Improvement of phytase efficacy in poultry through dietary fat supplementation

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

The work in Chapter 3 of the thesis has appeared in publication as follows:

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I contributed in generating ideas for the experiment and was responsible for preparing experimental design and protocol, conducting the experiment, conducting the samples and data analysis and the write up of the paper. The contributions of other authors were of supervision nature: H. Miller contributed in generating ideas for the experiment, critically reviewed and gave suggestions to improve on the experimental design, experimental protocol and the article.

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I contributed in generating ideas for the experiment and was responsible for preparing experimental design and protocol, conducting the experiment, conducting the samples and data analysis and the write up of the paper. The contributions of other authors were of supervision nature: Miller,H. and Kuehn, I., contributed in generating ideas for the experiment, critically reviewed and gave suggestions to

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Abstract

Phosphorus in phytate is largely unavailable to chickens unless they are provided with dietary phytase. Phytase was shown to increase phytate degradation in the crop and proventriculus-gizzard and very little phytate degradation occurred in the duodenum-jejunum or ileum. These previous investigations were conducted on chickens fed corn based diets but not with wheat based diet. Increase in digesta passage or mean retention time (MRT) along the gastrointestinal tract could enhance phytase efficacy as the prolonged reaction time between substrates and phytase may further facilitate phytate dephosphorylation. Dietary fat and fibre supplementation have been shown to influence intestinal MRT in chickens therefore it is expected that inclusion of both dietary fat and fibre could be manipulated to further improve phytase efficacy in broiler chickens. Three experiments and preliminary studies were conducted: 1) to assess the effect of methodology on estimation of phytate hydrolysis and P digestibility in young broilers and 2) to investigate whether or not dietary fat and fibre could further improve phytase efficacy in broilers fed diets containing wheat by delaying digesta MRT. In the preliminary study, in order to facilitate analysis of limited digesta samples, a sample preparation protocol involving H_2SO_4 digestion for sequential analysis of titanium and other minerals in feed and digesta was established. It was also found that blood protein interfered with the colorimetric analysis of P and myo-inositol, thus deproteinization is required. Continuous feeding prior to sampling was recommended to obtain adequate amount of digesta for digestibility related analyses. In the first study, feeding duration (1 h or 5 h) and 1 h feeding followed by feed withdrawal and refeeding prior to sampling significantly affected the concentration of phytate, inositol phosphates and measured degradation and digestibility of phytate-P in different segments of gastrointestinal tract, which could lead to overestimation or underestimation of degradation and digestibility values. Therefore, it is also recommended to collect digesta samples at least 3 h after the start of photoperiod and avoid sampling 4 h prior to dark period when lighting program is applied. In the second study, 5% fat inclusion had no significant effect on growth performance, while phytase supplementation at 1500 FTU/kg improved feed intake (FI) and body weight gain (BWG). However, interactions between both factors additively increased FI from 804g to 1,221g, BWG from 630 g to 904 g, ileal phytate degradation by 44% and ileal P digestibility by 17%. Adding cellulose as filler in pellet-crumbled diet has diluted the nutrient concentration in 5% fat diet that eventually led to poorer FCR. However, phytase supplementation eliminated the negative effect of nutrient dilution by improving performance of chickens as good as those fed those fed 5% fat diet without cellulose addition. In the third study, a combination of 1500 FTU/kg phytase supplementation with 5% fat increased crop digesta MRT and improved phytate-P degradation compared to with 1% fat, providing evidence for the role of fat in improving phytase efficacy in broilers. This thesis has shown the importance of methodology standardization in estimating phytate hydrolysis and P digestibility and the role of dietary fat and fibre in improving phytase efficacy in broilers.

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List of Abbreviations

ANOVA	Analysis of variance
Ca	Calcium
CD	Conversion degree
D	Dark
d	Day
DL	Dephosphorylation level
DM	Dry matter
EBW	Empty body weight
EDTA	Ethylenediaminetetraacetic acid
FR	Feed refeeding
FTU	Phytase activity unit
FW	Feed withdrawal
g	Gram
GIT	Gastrointestinal tract
GLM	Generalised linear model
h	Hour
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HPIC	High-pressure ion chromatography
HPLC	High-performance liquid chromatography
ICP-OES	Inductively coupled plasma optical emission spectrometry
$InsP_3$	Inositol-3-phosphate
$InsP_4$	Inositol-4-phosphate
$InsP_5$	Inositol-5-phosphate
$InsP_6$	Inositol-6-phosphate
InsPs	Inositol phosphates
KOH	Potassium hydroxide
L	Light
M	Molar
min	Minute

ml	Millilitre
mM	Millimolar
MRT	Mean retention time
nm	Nanometer
P	Phosphorus
PP	Phytate phosphorus
SLF	Submerged liquid fermentation
SSF	Solid state fermentation
TCA	Trichloroacetic acid
TDM	Total dry matter
Ti	Titanium
TiO_2	Titanium dioxide
x g	Centrifugal force

