Mucin layer thickness

No significant effect of wheat type on mucin layer thickness has been found in this study. However, mucin thickness was reduced with increased inclusion of HIGHPHY Wheat, which may be explained by the HIGHPHY wheat reducing viscosity of the digesta, ultimately reducing mucin secretion. It is well known that wheat contains Non-Starch Polysaccharides (NSPs) responsible for increasing the viscosity of digesta resulting in an increase in secretion of mucus, giving rise to an increase in the thickness of the unstirred water layer or mucin layer (Smits and Annison, 1996).

In the present study we can conclude that HIGHPHY wheat containing diets had no adverse effect on ileal mucin layer thickness of broilers. Decreased mucin layer thickness can result in compromised gut protective functions and affect nutrient transport to the villus. On the other hand, increased mucin thickness impairs the rate of nutrient absorption e.g. reduces glucose, linoleic acid and amino acid absorption thereby reducing nutrient availability for growth (Cerda *et al*., 1987; Reeds *et al*., 1999).

To attempt a better understanding of mucin function, in the present study a correlation between the amino acid digestibility and the mucin layer thickness has been demonstrated in figure 4.5. It can be seen that there is little correlation between these two measures, suggesting that there is little relationship between these measures, and mucin thickness is not influencing lysine digestibility.

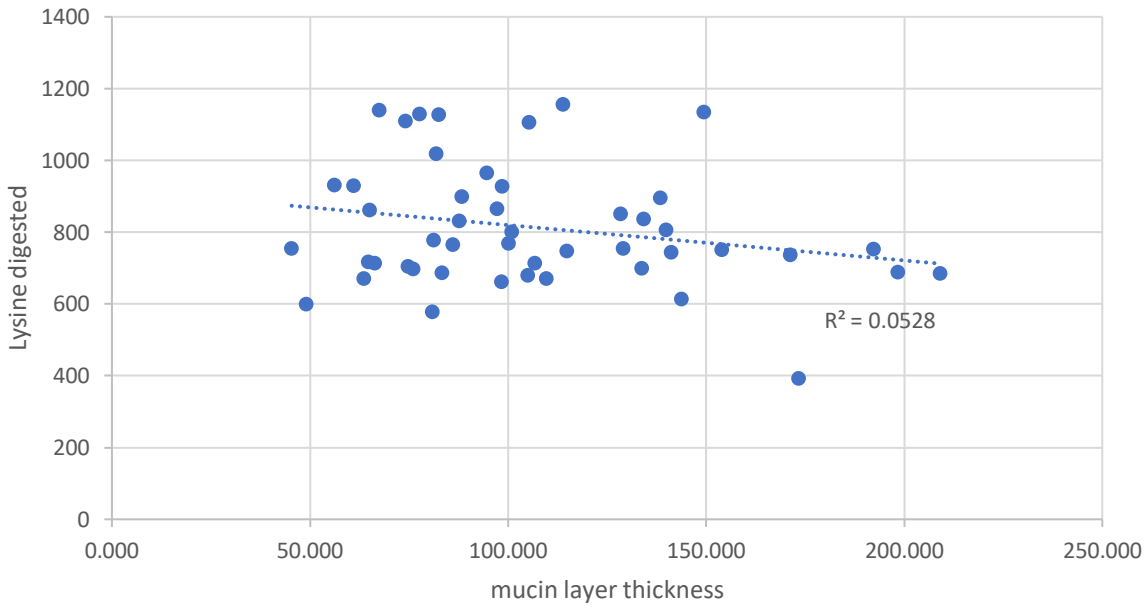


Figure 4.5: Correlation between mucin layer thickness and lysine digested

The mucin layer thickness is higher (106.76 µg/cm^2) compared to that of chapter 3 (42.33 µg/cm^2) in the 3rd week, which may indicate enteric inflammation increase during this trial period. For more understanding of how diet affects the mucin layer thickness, a feeding trial would need to be performed for longer time period, or with more commercially relevant diet formulations.

4.4.4. Amino acid digestibility

Though the broiler performance was the same between the two wheat types, the digestibility of certain amino acids was higher in the HIGHPHY Wheat containing diets compared to the standard wheat diets. There are no published papers about amino acid digestibility of a HIGHPHY Wheat containing diet, although several papers reported on the effect of exogenous phytase addition to broiler diets (Rodehutscord *et al*., 2004; Cowieson *et al*., 2006, Walk *et al*., 2013). Cowieson *et al.* (2017), reported some amino acids (cysteine, threonine, serine, glycine, and valine) showed higher responses, while some (arginine, glutamine, and methionine) showed lower responses with phytase addition. They presented the effect of phytase on apparent ileal amino acid coefficients in broiler from 24 published papers (1996-2015) where addition of phytase in control diets improved the digestibility coefficient of amino acid by an average of 4.1% (0.80-0.84, $P<0.001$) compared to that of control diets (0.80). Different varieties of phytase has been used on those trials such as RONOZYME P & HiPhos, Nanuphos, Axtra PHY, Optiphos, Phyzyme/Phyzyme XP, Quantum/Quantum Blu etc most of which belong to the category of Histidine acid phosphatase phytase. The bulk of mature grain phytase activity of HIGHPHY wheat used in this trial is related to purple acid phosphatase phytase (PAPhy). This seems to be responsible for improving the digestibility of certain amino acids which is beneficial for broilers without addition of those external sources of phytase available in the market. It can be concluded that PAPhy has the potential in improving amino acid digestibility in broilers and these effects originate from the removal of the antinutritional effects of phytic acid on protein digestibility.

Amerah *et al*., (2014), also reported improved amino acid digestibility with the inclusion of phytase in broiler diets where 2 levels (0 & 1000 FTU/kg of feed) of phytase (Axtra PHY, Danisco Animal Nutrition, Marlborough, UK) have been supplemented to diets containing 4 Ca:AvP ratios (1.43, 2.14, 2.86, and 3.57) and fed to ROSS 308 male broilers from 5-21 days.

The increased digestibility of some essential amino acids may have occurred because HIGHPHY wheat contains very high levels of phytase, which has been commonly shown to increase pre-caecal amino acid digestibly of amino acids, although the exact mechanism behind this effect is not yet established (Siegert *et al*., 2021). Protein solubility in HIGHPHY wheat was also investigated and shown to be higher in an *in-vitro* study compared to that of standard wheat, which may also be an indication of why the higher amino acid digestibility occurred in the present study (Table 4.16). As the pH of proventriculus and gizzard ranged from 0.5-4, therefore it indicates that protein of HIGHPHY wheat is more soluble in this part of digestive tract (at pH 1 and 3) which may increase the availability of protein and digestibility of amino acids in the small intestine. However, the protein solubility at higher pH needs to be investigated to find out the protein solubility in small intestine.

Table 4.16: Protein solubility of HIGHPHY wheat and standard wheat at specific pH

pH	Standard wheat	HIGHPHY wheat	SEM
pH1	0.546[b]	0.677[a]	0.00
pH2	0.451[a]	0.436[b]	0.00
pH3	0.398[b]	0.417[a]	0.00

[abc] Means on the same row having different superscripts are significantly (P<0.05) different

The increased digestibility of some essential amino acids may have occurred because the higher mature grain phytase activity influenced the hydrolysis of phytate complexes with nutrients, which liberates more protein and amino acids for digestion and absorption (Selle *et al*., 2007 & Onygango *et al*., 2004). There may be another possible explanation, which is that the lower viscosity of HIGHPHY wheat resulted in lower mucin secretions and increased apparent N retention by reducing endogenous protein losses, as mucin contains a significant number of glycoproteins (Mehri *et al*., 2010). However, in this study the viscosity and mucin layer thickness of the different diets were similar, which may be due to the effect of the xylanase enzyme added to all diets which reduces both the anti-nutritional effect as well as the viscosity.