Chapter 1
Literature Review

1.1. Introduction

Phosphorus has a critical role in cell metabolism, bone development and bone mineralization in animals and P deficiency could hinder the animals from attaining their optimum genetic potential in growth and feed efficiency as well as skeletal development. In the case of monogastric animals, particularly poultry, the P is present in plant-based feed ingredients and approximately 70% of it is in the form of phytate-P. Phytate is able to reduce the bioavailability of other nutrients, particularly calcium, proteins and starch in poultry. Due to the low availability of phytate-P to poultry, dietary P (inorganic P) is added to poultry diets in order to meet the P needs of the bird. It is a common practice in the commercial environment to overfeed dietary P exceeding the published requirement (Applegate & Angle, 2008). Calcium phosphate, a phosphate supplement in poultry diet, is produced from rock phosphate which is non-renewable and was predicted to decline in its production in near future (Ulrich and Schnug, 2013). Besides having higher cost of feeding due to inclusion of expensive inorganic P in poultry diet, the excess of soluble P from overfeeding of dietary P and undigested phytate-P may increase the total and soluble P content of excreta and litter. This will lead to a higher risk of environmental pollution (Angle *et al.*, 2002). Thus, the efficient approach to reduce feed cost and ecological hazards posed by P is by reducing or avoiding the use of inorganic P supplementation and increasing the bioavailability of phytate-P and other nutrients in the feed. This can be done via degradation of phytate using a phytase enzyme (inositol hexaphosphate phosphohydrolase) and solubilization of phytate at pH values below 4.5 (Graham *et al.*, 2009).

Several excellent reviews have appeared covering the use of microbial phytase in poultry nutrition in relation to P utilisation, the extra phosphoric effects of phytase and factors affecting phytase efficacy in phytate hydrolysis (Maenz, 2001; Kornegay, 2001; Selle and Ravindran, 2007; Selle *et al.*, 2010; Greiner and Konietzny, 2011).

This chapter will provide a brief review of *in vitro* phytate hydrolysis, phytate hydrolysis in the gastrointestinal tract (GIT) of broilers and factors that affect phytate hydrolysis which could be manipulated to enhance phytase efficacy in broilers.

A number of factors have been identified to influence the efficacy of phytases and these factors could be manipulated in order to enhance the positive responses in broilers. However, one has to understand the avian digestive system, the condition of the GIT of broilers and nature of phytate and phytases in order to formulate strategies in improving phytase efficacy.

1.1.1. Gastrointestinal tract of chickens

Avian digestive system consists of elementary canal from beak/mouth to cloaca/vent, liver and pancreas. Feed enters beak into mouth and passes esophagus into crop, proventriculus and ventriculus/gizzard. Digesta in the gizzard is discharged through the pylorus into the duodenum, passes into lower small intestine (jejunum and ileum) and finally faecal materials is discharged at the cloaca via the large intestine or colon. The retention time (RT) of feed/digesta is the time taken for feed to retain in each segment of gut before pass through the GIT.

As chickens consume feed, saliva in the mouth moistens the feed for easy swallowing and also initiates digestion. The moistened feed moves along the esophagus, a flexible tube that connects mouth to crop and from crop to proventriculus. Swallowed feed and water are temporarily stored in the crop, an out-pocketing of oesophagus (Figure 1.1).Usually the pH of crop content is similar or close to the pH of the feed which is between 4.5 to 5.9 and digesta remains in the crop for 30 to 40 min (Svihus 2011a). As the feed enters the proventriculus or true stomach, the pH of the digesta is drastically reduced to as low as pH 2.0 due to secretion of hydrochloric acid by submucosal glands in the proventriculus. The proventriculus also secretes a pepsinogen for protein digestion.

The feed materials, then passes into the gizzard or ventriculus. Gizzard is also referred as mechanical stomach and functions as ‘the teeth’ to grind, mash and mix feed consumed by chicken. Gizzard also has a thick lining that serves as a protecting layer for the muscles against highly acidic feed from proventriculus. The feed is ground and mixed in the gizzard and the pH of gizzard content was reported to be

highly variable, ranging from 1.9 and 4.5, with an average value of 3.5 (Svihus2014). After 30 to 60 min in the proventriculus and gizzard, the digesta is peristaltically moved into the small intestine.

Small intestine starts from the exit from gizzard to the end of small intestine at the junction of ileum, caeca and colon. It consists of duodenum, jejunum and ileum. Duodenum can be easily recognised by loop structure with pancreas in the middle, while jejunum and ileum is separated at Mickel's diverticulum (Figure 1.1). Meckel's diverticulum is a residual tiny sac after the yolk sac is taken into the navel cavity of the embryo right before hatch. Digesta passes through duodenum within a short time (<5 min, Chee *et al.*, 2010) but the pH of duodenal content is increased from 2.0 to more than 6.0 as duodenum receives pancreatic juice that contains sodium bicarbonate for hydrochloric acid neutralization. Besides stopping the activity of gastric pepsin and stomach acid, it also prepares a more conducive environment for further enzymatic digestion by pancreatic amylase, trypsin, chymotrypsin, elastase and lipase. Duodenum also receives bile, a detergent for lipid digestion and absorption of fat-soluble-vitamins, from the liver via gall bladder.