4.4.5. Hydrolysis of phytate

There were no effects of wheat type on the inositol phosphate hydrolysis of broilers. Zeller *et al.,* (2015) reported no effect of phytase supplementation on InsP6 hydrolysis of broilers, which is very similar to our findings. Moreover, the samples of present study were collected from only ileal sites, but the ideal site of phytate hydrolysis is in the gizzard as the enzyme phytase is more active in the gizzard. In future studies gizzard samples should also be analysed to get a better indication of phytate hydrolysis, during the full length of the GIT.

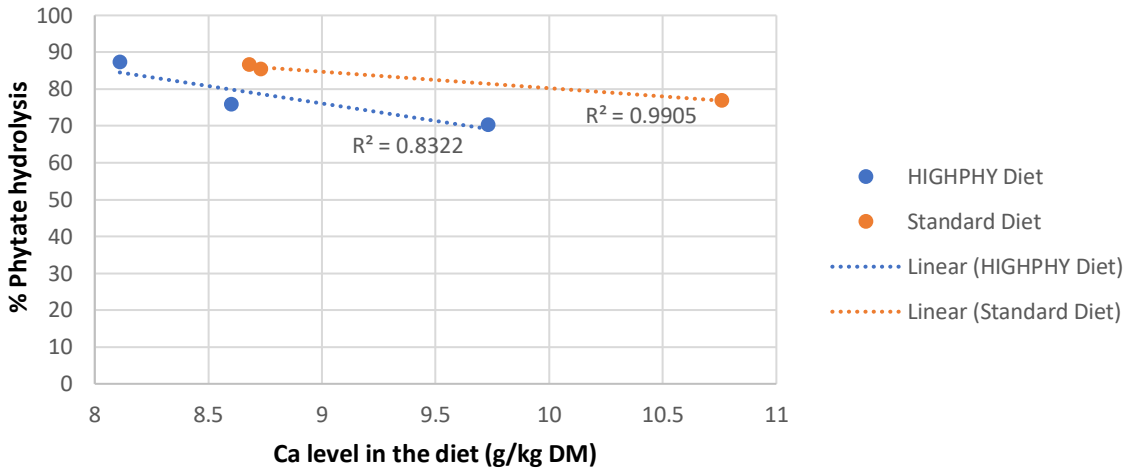


Figure 4.6: Correlation between dietary Ca level and phytate hydrolysis

Another possible explanation of reduced inositol phosphate hydrolysis can be the dietary calcium level in the diet (Figure 4.6). In the present study the dietary Ca levels were higher with an increased inclusion of both of the wheats. Calcium is important because high dietary levels reduce phytate digestibility in chicks (Selle *et al*., 2007); with Ca concentrations of less than 5g/kg able to increase ileal digestibility of phytate-P by up to 70% (Tamim and Angel, 2003; Tamim *et al*., 2004). When calcium and phytate levels exceed a critical concentration, salt formation and precipitation occur which reduces the amount of Ca available in the intestine for absorption. It has been reported in other studies that dietary Ca levels have a significant effect on the response of supplemental phytase (Sebastian *et al*., 1996); with phytate breakdown and phytase efficacy increasing when diet Ca levels are reduced to 0.75-0.85 % (Ballam *et al*., 1984; Perney *et al*., 1993). Ideally, dietary Ca level should keep to a minimum to facilitate maximum phytase efficacy, without compromising skeletal development.

4.5.6. Ca and P digestibility

Although there was no effect of wheat type on the Ca digestibility coefficients of broilers, the P digestibility coefficient was higher in HIGHPHY Wheat containing diets compared to standard wheat diets. The higher coefficient of digestibility of P indicates that HIGHPHY MGPA has a significant impact on the amount of P digested in the ileum at day 20. Higher P digestibility was also reported by Scholey *et al*. (2017) when they fed the birds the control diet with varying replacement levels of HIGHPHY wheat until day 21. However, that study also reported higher Ca digestibility with a HIGHPHY wheat containing diet which contrasts with the results of the present study. The dietary calcium percentage of the present study was higher and therefore may have exceeded a critical concentration where salt formation with phytate and precipitation occurs, thereby reducing the amount of Ca available in the intestine for digestion and absorption (Tamim *et al*., 2004, Plumstead *et al*., 2008).

However, it is important to note that the diets in this study were not formulated to be ideal for investigating Ca and P digestibility, as the diets were not limiting in Ca and P content.

4.5. Conclusion

Although there were no significant effects of wheat type in broiler performance, intestinal pH, mucin layer thickness or inositol phosphate ester hydrolysis, the coefficient of digestibility of some of the essential amino acids (Threonine, valine, Isoleucine, leucine, lysine, and histidine) and P digestibility were all higher in HIGHPHY Wheat compared to that of standard wheat. The traditional performance measures mainly focus around BWG, FI ad FCR, but we need to think about other important parameters like digestibility of nutrients. Therefore, HIGHPHY wheat may be a good alternative to standard wheat in terms of improving P and amino acid digestibility and reducing the cost of addition of external phytase in diets. Moreover, HIGHPHY wheat has the potential to replace the addition of external amino acids in poultry diets and formulation of organic feed in poultry.

Chapter 5: In vitro evaluation of phytase and susceptible phytic acid content in grains for poultry feed

This chapter considers in two distinct parts the key elements required for successful utilisation of plant-derived phosphorus from grain: firstly, the proportion of phosphorus locked into phytate that may be freed by phytase enzyme for use by poultry, and secondly the level of residual phytase activity within wheat after heat processing.

5.1. Comparative investigation of the phytate content of seeds from Australia and UK and Bangladesh and their susceptibility to denaturation with phytase enzymes

5.1.1. Introduction

Poultry feed costs are the largest cost involved in the production of poultry, and phosphorous stands third in the most expensive feed constituents after energy and protein. The majority of the plant feed P are present in the form of phytate which is unavailable in poultry without the presence of phytase. Phytase is commonly added to broiler diets to denature phytate, although grain phytases are also present, they are less efficient than microbial phytase due to their low activity levels; particularly after heat-treating poultry feed (Rapp *et al.*, 2001). Phytase has the capacity to release phytate bound phosphorous and has resulted in a reduction of inorganic phosphate additives to broiler diets (Humer *et al.,* 2015).

Nutritionists can use the total phytate level in grain to calculate the potential organic phosphorous levels in a feed formulation (with the addition of exogenous phytase) and adjust inorganic phosphate levels to compensate for the short fall. There is a practical problem, as only approximately 35% of dietary phytate is hydrolysed by exogenous phytase at the ileal level in broilers (Selle & Ravindran, 2007). Therefore, it is important to know not only the total

phytate of feed ingredients but also the level of phytate susceptible to degradation by phytase enzyme under normal GIT conditions. Despite the potential importance, of the phosphorus contribution from phytate, there are relatively few reports on total phytate content of feed ingredients and only one or two reports on susceptible phytate of feed ingredients are available. There is scientific literature available for determination of total phytate found 45 measurements for maize, 62 for soybean meal, 6 for distillers dried grains with soluble, 106 for wheat, 23 for canola meal and 6 for wheat middlings (Nelson *et al*., 1968; Kirby and Nelson, 1988; Eeckhout and de Paepe, 1994; Ravindran et. al., 1994; García-Estepa *et al.*, 1999; Hidvégi and Lásztity, 2003; Selle *et al*., 2003; Lumpkins and Batal, 2005; Steiner *et al*., 2007; Liu and Han, 2011). These total phytate P determination have been made over 37 -years period in different places around the world including North and South America, Europe, Australia, and Sri Lanka and hence their applicability in current fed formulation at any particular origin is questionable.

Though determination of total phytate content of feed is useful, not all the phytate present in poultry feed ingredients are phytase susceptible as there is still a portion of phytate which is phytase resistant. So the determination of phytate P availability and the amount of phytase and inorganic P needed to supply based on total phytate content of feed is deceptive, as total phytate does not indicate the substrate availability for phytase (Dayyani *et al*., 2013). This concept leads to the idea to consider susceptible phytate content of a feed ingredient or diet when formulating a diet using phytase. Moreover, Morgan *et al*., (2016) have reported improved feed conversion ratio and body weight gain in broiler when they fed diet containing high susceptible phytate compared to diet with lower susceptible phytate. They also observed higher phytate degradation and P solubility in gizzard, jejunum and ileum with diet containing

higher susceptible phytate. Additionally, better bone mineralization has been found with a high susceptible phytate containing diet as Ca and P content in femur and P content in tibia of broiler improved compared to a low susceptible phytate containing diet.

But literature on susceptible phytate content of feed ingredients is scarce. It is therefore important to collect and analyse the commonly used poultry feed materials from different geographical origins to examine the variation in total and susceptible phytic acid content of feed materials. Early evidence (Morgan *et al*., 2016), indicates that phytate susceptibility to degradation by phytase may vary both between feed materials and between batches of material. The susceptible phytate content is measured by putting the feed ingredients into conditions that replicate the pH and temperature identical to that of the proximal part of the digestive tract for phytase activity and quantifying the amount of phytate P released.

The aim of this study is to determine how much variation exists among the susceptible phytate content of different feed samples from different countries of origin.

5.1.2. Procedure

The feed samples were selected from diverse global regions after a global call for volunteers to supply samples from areas where poultry production was a major part of local agriculture. The study was set up as a preliminary exploration to quantify global variation. It was a descriptive study. Different feed samples were collected from Australia and Bangladesh (BD), Nigeria and Tanzania. Five samples (for each feed) of wheat, soybean meal, rapeseed meal, barley, maize and sorghum were collected from multiple sites across Australia. Three samples (for each feed) of

commonly used feed ingredients e.g., Whole maize, broken maize, whole wheat, whole soya and soybean meal were collected from different feed mills across Bangladesh. Also, local feed ingredients typically used in poultry across Nigeria (e.g., cowpea, epa rode, ewalbile, groundnut, yellow and white maize, millet, otili, white and red sorghum, soybean meal and wheat) and Tanzania (e.g. cowpea kunde, dengu, mbazi beans, finger millet, sesame seed-simsim and, soyabean whole) collected from different sites. The total phytate content of these feed samples were determined by using Megazyme^{TM} method through the determination of free phosphorous and phytic acid concentration. The method is explained in detail in chapter 2 section 2.5.15.

The susceptible phytate contents of the feed samples were determined by slight modification of the Megazyme^{TM} method. The modification involved digesting the samples in warm buffer of acetate which have a P^{H} of 4.5 for 10 minutes to mimic the environment inside a chicken's digestive tract (Morghan *et al.*, 2016). The details of the method to determine susceptible phytate content is documented in chapter 2 section 2.5.16.

5.1.3. Results

The typical feed ingredients used in poultry varied considerably among different countries. Table 5.1 and 5.2 show that the total phytic acid and susceptible phytic acid content of feed ingredients varied considerably among country of origin and between batches of ingredients.

The total phytic acid content of all the feed ingredients from different countries were higher compared to their susceptible phytic acid content. In Bangladesh (BD) approximately 47% of total phytic acid of whole maize was susceptible to an external phytase enzyme which reduced to 38% in broken maize. In Nigeria 2 types of maize samples were analysed where white variety of maize had higher susceptible phytic acid % (99%) compared to the yellow variety (77%) and both of these values were higher compared to the maize samples collected from BD. The susceptible phytic acid % of wheat and soyabean meal samples are also higher in Nigeria (71% in wheat & 58% in soyabean meal) compared to BD (34% in wheat & 34% in soyabean meal). Whole soya is also a common feed ingredient in BD and Tanzania but their susceptible phytic acid % varied greatly which was 18% in BD and 81% in Tanzania.

Table 5.1: Total phytic acid and Susceptible phytic acid content of feed ingredient fed to poultry in Bangladesh, Nigeria and Tanzania

	Total phytic acid (g/100g)	Susceptible phytic acid (g/100g)	%Susceptible phytic acid
Bangladesh			
Broken Maize	0.765	0.295	38.404
Whole Maize	0.788	0.375	47.621
Whole Wheat	0.625	0.216	34.512
Soyabean meal	1.538	0.490	34.796
Whole soya	1.356	0.250	18.453
Nigeria			
Cowpea	0.441	0.214	48.572
Epa rode	0.713	0.435	60.999
Ewalbile	0.750	0.560	74.661
Groundnut	0.867	0.295	33.984
Maize-yellow	0.632	0.489	77.378
Maize-white	0.591	0.585	99.070
Millet	0.488	0.532	108.989
Otili	0.788	0.423	53.715
Sorghum-white	0.595	0.608	102.200
Sorghum-red	0.626	0.650	103.841
Soyabean meal	1.284	0.747	58.201
Wheat	0.781	0.560	71.613
Tanzania			
Cowpea kunde	0.844	0.704	83.422
Dengu	0.439	0.348	79.310
Mbazi beans	0.730	0.449	61.469
Millet-finger	0.439	0.375	85.369
Sesame seed - Simsim	1.820	1.282	70.469
Soyabean whole	1.139	0.931	81.721

Table 5.2 shows the comparison of total phytic acid and susceptible phytic acid content of feed ingredients from UK (Morgan, *et al*,2016) and Australia and Bangladesh. The total phytic acid P concentrations of all the feed ingredients were higher in the samples from Australia compared to UK. The Australian feed ingredients also showed more variation in susceptible phytic acid P levels between ingredients with Barley and wheat showing 38.97% and 34.89% difference respectively in their range of susceptible phytate-P. The Australian samples show phytase more readily hydrolysed phytate in Maize, Soybean meal and sorghum in GIT

conditions though the highest total Phytate is found in Rapeseed meal. Whereas the UK samples show less than a 10% difference in the average susceptible phytate level. Sorghum was interesting as although its levels of phytate are relatively low compared to soybean and rapeseed meals, its susceptibility to exogenous phytase averages at 94.45%. Unfortunately, only Australian samples for Sorghum were tested so no UK and BD comparison can be made. The total phytic acid contents of maize, soyabean meal and wheat are higher in BD and Australian samples compared to that of UK samples but the susceptible phytic acid contents were lower in BD samples compared to both Australian and UK samples. This indicates the rate of phytate hydrolysis by external phytase is lower in BD samples compared to UK and Australian samples.