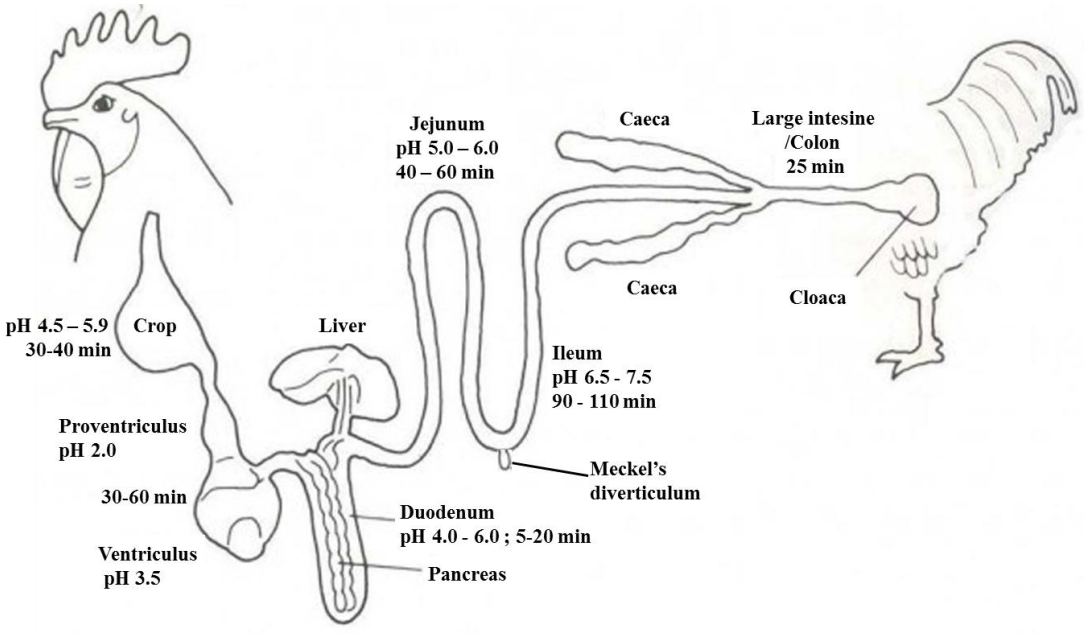


Figure 1.1 pH and retention time of the digestive content along gastrointestinal tract of chicken.

The digesta then enters the jejunum, the site for digestion and absorption of fat, starch and protein with mean RT of 40 to 60 min (Weurding *et al*., 2001, Chee *et al*., 2010). Although the length of the ileum is about the same as that of the jejunum (Svihus, 2001), digesta passage through the ileum is slower ranging between 90 and 110 min (Weurding *et al*., 2001, Chee *et al*., 2010). In addition to some major nutrient digestion and absorption, minerals and water are thought to be mainly absorbed in the ileum. In the lower small intestine, digesta pH is less variable in comparison to those in the crop and gizzard with an average pH of 6.5 to 7.5 (Svihus, 2011a). About 18% of ileal digesta dry matter enters caeca, 2 blind pouches that located between end of ileum and before large intestine, and the rest passes into large intestine. According to Svihus *et al*. (2013), only finely-ground particles and/or soluble, low molecular weight and non-viscous molecules enter caeca. In caeca, some of the water and electrolytes in digestive waste are reabsorbed. Uric acid and soluble carbohydrates are fermented by caecal microorganisms and produce urea, volatile fatty acids and vitamins but very little (11%) of these nutrients are absorbed and available for chicken (Svihus *et al*., 2013). Pasty caecal content or dropping leaves caeca and enters large intestine 2 to 3 times a day. Ileal digestive waste and caecal dropping pass through short large intestine or colon, which is the last water re-absorption site, before being discharged via cloaca. In cloaca, the digestive waste and caecal dropping are mixed with the waste of urinary system (urates) to form faecal material coated with while pasty material (uric acid crystals).

1.1.2. Early development of digestive tract

For fast growing meat chicken, early development of digestive tract particularly intestine and accessory organs including pancreas, liver and gall bladder, is critical to ensure adequate nutritional supply to support its growth. The size of GIT and digestive organs of very young chick limit feed intake. Immediate access to feed and water stimulates the growth and maturation of proventriculus, gizzard and small intestine until their maximal growth is achieved between 7 to 10 days post-hatched. Villus height, villus surface and crypt depth are also increased with age (Ravindran, 2003). Functionality of pancreas and brush border of the small intestine is also undergo rapid maturation in the first week of life with the increase of lipase, amylase,

protease and other digestive enzymes and secretions. Delaying the access of post hatched chicks to feed was shown to reduce the rate of intestinal development and decrease the growth performance (Yegani and Korver, 2008). Body weight of 7 days old chicks was shown to influence the growth performance of 42 days old broiler (Lilburn, 1998) and body weight of 7 day old chicks is depend the quality and amount of feed intake. Therefore, providing the right form of nutrients at the right time to chicks after hatch is critical.