Table 5.2. Comparison of total phytic acid and susceptible phytic acid content of feed ingredients from UK (Morgan, *et al*,2016) and Australia and Bangladesh

	UK			**AU**			**BD**		
Ingredients	**Total phytic acid g/100g**	**% Sus. Phytic acid**	**Range in susceptible phytic acid (%)**	**Total phytic acid g/100g**	**% Sus. Phytic acid**	**Range in susceptible phytic acid (%)**	**Total phytic acid g/100g**	**% Sus. Phytic acid**	**Range in susceptible phytic acid (%)**
Barley	0.434	55.82	52.68-61.45	0.654	33.36	17.29-56.26			
Rapeseed meal	0.833	50.81	46.84-56.16	2.391	18.23	14.25-24.57			
Maize	0.501	57.44	51.82-60.78	0.685	64.92	59.16-71.90	0.788	47.62	45.6-49.4
Soybean meal	0.339	48.55	43.62-53.34	1.933	70.55	56.10-85.98	1.538	34.80	28.7-35.08
Sorghum	-	-	-	0.697	94.45	75.95-102.72*			
Wheat	0.243	57.45	49.51-63.45	0.700	39.62	29.84-64.73	0.625	34.51	32.5-36.68

5.1.4. Discussion

The total phytate content of maize, soybean meal and wheat were previously reported as 0.66, 1.4 and 0.89 g/100g cereal respectively in the United States (Tahir *et al*., 2012). In contrast, the National Research Council (NRC, 1994) had previously documented total phytate content of maize and soybean meal were 0.78 and 0.87 g/100g cereal respectively. This suggests a highly relevant increase of 12.5% for phytate P in soybean meal over the past 2 decades, when 114 samples from United States were measured (Tahir *et al*., 2012).

The current study suggests that the rate of change may have slowed over the last 10 years; the total phytate content of maize from BD of 0.79 g/100g cereal is similar to that reported by the NRC (1994) and, similarly, maize from Nigeria show total phytate levels of 0.63 and Australia is 0.685 g/100g cereal, which are similar to that of United States (0.66 g/100g cereal reported by (Tahir *et al*., 2012). In contrast, the total phytate content of maize samples from UK (Morgan, *et al*, 2016) is 0.501 (g/100g), which is considerably lower than values for Australia, Bangladesh, Nigeria, NRC and United States. Similarly in the case of soybean meal (SBM), the total phytate content from BD (1.54 g/100g cereal) and from Nigeria (1.284 g/100g cereal were both close to that reported by Tahir *et al*., (2012), (1.4 g/100g cereal) but when compared to NRC values; the total phytate content of soybean meal is 77% higher in BD and 49% higher in Nigeria. The total phytate content of soybean meal from the UK is the lowest and Australian sample (Table 5.2) showed the highest value among Australia, Bangladesh, Nigeria, NRC and United States. In contrast to the findings for both maize and SBM, the current study showed greater diversity in total phytate content of wheat: BD samples showed a mean value of 0.62 g/100g cereal in contrast to 0.78 g/100g cereal for samples from Nigeria.

Therefore, while the Nigerian samples indicates levels closer to the values of the United States samples reported by Tahir *et al*., (2012), the BD wheat samples were 30% lower than reported US values. The total phytate content of wheat from Australia is close to the value of Nigeria but still 27% lower than the United States value. The total phytate content of maize samples from UK is 0.243 g/100g cereal which was lower than to Australia, Bangladesh, Nigeria, and United States. This suggests geographical location of production affects the total phytate levels of all grains considered in this study.

In most of the countries the susceptible phytate content of cereal grains are higher compared to that of oilseeds and oilseed meals, only exception is soybean meal which has a higher susceptible phytate of 70.55% in AU and soybean whole with a susceptible phytate content of 81.721 in Tanzania. The variability in susceptible phytate content is less (within 10%) in maize and rapeseed meal between batches of ingredients within same country, but country to country variation is higher and not consistent among different countries for most of the feed ingredients. The practical implications of this in diet formulations are that in order to maximise the level of phytate bound phosphorus released from poultry feed materials, the level of the susceptible phytate content of diet materials needs to be measured. This would allow optimal use of phytase and minimal use inorganic P. This type of precision approach is becoming more widely accepted as the poultry sector strives to minimise the environmental impact of meat production.

The variations in feed ingredient composition such as total phytate content can be influenced by various factors including the particular cultivar grown, the growing conditions such as

moisture, rainfall, water holding capacity of the soil, fertilization rates etc and also the technological processes involved in harvesting and storage of the crop (Raboy, 2000). The phytate P content in plant based feed ingredients is affected particularly by the phosphorous fertilization rates (Zhou *et al*., 1992). These factors affecting the total phytate content may also affect the susceptible phytate content and hence explain the variation in susceptible phytate content found among differing batches of ingredients and countries of origin in the current study.

Selle & Ravindran, 2007 reported that only approximately 35% of dietary phytate is hydrolysed by exogenous phytase at the ileal level in broilers. The estimation of 35% of total phytate P being available for degradation by phytase in general is not a good indicator for evaluating phytase efficacy. From the *in vitro* study reported here, it has been evident that if we consider 35% phytate P is available from all the feed ingredients in general we are underestimating the value as in most of the feed ingredients the susceptible phytate % is much higher than that value and few of them are nearly similar; only exception is 18.23% susceptible phytate is found in rapeseed meal in AU and 18.453% in whole soya in BD. So, it is practical to calculate the susceptible phytate content of each diet to estimate how much phytate P is available to poultry from that specific diet.

5.1.4. Conclusion

The cereal grains contain higher susceptible phytate content compared to that of oilseeds and oilseed meals. The general perception of only 35% dietary phytate of all feed ingredients hydrolysed by phytase underestimate the value as majority of feed ingredients have higher susceptible phytate content. Therefore, it would be more accurate to formulate diets by taking into consideration the susceptible phytate level of the batches of ingredients present in that particular diet. This would give a more accurate level of plant-derived P available to the bird and reduce the need for excess inorganic P addition. There are, however, large variations in the amount of the total and susceptible phytate levels among feed ingredients from different countries of origin and also between batches of ingredients within same country.

5.2. Effect of processing on phytase activity of HIGHPHY Wheat

5.2.1. Introduction

Feed processing technologies, especially those involving heat treatment often denature the endogenous phytase enzymes naturally occurring within cereals such as wheat, thus reducing phytase activity and ultimately limiting hydrolysis of phytate (McCance and Widdowson 1949). Extrusion cooking is a typical example of heat treatment now well established as reducing the phytase activity of cereal products such as bran, and subsequently decreasing zinc, magnesium and phosphorous absorption when compared to a raw bran meal. A similar effect can be seen in the stomach and small intestine of humans, with decreased hydrolysis of phytate due to heat treatment being responsible for a decrease in the absorption of different nutrients' (Sandberg, 1991).

Phytase is present in most of the cereals fed to poultry, but the activity varies widely among the cereals (Bartnik and Szafranska, 1987). Different types of feed processing have different impacts on the phytase activity of cereal grains. Phytase activity differs significantly among different wheat varieties during different processing conditions. Sushma (2021) reported that the wheat phytase activity was increased while soaking and germination, but it decreased during kilned, roasted and ground seeds. They compared 4 wheat varieties and found that all 4 wheat phytase activities differed greatly during the different processing conditions. Germination has been used to induce phytase activity in cereals (Senna *et al*., 2006) as phytase activity usually increases on germination (Sung *et al*., 2005). The purpose of the present investigation was to observe how pelleting may affect the phytase activity of a novel variety of wheat called HIGHPHY wheat.