1.1.3. Feed components, particle size and form

Carbohydrate, protein, fat, mineral, vitamin and water are the main components of the feed that provide energy and nutrients to the chickens. Energy is required for performing normal body functions such as walking, breathing and maintaining general metabolism of the chicken. Nutrients are necessary for the development of muscle and bone, reproduction and health. Corn, wheat and barley are some of the major carbohydrate sources that used in chicken feed as the source of energy. Most carbohydrates in the form of starch are readily digestible in young chicken. However, other type of carbohydrate known as non-starch polysaccharide or fibre is less digestible and some of them are resistance to digestive enzyme such as cellulose. Fibres such as β-glucan and arabinoxylan become antinutrients that interfere with other nutrient utilization by creating viscous environment that reduces the nutrient absorption in the small intestine and consequently detrimentally affects the performance of the chicken.

Fat on the other hand provides higher calories per gram carbohydrate compared to cereal grains. Usually fat is added into feed to increase the overall energy concentration of the feed and also required for the utilization of fat-soluble vitamins by chicken. Fat is also included in the chicken feed as a source of linoleic acid, the essential fatty acid for growth and reproduction (Balnave, 1981). Besides reducing grain dust during feed processing, fat also improve the palatability of the feed. The presence of fat in digesta that enters duodenum greatly accelerate fat digestion by stimulating cholecystokinin secretion that consequently regulates pancreatic juice and bile secretion and stimulates bile release from the gall bladder (Ravindran *et al*., 2016). Supplementation of fat also reduce food passage along the

digestive tract that warrant better digestion and nutrient absorption (Mateos and Sell, 1980). Nevertheless, digestibility of fat is considered low in very young chicken due to the low lipase activity and inadequate bile secretion but fat digestion increased with age (Ravindran, 2003).The digestibility of saturated fat with high amount of free fatty acids is even lower than unsaturated fat (Leeson, 1993), thus source of fat such as soybean oil and corn oil are more readily digestible compared to tallow or animal fat.

Soybean meal, corn gluten meal and fishmeal are among the common protein sources used in chicken feed to provide amino acids required for body protein synthesis and construction of body tissues including muscles, nerves and cartilage. Dietary lysine and methionine are among the essential amino acids that must be supplied in the feed because inadequate of both amino acids leads to a significant drop in health and productivity of chicken flock.

Minerals, inorganic component of the feed are usually categorised as macrominerals and microminerals based on the amount required by chicken. Limestone and oyster shells are the source of calcium and dicalcium phosphate is the source for both calcium and phosphorus. Calcium and phosphorus are essential for the formation and formation of bones. Deficiency of either Ca or P in young chick results in abnormal bone development. Sodium, potassium, chloride and magnesium are required for maintaining osmotic balance and pH in the body of the chicken. Macrominerals also essential in many metabolic and muscle functions. Microminerals, also called as trace elements, are essential for metabolism in the body and usually functions as a part of other larger molecules, for example Fe with haemoglobin and iodine with thyroxine. Although present in most feed ingredients, supplementation of trace elements including copper, iodine, iron, manganese, selenium and zinc in the feed is necessary in order to ensure adequate intake by the chicken.

Vitamins are required for normal body function, growth and reproduction of chicken and inadequate intake of one or more vitamins can increase susceptibility of chicken to diseases and syndromes. Fat-soluble vitamins are A, D, E and K, meanwhile water-soluble vitamins are vitamin C and B (includes niacin, biotin and riboflavin). Some of these vitamins are produced by intestinal microorganisms of chicken or by the chicken itself and some of them present in the feed ingredients but

supplementation of a vitamin premix in the feed is still necessary to ensure all vitamins are available for chicken in adequate amount.

In the effort to provide a highly digestible feed to post hatched chicks, ingredients with high energy and high protein content are used and consequently the feed contains very low crude fibre (CF). On the other hand, chickens fed on diets with very low CF were found to have poor GIT development (Gonzales-Alvarado *et al*., 2008). According to Mateos *et al*. (2012), about 2-3% of insoluble dietary fibre (DF) with particle size of more than 1 mm is required to stimulate a proper development of GIT. The examples of insoluble DF are oat hulls, sugar beet pulp, soybean hulls and sunflower hulls. Although microcrystalline cellulose is also insoluble DF, it does not affect the development of GIT and growth performance. It was thought to be due to its lack of physical structure (Jimenez-Moreno *et al*., 2010). On the other hand, fine DF may accumulate in gizzard and reduce the passage of digesta through the GIT. The passage rate of digesta containing fine DF may be further reduse with the presence of coarse fibre (Mateos *et al*., 2012).

Use of whole wheat in chicken diet also contributes in development of GIT, particularly gizzard and improves ileal nutrient absorption (Hetland *et al*., 2002). Increase in pancreas and liver secretions may also contribute in more efficient digestion and absorption of diet with whole wheat compared to diet ground wheat which indicates the role of wheat form (whole or ground) in development of digestive functions (Svihus *et al*., 2004). According to Amerah *et al*. (2015), the effect of feed particle size is more critical on growth performance and development of GIT when chickens fed on mash feed compared to pelleted feed. Chickens fed on mash feed with coarse particle size have higher body weight gain and large size of gizzard than those fed on fine particle size. Meanwhile, pelletization of the feed reduces the performance gap between different particle sizes. On the other hand, less developed gizzard was observed in chickens fed on crumble-pellet feed although the growth performance was better than those fed on mash feed. During pelletizing, feed ingredients are finely ground, mixed and mechanically pressed to form ‘artificial grain’. Besides having well balanced nutrients, pelleting improves palatability, reduces selective feeding and feed wastage and consequently improves feed intake, body weight gain and feed conversion ratio. However, the form of pellet feed is more readily disintegrate as feed enters the mouth and further breaks up due to grinding

activity by gizzard muscles. The retention time of small particle feed in the gizzard is shorter than coarse feed and less mechanical stimulation leads to size reduction of digestive organs.

The positive effect of pelleting observed in chickens basically due to the improved ingestion of feed but the effects may vary depending on the quality of feed ingredients and how the feed is processed. Pelleting improves the quality of low or medium energy feed with better pellet quality. In addition, benefit of pelleting toward growth is more pronounced in chickens fed low energy pelleted feed compared to high energy mash feed (Trevidy, 2005).

Although early development of digestive system in chicken is critical in ensuring adequate nutrients intake that necessary for growth, the process of feed digestion is not 100% efficient. This is due to the lack of or very low activity of specific enzymes in the GIT to break down certain components of the feed. The presence of indigestible anti-nutritive factors in most of feed ingredients such as NSPs and phytate also interfere the digestion process. Feed enzymes are used to reduce the adverse effects of anti-nutritive factors by breaking down fibre or phytate and improving the availability of nutrients including starch, amino acids, calcium and phosphorus from the feed.

Broiler chicken is one of the fastest growing farmed animals and presently the chicken can reach a weight of approximately 2kg in 35 days while consuming only 3.2kg of feed. broiler growth rates have increased about 300% over the last 50 years of production intensification and genetic selection. Leg disorders are considered as welfare issues and have been a considerable problem to broiler industry. Leg-bone abnormalities can lead to severe walking problems and lameness and even death due to starvation and dehydration. There is evidence that indicates the importance of early nutrition on chick development to prevent initiation of bone defect in a very young chicken (Fleming, 2008). Rickets is commonly observed in young broilers, which indicates deficient or imbalanced in dietary calcium, phosphorus, or vitamin D_3. Tibial dyschondroplasia, characterized by abnormal cartilage mass in the proximal head of the tibiotarsus, is another common disease related to imbalance calcium: phosphorus ratio. The diet phosphorus level is relatively higher than

calcium. The deformation of bone can be prevented or alleviated with the balance supply of Ca and P at 2:1 ratio in the starter diet.

1.1.4. Phytate

Phytate is a salt form of phytic acid or myo-inositol-6-phosphates (InsP6), which is a major storage form of P and myo-inositol in mature plant seed. It was reported that the biosynthesis of phytate begins soon after flowering and during development of seed (Bohn *et al*., 2008; Woyengo and Nyachoti, 2011). Almost all P that taken up by the root of a crop is translocated to the seed and usually they are more than required for cellular function. Phytate is synthesized via 2 possible pathways that is via lipid independent pathway (Raboy, 2009) and lipid dependent pathway to yield phytic acid (Loewus, 2002) as shown in Figure 1.2 shows both possible pathways of phytate biosynthesis.

Phytate is present in major plant feedstuffs in the form of phytate-mineral complexes, mainly Mg-K-phytate (Shears and Turner, 2007; Lott *et al*., 2000) (Figure 1.3). Due to its chemical structure, InsP6 is capable of binding to positively charged molecules and nutrients to form a very stable insoluble complex, which is a main antinutritive character of InsP6.It has 12 protons or reactive sites at 6 phosphate groups that are located on the 6-carbon myo-inositol ring, 6 of these are strongly acidic (pKa values of 1.5-2.0), 3 have pKa values between 5.7 and 7.6 and the other 3 are basic (pKa values greater than 10) (Woyengo and Nyachoti, 2011). Besides Mg and K, phytate can also forms complexes with other minerals including Cu, Zn, Ni, Co, Mn, Fe and Ca but Cu and Zn have the strongest binding affinity (Cheryan, 1980).

Besides the type of cations being involved during the formation of phytate-mineral complexes, phytate solubility is also dependent on pH. Phytate is more soluble at lower pH values than at higher pH values. Na-phytate and K-phytate are soluble at all encountered pH values. Zn-phytate, Ca-phytate and Mg-phytate are insoluble above pH values of 4.3, 5.5 and 7.2, respectively. Meanwhile, Fe-phytate is insoluble at pH below 3 but slowly becomes soluble as pH value increases above 4 (Selle and Ravindran, 2007; Kumar *et al.*, 2010).