5.2.2. Procedure

Following the positive digestibility data derived from the digestibility trial reported in chapter 4, a commercial feed mill undertook a pelleting assessment of the HighPhy wheat. HIGHPHY wheat samples were collected from the Bury St Edmunds feed mill of Forfarmers UK Limited. The wheat samples were collected before and after processing such as raw, post grinding, post conditioning, warm pellet and cold pellet. The pelleting conditions applied while pelleting HIGHPHY wheat samples were as follows:

- Moisture content of whole wheat: 12.23%
- Hammer mill grinding speed: 3000 rpm
- Hammer mill screen size: 3.0 mm
- Pellet press: CPM 7932
- Die size: 3.5 mm x 60/35
- Conditioning steam temperature: 60^{o}C
- Post pelleting, pellet temperature: 77^{o}C

After pelleting, the phytase activity of those wheat samples were determined by a slight modification of the direct incubation method, or quantification of the liberated phosphate method described by (Greiner and Egli, 2003). The detail of the method is described in Chapter 2, Section 2.5.13. In short, samples (0.1 g) of dry-milled HIGHPHY wheat were suspended in 20 mL of 100 mM sodium acetate buffer, pH 5.0, containing 100μmol of sodium phytate preincubated at 37 and 45 °C, respectively. After certain time intervals at the given temperature, 400 μL portions of the incubation mixtures were removed, and the liberated phosphate was measured by the ammonium molybdate method (Heinonen and Lahti, 1981) with some modifications. Added to the assay mixture were 1.5 mL of a freshly prepared

solution (2:1:1 v/v) of pure acetone/ 5 Molar H_2SO_4 / 10 Millimolar ammonium molybdate with 100 μL of 1.0 Molar citric acid. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. To calculate the enzyme activity, a calibration curve was produced over the range of 5-600 nmol of phosphate. Blanks were run by addition of the ammonium molybdate solution prior to adding the enzyme to the assay mixture. Activity (units) was expressed as 1 µmol of phosphate liberated per minute.

5.2.3. Results

The total phytase activity of the acid phytate-degrading enzymes present in the HIGHPHY wheat were determined at five different stages of processing while pelleting. An incubation temperature of 45^0C was chosen, which is close to the temperature optimum of cereal phytate-degrading enzymes and a linear release of phosphate with time at this temperature has been observed (Table 5.3). The liberated phosphate was linearly increased with time until 25 minutes and after 25 minutes the amount of liberated phosphate were similar (Table 5.3). The amount of liberated phosphate was higher in raw wheat compared to wheat after processing, as 131.46 µmol phosphate was released from a g of grain while it was raw and gradually it was reduced to 73.65 µmol after pelleting.

Table 5.3: Direct incubation (Quantification of the liberated phosphate)

	Liberated phosphate (µmol/g of grain)				
Time	RAW HPW	GROUND HPW	HPW POST CONDITIONING	HPW PELLET WARM	HPW COLD PELLET
10	48.28	40.67	36.76	29.45	28.27
15	65.78	58.96	53.58	46.35	41.56
20	92.53	85.22	72.98	54.89	56.78
25	131.46	101.3	93.29	78.9	73.65
30	131.02	101.05	92.89	77.67	73.34

The phytase activity were reduced during processing when compared to raw HIGHPHY wheat (Table 5.4 and figure 5.1), as it was 4675.5 (mU/g of grain) in raw wheat and reduced gradually as processing advanced and a final level in cold pellets of HIGHPHY wheat of 2705.3 (mU/g of grain). The major reduction in phytase activity has been observed after grinding and conditioning which is about 18.67 and 26.34 % respectively. About 39% reduction in phytase activity was recorded after pelleting compared to raw wheat, although there was not much difference in phytase activity between warm and cold pellet. Overall, the phytate degrading activity of phytase was reduced 18% after grinding and 42% after pelleting HIGHPHY wheat.

Table 5.4: Phytate-degrading activity (mU/g of grain)

Different form of HIGHPHY wheat at processing	phytate-degrading activity (mU/g of grain)	% Reduction in phytase activity
Raw wheat	4676.5	
Post grinding	3803.3	18.6
Post conditioning	3444.5	26.3
Warm pellet	2842.6	39.2
Cold Pellet	2705.3	42.2

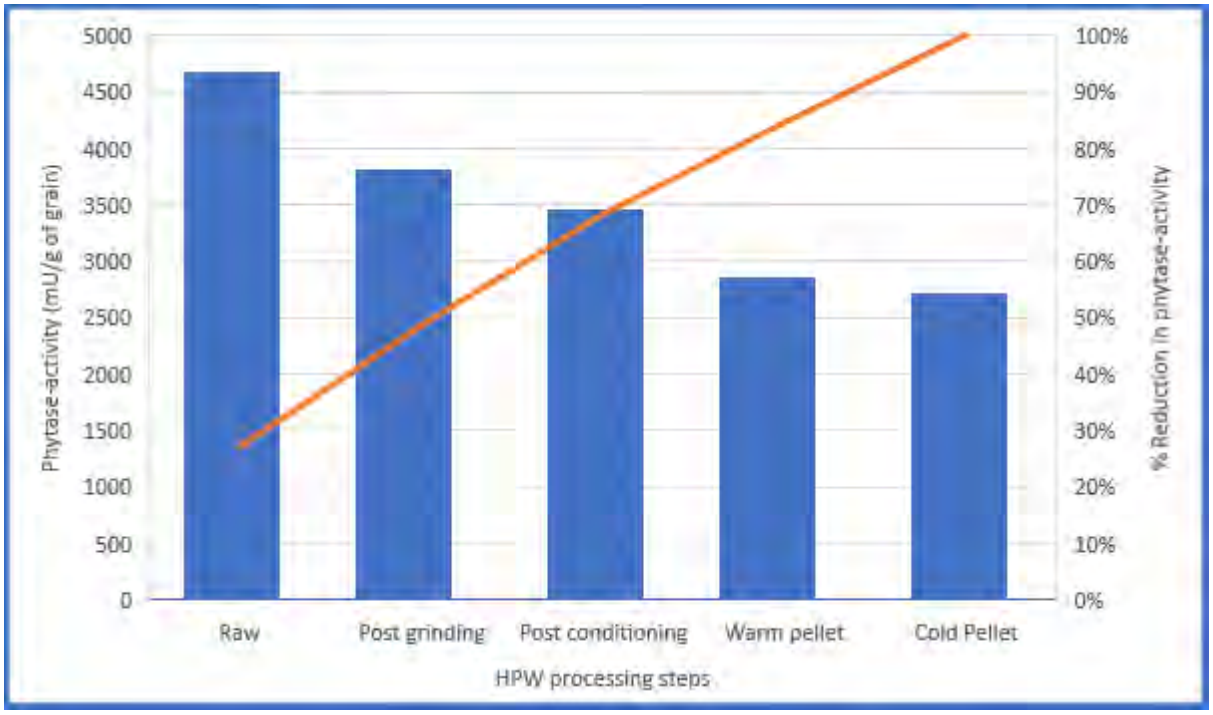


Figure 5.1: Effect on phytase activity level with cumulative processing

5.2.4. Discussion

The phytase activity of the HIGHPHY wheat at different processing conditions has not been studied yet. The phytase activity of HIGHPHY wheat has been recorded by only a few scientists which were 6200 FTU/Kg (Brinch-Pedersen *et al*., 2012) and 6196 FTU/kg (Scholey *et al*., 2017). The phytase activity of raw wheat determined in the present study was 4676.5 (mU/g of grain), which is slightly different from the previous study. The difference in the phytase activity may be due to the use of different method for determining phytase activity as in the present study a direct incubation approach has been practiced.