With reference to other monogastric animals, a study on phytate solubility in pigs conducted by Schlemmer *et al*. (2001) has shown that InsP6 and less phosphorylated inositol phosphates (lower InsPs) have different levels of solubility, where the lowest InsPs has the highest solubility. It was also observed that the complexes formed between lower InsPs with minerals were proportionately weaker, suggesting that the hydrolysis of phytate to at least InsP3 is necessary in order to achieve higher solubility of both minerals and InsPs at higher pH (Cowieson *et al.*, 2011).

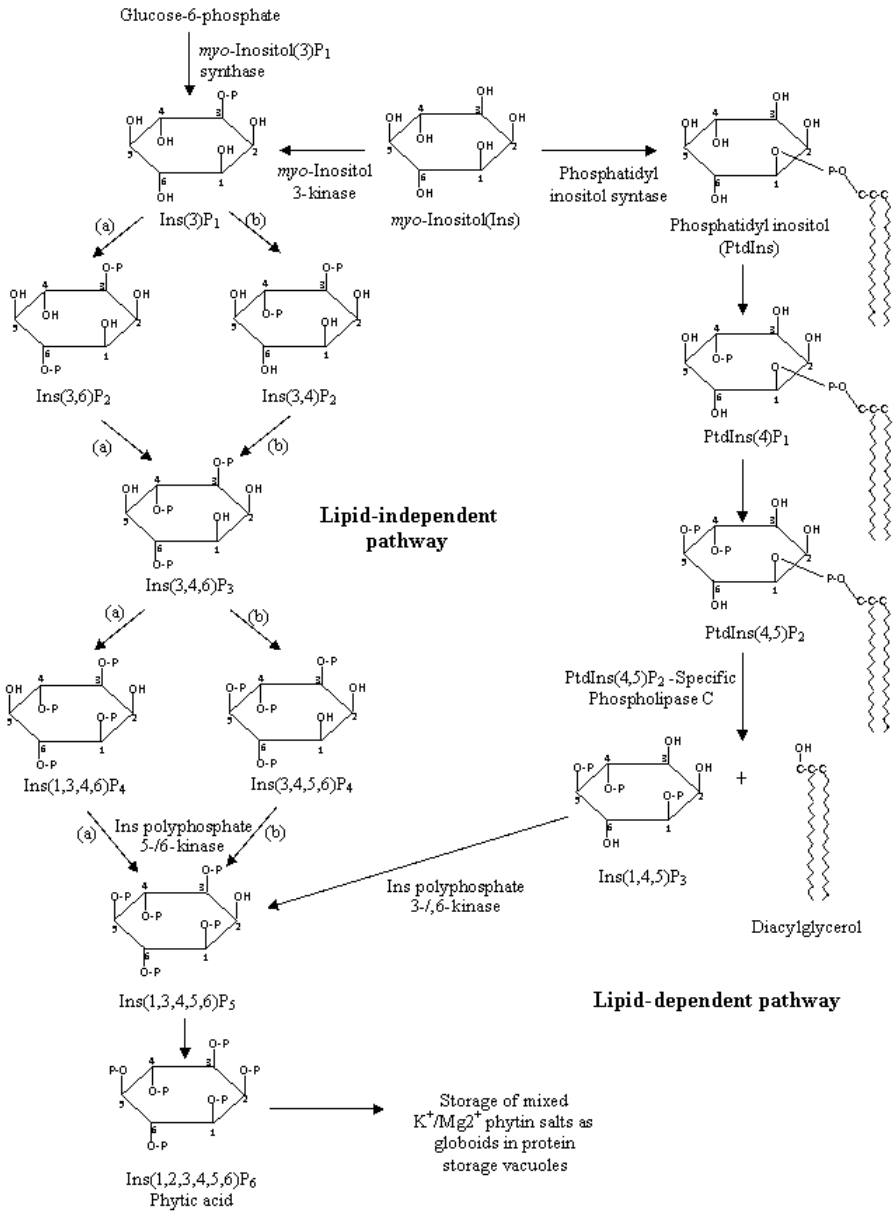


Figure 1.2 Phytic acid synthesis. Lipid indipendent pathway (left) as in; (a) *Dictyostelium* (slime mold), (b) *Spirodela polyrhiza* (duckweed). Lipid dependent pathway as in Arabidopsis

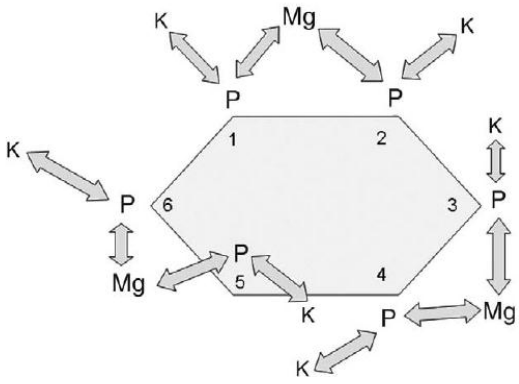


Figure 1.3 Schematic diagram of the Mg-K-phytate axis, as proposed by Lott *et al.* (2000). Adopted from Bedford and Partridge (2001)

Although most of the phytate-P present in feed ingredients is in the form of Mg- and K-phytate, Ca-phytate was shown to play a crucial role in phytate-P bioavailability in poultry. The formation of Ca-phytate along the GIT of chicken was assumed to be important and was described in detail by Selle *et al. (*2009). It is known that pH has a significant effect on the solubility of phytate and pH of the digesta ranges from acidic (pH 4.5) in the crop of poultry, highly acidic (pH 2.5) in the stomach (proventriculus) and gizzard (ventriculus) to approaching neutrality in the small intestine (pH 6.5) (Figure 1.2). It was reported that the formation of Ca-phytate complexes occurs over a broad pH range, between pH 2 to pH 12, and the affinity of phytate for Ca ion increases with pH (Marini *et al*., 1985). The Ca-phytate complexes were soluble below pH 4 and became insoluble at pH above 5 (Grynspan and Cheryan, 1983). It was concluded that pH 5 and pH 5.4 are critical for the formation of Ca-phytate complexes from phytate/ InsP6 and InsPs (InsP1 to InsP5), respectively (Selle *et al*., 2009).

Due to its strong negative charge, phytate is also capable of interacting with proteins to form binary phytate-protein complexes or ternary mineral-phytate-protein complexes (Figure 1.4), which was thought to be mediated by pH values in the gut (Singh, 2008). Binary phytate-protein complex forms at pH values below 5 through strong electrostatic interaction between negatively charged phytate and positively charged protein, which only redissolves at pH values below 3 (Dersjant-Li *et al.*, 2014). At pH above 7, the formation of completely insoluble ternary mineral-phytate-protein complexes occurs via involvement of multivalent cations in the interaction (Dersjant-Li *et al*., 2014). Phytate can also directly bind with starch via hydrogen

bonds or indirectly via proteins associated with starch or with starch (Truong *et al.*, 2015) and fat in the presence of Ca to form faecal soap fat (Atteh and Leeson, 1984).

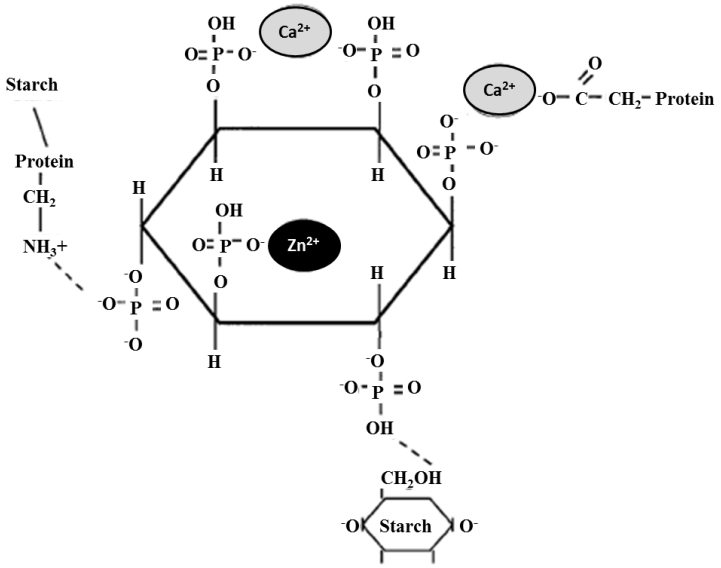


Figure 1.4 Structure of phytate and possible bonds (after Thompson, 1988). Adopted from Kornegay (2001)

The presence of these complexes along the GIT will lead to a reduction in amino acids, energy and nutrients digestibility and reduction of protein functionality, especially with regards to a number of digestive enzymes in chickens (Selle and Ravindran, 2007).

1.1.5. Phytase

Phytate, an antinutrient that presents in most plant based feed ingredients but yet it is useful source of nutrients. Degradation of phytate could leads to the release of P and other bound nutrients and makes them available for absorption in poultry. The most practical and effective approach to breakdown phytate in poultry feed is via supplementation of exogenous microbial phytases. Phytases (inositol hexaphosphate phosphohydrolase), a subgroup of phosphatases, are important enzymes that capable of hydrolyzing of phytate and release phytate bound phosphorus (phytate-P) in stepwise manner. Phytases have been identified in plants, microorganisms and in some animal tissues. In most feed ingredients, the detected phytase activity is considerably low and due to a narrow pH spectrum of activity, plant phytases become less effective at a low pH, more susceptible to proteolytic digestion and

thermal destruction during feed processing. Animal phytases are produced by intestinal mucosa and are largely found in the duodenum. Their productions seem to be regulated by the presence of phytate and products of its hydrolysis (Woyengo and Nyachoti, 2011). Meanwhile, microfloral phytases, produced by the hindgut microbial population, are considered to have some influences in further hydrolyzing the undigested phytate-P as concluded by Kerr *et al*. (2000). In comparison to the intestinal mucosa phytases, microfloral phytases are more capable of hydrolyzing phytate-P.