Though there is no previous study on the effect of processing condition on HIGHPHY wheat but in -vitro evaluation of heat stability of phytase in HIGHPHY wheat has been observed by Brinch-Pedersen *et al*., 2012. They reported a residual phytase activity of 70%, 42% and 22% after incubating the HIGHPHY wheat flour for 10, 20 and 40 minutes respectively at 80^0C and in a relative humidity of 100%. Though the experimental condition is different compared to present study, the previous study showed that phytase activity reduced with the time of incubation which is similar to the present study. Poultry feed manufacturers commonly use high conditioning temperature (>80%) for minimum retention time of 30 seconds and maximum three or four minutes to obtain high quality pellet (Cutlip *et al*., 2008; Abdollahi *et al*., 2013). Naghshabandi, Moghimi and Latif, 2018 reported that phytases should maintain their activities at 80-90^0C for 3 -5 minutes during the pelleting process. So, the residual phytase activity of 70% at 80^0C for 10 minutes for HIGHPHY wheat flower indicates that HIGHPHY wheat's phytase activity will sustain in a good amount under pelleting process.

Grinding has significant effect on phytase activity of four different wheat varieties and fine grinding of wheat resulted in further decrease in phytase activity compared to that of course grinding (Sushma 2021). Though the wheat varieties (LOK-1, DBW-17, HD-2894, HUW-234) are different from the present study (HIGHPHY wheat) but grinding reduced the phytase activity which is similar to the findings of the present study. They also reported with increasing temperature of roasting or heat treatment, the phytase activity also reduced gradually and varies among different wheat varieties (Sushma 2021). In the present study phytase activity of HIGHPHY wheat was reduced further after conditioning and pelleting which may be due to the specific temperature or heat treatment involved during the pelleting process.

In the current study, the residual phytase activity after pelleting of the HIGHPHY wheat was 58%. This sharply contrasts with common wheat cultivars as, although there is some variation from country to country, Jonbloed *et al*. (1990) reported that when pelleting was conducted above 80^0C endogenous phytase was wholly inactivated in wheat. However, on the contrary, Peers (1953) found just partial inactivation of wheat phytase when heated at 80°C for 10 mins and Ma and Shan (2002) reported that phytase activity was reduced not more than 10.53% when wheat (NEAU123) was heated at 100^0C for 1 hr. A similar result was reported by Ranhotra *et al*., (1975) where just 25% reduction in wheat phytase activity was found when wheat was heated at 100^0C for 1.5 hours, although the authors also reported the cereal finally lost nearly 90% of its phytase activity when heated at 100^0C for 3 hrs. These contrasting results relate to whether moisture is introduced alongside heat: the latter studies undertook dry heating of the samples while processing, in contrast to the substantial steam input associated with the pre-conditioning process of pelleting.

Enzymes are more stable in dry condition compared to that of wet condition (Ma and Shan, 2002) but current commercial requirements for high quality pellets that do not deteriorate during transportation means introduction of moisture alongside heat is an unavoidable part of pelleting for poultry feed (Abdollahi *et al*., 2019). In the present study, water vapour or steam heating was applied while pelleting which is also applied for the samples reported by Jonbloed *et al*. (1990) and Peers (1953) who found partial or complete inactivation of wheat phytase above 80^0c. Sushma (2021) reported processing of different varieties of wheat at 60^0C reduced the phytase activity from 624-645 nM/min/g to 70-90 nM/min/g which means the remaining phytase activity was approximately 11-14% after heat treatment. In the present study, the remaining phytase activity of HIGHPHY wheat was 58% when the conditioning

temperature was 60^0C while pelleting. So HIGHPHY wheat certainly showed great potential to survive it's phytase activity compared to other wheat variety. The only practical limitation is that to keep this wheat separate from the rest at a feed mill, separate storage space or storage unit might be needed.

HIGHPHY wheat has a great potential in plant-based or organic production system as it a non-GM product and there is no non-GM phytases are available in the market for organic production. Moreover, HIGHPHY wheat can be used as an alternative source to external phytase in those countries where the energy supply is insufficient, or they have limited biotechnical capacity as growing bacterial or fungal phytase require bioreactors which need huge energy supply.

5.2.4. Conclusion

Higher phytase activity facilitates maximum hydrolysis of phytate of cereal grains and therefore makes phytate P and other nutrients more available to poultry. Plant-derived phytase activity is rarely considered in feed production as little plant-derived phytase maintains activity after heat processing. However, this investigation indicates phytase activity levels of nearly 58% are retained after pelleting HIGHPHY wheat.

Chapter 6: General Discussions, Conclusions and Recommendations

6.1. Introduction

This chapter has been divided into three sections to discuss how the efficacy of phytase can be improved in broiler diets. Firstly, the key findings from the two broiler trials and the in vitro studies are reviewed and contrasted in the context of present poultry production practices worldwide. Secondly, the recommendations based on this project for the benefit of the poultry industry and poultry nutritional research are discussed. Finally, the areas of focus for future research and the overall impact of the findings of this project are outlined.

Global poultry production faces several challenges, among which ensuring the maximum availability of nutrients from the plant-based feed materials by ensuring proper gut health of poultry is gaining importance. Phytate is a major storage form of plant P which is considered an antinutritional factor for its adverse effect on the availability of not only plant P, but also its impact on other nutrient availability in poultry. Phytase enzymes have been used for decades in poultry feed to reduce the antinutritional effect of phytate by hydrolysing phytate. Research is continuously carried out on the use of phytase in poultry diets, not only to improve the phytate P utilization, but also to reduce the use of costly finite mineral phosphate. The impact of phosphorous from the scarce reserve of finite mineral phosphate and its residual effect on the environment is becoming a global problem. Phytase when used in conventional amounts improves broiler performance and further increasing the dose also improves broiler performance beyond that of conventional doses. Superdosing has the potential for further improving broiler performance, but several factors need to be explored

to get a better understanding of how it affects performance and gut health of broilers. Several factors are associated with how efficiently phytase is used in broiler diets. These include susceptible phytate content of feed ingredients, processing effects on phytase activity and the mature grain phytase activity of raw materials. Also, use of novel feed ingredients with high mature grain phytase activity may be an alternative to using external phytase enzymes, with the potential to improve phytase efficacy in poultry. Thus, this thesis included a broiler trial to examine how superdosing of phytase affects broiler performance with a focus on whole-body mineralization. This is followed by a study investigating the use of a novel wheat variety, HIGHPHY wheat, as an alternative to external phytase usage in broiler diets. Furthermore, this thesis quantifies how susceptible phytase of feed ingredients from different origin differ and how processing affects the phytase as part of a holistic approach to a better understanding of phytase efficacy in poultry.

6.2. Key findings, contrast, and critique of investigation

6.2.1. Superdosing phytase

The first study investigated the the effect of super-dosing phytase supplementation on performance and whole body phosphorus content of broilers through examining the relative concentration of different inositol-esters within the broiler digestive tract. Though superdosing of phytase in broiler diets increased the generation of myo-inositol content in crop at day 7 and ileum and jejunum at day 21 and 35 by improving phytate degradation compared to that of standard dose of phytase, this did not translate to any benefit on performance of broiler compared to other diets. The result of this study contradicts the findings of other studies, as most report a positive response on broiler performance following

high levels of phytase inclusion (Walters *et al*., 2019, Santor *et al*., 2014, Powell *et al*., 2011, Singh 2008, Ravindran *et al*., 2008, Selle and Ravindran 2007, Onyango *et al*., 2004). It was therefore most unexpected that the positive control diet showed no improvement over the negative control diet. The lack of performance response to super-dosing is a possible indication that adequate digestible P level was present in the diets without the need for phytase to make more P available. Therefore, focussing on ensuring a minimal level of P in the diet without causing harm to the bird in future studies is more likely to allow accurate assessment on the effect of super-dosing on performance. So, superdosing has no effect on the performance of broilers in the present study and the performance response to super-dosing in other studies can't be compared with the present study because of the difference in experimental design, diets, environments etc.

Improved tibia ash percentage has been observed with superdosing of phytase at day 21 and 28 in this study, which is in agreement with the findings reported by Walters *et al*. (2019). However, the tibia and whole-body Ca and P content were not affected by superdosing of phytase compared to standard dose. This is contradictory to the study reported by Walters *et al*. (2019), where superdosing of 2000 and 3000/1000 FTU/kg phytase improved tibia P beyond the conventional doses of phytase. There was no effect of phytase superdosing on mucin layer thickness, ceca and digesta weight in this study, but superdosing reduced the digestive tract weight in broilers at day 21 and 28 compared to diet containing negative control and standard dose of phytase respectively. Reduced intestinal weight is considered to be beneficial as improved nutrient retention is observed because of the thinning of intestinal wall (Huyghebaert *et al*., 2011, Sharifi *et al*. 2012).

The WBP: Tibia P ratio was not constant in broilers for each age assessed, which may indicate that tibia P is not a reliable estimate of WBP content. WBP is very laborious to measure but future studies could consider quantifying the WBP content together with P digestibility to estimate a more complete picture of the P retention in broilers.

There was no effect of phytase superdosing on cecal microbial diversity compared to other diets, which indicates there is little effect of superdosing on gut microbiome. This result contradicts the findings of a study where inclusion of megadose of phytase (3000 FTU/kg) in broiler diets resulted in a change of the cecal microbiota population in broilers (González-Ortiz *et al*., 2020). They reported a decrease in proportion of Bifidobacterium and an increase in Faecalibacterium, unclassified Clostridiales and unclassified Mollicutes (RF9 group). Though superdosing reduced the relative abundance of genus Anaerostipes and Pygmaiobacter, there was lack of information on the effect of phytase on these two genersa, so future studies should investigate this finding further. Also, in future studies measuring the viscosity of digesta without xylanase addition in diets and measuring volatile fatty acid production in cecal digesta may help further to determine the beneficial effect of phytase superdosing in broiler diets. The beneficial effects of SCFA are well evident as they increase the growth of epithelial cells, stimulate mineral absorption, inhibit the growth and adherence of pathogenic microbes by decreasing P^H (Walugembe et al., 2015).

6.2.2. Novel HIGHPHY wheat with high Mature Grain Phytase Activity

The second study was conducted to investigate and compare the amino acid digestibility and physiological response of broilers fed diets containing graded levels of a standard wheat or a novel strain of HIGHPHY wheat containing high levels of phytase. Although there were no significant effects of wheat type in broiler performance, intestinal pH, mucin layer thickness or inositol phosphate ester hydrolysis, the coefficients of digestibility of some of the essential amino acids (threonine, valine, Isoleucine, leucine, lysine, and histidine) and P digestibility were all higher in HIGHPHY Wheat compared to standard wheat. There are only a few research papers published on HIGHPHY Wheat; in one study, it was reported that broiler performance was unaffected between HIGHPHY wheat and standard wheat groups, which is similar to our results (Scholey *et al.,* 2017).

Therefore, HIGHPHY wheat may be a good alternative to standard wheat in terms of improving P and amino acid digestibility. Moreover, HIGHPHY wheat has the potential to replace the addition of external amino acids in poultry diets particularly in formulations of organic feed in poultry, as the wheat is not delivered using Genetic Modification or Gene Editing processes, this route is a viable option. Higher P digestibility was also reported by Scholey *et al*. (2017) when they fed the birds a control diet with varying replacement levels of HIGHPHY wheat until day 21. However, that study also reported higher Ca digestibility with a HIGHPHY wheat containing diet, which is in contrast to the results of the present study. The dietary calcium percentage of the present study was higher and therefore may have exceeded a critical concentration where salt formation with phytate and precipitation occurs, thereby reducing the amount of Ca available in the intestine for digestion and absorption (Tamim *et al*., 2004, Plumstead *et al*., 2008). However, it is important to note that the diets in this study

were not formulated to be ideal for investigating Ca and P digestibility, as the diets were not limiting in Ca and P content.

6.2.3. Susceptible phytate and the effect of pelleting on phytase activity

Different feed ingredients from different countries of origin were collected and susceptible phytate content of those feed ingredients were analysed in vitro with a huge variation in susceptible phytate values recorded. The cereal grains contained higher susceptible phytate content compared to oilseeds and oilseed meals. The findings of the present study contradict the general perception of only 35% dietary phytate of all feed ingredients being hydrolysed by phytase (Selle & Ravindran, 2007), which may underestimate its value as the majority of feed ingredients have a higher susceptible phytate content in the present study. Therefore, it would be more accurate to formulate diets by taking into consideration the susceptible phytate level of the batches of ingredients present in that particular diet. This would give a more accurate level of plant-derived P available to the bird and reduce the need for excess inorganic P addition. There are, however, large variations in the amount of the total and susceptible phytate levels among feed ingredients from different country of origin and also between batches of ingredients within the same country. And the NIR technology can be used as a more practical tool to facilitate the rapid and cheap analysis of phytate in different batches of feed ingredients. NIR is used widely to find out the nutritional profile of a feed which is very cheap and very practical compared to laboratory analysis. Future studies will be required to analyze the total and susceptible phytate content using NIR technology.

HIGHPHY wheat has the great potential to improve nutrient availability especially phytate P as it contains higher phytase activity which facilitates maximum hydrolysis of phytate of cereal

grains and therefore makes phytate P and other nutrients more available to poultry. Plant-derived phytase activity is rarely considered in feed production as little plant-derived phytase maintains activity after heat processing. However, this investigation indicates phytase activity levels of nearly 58% are retained after pelleting HIGHPHY wheat.

6.3. Critique of the project

Some weaknesses during the trials and laboratory analysis of the samples were identified which need to be considered during future studies.

- Feed spillage occurred during the first trial as the feed system (open trough) encourages the chicks to climb in the feeders, spill feed and dust bathe. Therefore, measures for feed intake for this study where inaccurate, so that overall efficiency could not be measured.
- The diets for the first trial were supplied by the industrial sponsor without any marker in the diet. The lack of marker meant that digestibility measures could not be related to the performance measures for the trial to create a deeper understanding of bird response. Also, it was not possible to analyse the hydrolysis effect of phytase or digestibility. An analysis of acid insoluble ash of the diets as an alternative to external marker was performed but without added AIA, the measured values were so small they were not meaningful to use as a marker.
- The pH of the different part of the broiler gastrointestinal tract were not measured in first broiler trial, as there was a later realization as to the importance of this and how much it is related to the effect of phytase.

6.4. Recommendations for practical application

1. Though there was little difference in feed conversion ratio when superdosing phytase, the birds did have the higher tibia ash percentage compared to other treatments. Therefore, superdosing can be beneficial for the better bone mineralisation, which may be useful to reduce lameness in heavier weight broilers or broiler breeders. The economic loss from culling broiler breeders due to lameness is huge and superdosing has great potential to reduce the economic loss by reducing lameness in broiler breeder. Moreover, Superdosing adds more benefit than cost and it is affordable.

2. In the present study there was no significant effect of superdosing on caecal microbiota which suggests that superdosing may not have a beneficial effect on gut health problems and not have any detrimental effect on broiler gut health. Though it was hypothesised that superdosing might increase the nutrient utilization and thus affect the microbiome in the gut.

3. The WBP: tibia P ratio was not constant in broilers across the ages assessed, which indicates that tibia P might not be a reliable estimate of WBP content. Typically, use of tibia phosphorous as an excellent indicator of whole body phosphorous content didn't show in the present study. When modelling relationships between tibia phosphorous and whole body phosphorous, scientists should keep in mind that there is not always a linear relationship between those two parameters.

4. Incorporating HIGHPHY wheat in broiler diets improves the amino acid digestibility of some essential amino acids, which indicates the potential use of HIGHPHY wheat in organic poultry production. In organic production external phytase and additional amino acids use is not permitted because the production of phytase involves biogenetic modification. Improved AA

digestibility didn't reflect in growth because the dets were synthetic diets (not standard) which were designed to find out digestibility ad were not expected to increase growth.

5. Organic production currently has a higher phosphorous use which relies on mineral phosphates and leads to high P excretion. So, using HIGHPHY wheat may offer a route to improve environmental footprint of both N and P.

6. While making diets, poultry producers should take into consideration the susceptible phytate content of the feed ingredients when calculating phytase inclusion, as susceptible phytase varies greatly from country to country and batches of feed ingredients. In that case rapid testing of phytate in feed batches using NIR might have the potential and easier way compared to that of costly and laborious lab analysis.

7. After pelleting HIGHPHY wheat there is sufficient residual phytase activity left which makes this novel wheat variety viable to be used as a practical feed ingredient in commercial poultry production.

6.5. Future research and potential impact of the project

- Feed ingredients with high mature grain phytase activity need to be assessed to make more sustainable diet for poultry, while also evaluating how the phytase activity is affected by processing of these particular feed ingredients.
- In future experiments, broilers can be reared in challenging environments, such as with reduced level of inorganic P in the diet or with other environmental stressors, to assess the effect of phytase in conditions which are suboptimal, while evaluating superdosing effects.
- The susceptible phytate content of different feed materials need to be assessed regularly because they vary from origin to origin and also batches of feed. This requires a rapid, cost-effective analysis method to be developed.
- The value of HIGHPHY wheat in broiler diets should be holistically considered in future research or commercial use. HIGHPHY wheat not only improves the amino acid digestibility but also it improves the phytate P utilization, so it has potential for use in organic production to mitigate environmental effects and improve nutritive content of the diets.

6.6. Conclusions

Continuous research to maximize efficacy of phytase is essential to both reduce usage of our diminishing global reserves of mineral phosphate and also to reduce environmental impact of excess nutrients from poultry manure. Superdosing of phytase has the great potential to improve phytase efficacy and to reduce the use of mineral phosphate in the diet. The findings of the present studies provide a better understanding of using phytase in broiler diets at higher levels and contribute to the current knowledge of using phytase as a feed additive. The results of the present study demonstrate that superdosing of phytase improves the relative concentrations of inositol phosphate within broiler digestive tract but that didn't always translate in improving broiler performance, which doesn't add up to the suggestion that improvement in inositol phosphate will improve body weight gain in broiler. But the finding is still very important as increased concentration of myoinositol indicated improved phytate breakdown and thus we can reduce the addition of inorganic P in the diet which will reduce environmental impact. The results of the present study also gave a novel indication of no effect of phytase superdosing on whole body phosphorus content of broiler and tibia P is not a reliable estimate of WBP content. It also adds further understanding of superdosing doesn't have any effect on caecal microbiome of broiler.

The results of HIGHPHY wheat trial provides information on using new novel feed ingredients in broiler diets to efficiently utilize intrinsic feed phytase. It demonstrates the beneficial effect of this novel wheat on improving the essential amino acid digestibility, which is a novel finding and adds weight to the suggestion that feed ingredient with high mature grain phytase activity improves the nutrient availability in broiler. This result also proves the potentiality of using

HIGHPHY wheat in organic broiler production. And this study also provides an insight into how susceptible phytate content vary from origin and batches of feed ingredients and the important of considering phytate susceptibility in a di*et* alongside phytase dose and efficacy.

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Appendix

Appendix A: Trial 1- Effect of sex on Whole body mineralisation (g/kg) of broiler at different ages

Parameter	Male	Female	P-value
D7 WBCa:P	0.577	0.677	0.172
D14 WBCa:P	1.028	1.135	0.003
D21 WBCa:P	1.077	1.102	0.399
D28 WBCa:P	0.539	0.516	0.350
D35 WBCa:P	1.070	0.968	0.027
D7WBCa	6.863	8.199	0.290
D7WBP	10.938	11.694	0.245
D14WBCa	12.067	15.519	0.012
D14DBP	11.690	13.352	0.012
D21WBCa	13.067	13.771	0.403
D21WBP	12.065	12.347	0.539
D28WBCa	10.390	9.376	0.292
D28WBP	18.810	17.806	0.273
D35WBCa	10.546	8.675	0.012
D35WBP	9.673	8.829	0.015

Appendix B: Trial 1- Weekly Bone Ash % (% dry bone weight) of male and female broilers

Weekly bone ash %	Male	Female
D0 Tibia ash %	23.283	23.753
D7 Tibia ash %	37.944	38.876
D14 Tibia ash %	37.841	38.207
D21 Tibia ash %	38.833	39.934
D28 Tibia ash %	33.322	36.448
D35 Tibia ash %	35.660	36.142
D0 Femur ash %	21.541	22.660
D7 Femur ash %	37.430	39.967
D14 Femur ash %	36.718	37.482
D21 Femur ash %	38.760	37.593
D28 Femur ash %	33.342	37.035
D35Femur ash %	34.172	33.641
D0 Feet ash %	13.002	13.894
D7 Feet ash %	16.556	16.024
D14 Feet ash %	15.569	16.009
D21 Feet ash %	15.617	15.919

APPENDIX C: Polynomial analysis of performance parameters of trial 1

Model	Dependant Variable	Age	R^2	P-value		
				X	X^2	X^3
Linear	FI	0-21	0.006	0.642		
		21-35	0.069	0.121		
		0-35	0.049	0.1915		
	BWG	0-21	0.007	0.621		
		21-35	0.017	0.442		
		0-35	0.049	0.429		
	FCR	0-21	0.146	0.021		
		21-35	0.008	0.589		
		0-35	0.047	0.203		
Quadratic	FI	0-21	0.006	0.939	0.909	
		21-35	0.069	0.953	0.945	
		0-35	0.049	0.986	0.929	
	BWG	0-21	0.007	0.849	0.880	
		21-35	0.019	0.842	0.793	
		0-35	0.006	0.840	0.813	
	FCR	0-21	0.146	0.761	0.912	
		21-35	0.013	0.667	0.698	
		0-35	0.051	0.625	0.700	
Cubic	FI	0-21	0.006	0.939	0.909	NA
		21-35	0.069	0.953	0.945	NA
		0-35	0.049	0.986	0.929	NA
	BWG	0-21	0.007	0.849	0.88	NA
		21-35	0.019	0.842	0.793	NA
		0-35	0.006	0.84	0.831	NA
	FCR	0-21	0.146	0.761	0.912	NA
		21-35	0.013	0.667	0.698	NA
		0-35	0.051	0.625	0.7	NA