Another type of phytases, that is microbial phytases are currently used in animal feeds and these enzymes are produced using fungi (e.g *Aspergilus niger*), bacteria (e.g. *Escherichia coli*) and yeast. Most of them are derived by over expressing phytase genes in a suitable host. Phyzyme XP (Danisco Animal Nutrition, Marlborough, UK), Ronozyme P (DSM Nutritional Products, Basel, Switzerland), Quantum (AB Vista, Marlborough, UK), Natuphos (BASF, Germany) and OptiPhos (Phytex LLC, Sheridan, IN) are some of the commercially available phytases for animal feed. The biochemical properties and relative catalytic performances of selected commercialized phytases were reported by Menezes-Blackburn *et al.* (2015) (Table 1.1).

There is a series of quality criteria that should be fulfilled before an enzyme product can be considered as “ideal” for animal feed applications. Those criteria include the effective release of phytate phosphate in digestive tract, product stability during feed processing and storage and the economical production of the enzyme product. Many studies have demonstrated the differences between commercially available phytases and the understanding of those differences is necessary to secure the optimal animal performance. However, the properties of each phytase product could be used as guidelines on protential functionality in animal feeds and digestive systems.

Table 1.1. Enzymatic properties of selected commercially available phytases (Adopted from Menezes-Blackburn *et al.*, 2015)

Trademark	Quantum	Quantum Blue	PhyzymeXP	AxtraPHY	Ronozyme Hiphos	Ronozyme NP	Natuphos
Supplier	AB Vista	AB Vista	Danisco	Danisco	Novozyme/ DSM	Novozyme/ DSM	BASF
Recommended dosage for broiler	500 FTU/kg	500 FTU/kg	250 FTU/kg	250 FTU/kg	500 FTU/kg	1500 FTU/kg	500 FTU/kg
Donor organism	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*	*Buttiauxella sp.*	*Cytobacter braakii*	*Peniphora lycii*	*Aspergillus niger*
Production organism	*Trichoderma reesei*	*Trichoderma reesei*	*Schizosaccaromycespombe*	*Trichoderma reesei*	*Aspergillus oryzae*	*Aspergillus oryzae*	*Aspergillus niger*
pH range (80% of optimal activity)	4.0 – 5.0	3.5 – 5.0	3.0 – 5.0	3.0	3.0 – 4.5	4.5 – 5.5	4.5 – 5.5
Phytase activity at pH 3.0[a] (%)	92.5	101.3	82.8	235.1	145.7	12.5	64.2
Phytase activity at pH 7.0[a] (%)	0.8	2.2	1.7	.5	.6	7.8	7.0
K_M (µM) for phytate at pH 5.0 and 37°C	228	142	285	272	364	75	35
K_{cat} (s^{-1}) for phytate at pH 5.0 and 37°C	1545	1821	1327	1054	1478	1532	318
K_M (µM) for phytate at pH 3.0 and 37°C	257	178	302	311	427	98	142
K_{cat} (s^{-1}) for phytate at pH 3.0 and 37°C	1012	1274	984	768	1061	824	170
Residual activity (%) (pH 3.0, 37°C, 45 min)							
Wihout pepsin	95	98	92	87	93	58	81
With 3000 U pepsin	93	98	92	85	92	34	47
Optimal ionic strength (mMNaCl)	50-100	50-200	100-200	50-200	50-200	50-200	50-600
Phytase activity needed to achieve % of maximum reachable values							
50% reduction of $InsP_6$	326 (0.95)	319 (0.86)	395 (0.92)	323 (0.92)	445 (0.87)	418 (0.80)	586 (0.89)
50% reduction of $InsP_6$	2194 (0.84)	955 (0.94)	1159 (0.93)	952 (0.94)	2200 (0.97)	2606 (0.89)	2398 (0.93)

[a] phytase activity at pH 5.5 was taken as 100%; [b] Values (U/kg) obtained by non-linear fit of the observed data; coefficient of determination in parentheses.

Greiner and Konietzny (2011) have extensively described the classes of phytases based on the initiation sites for dephosphorylation of phytate and proposed the phytate degradation pathways based on four major classes of phytases (Figure 1.5). The phytases that preferentially initiate phytate dephosphorylation in position 3 are called 3-phytase, whereas, 6-phytases initially remove phosphate residue from position 6. Currently, these two classes of phytases are extensively studied in poultry nutrition. Other classes of phytases were also reported (2-phytases, 4-phytases and 5-phytase) with initiation site of phytate dephosphorylation at position 2, 4 and 5 respectively. 3-phytases were predominantly observed in microorganisms such as *Aspergillus* sp., *Bacillus* sp., whereas 6-phytases were found in *E.coli* and *P. lycii*. Most of plant phytases are 4-phytases except lupin (3-phytase) and lily pollen (5-phytase) while 2-phytases (intracellular phytases) were observed within animal cells. There is no report on the existence of 1-phytases as yet.

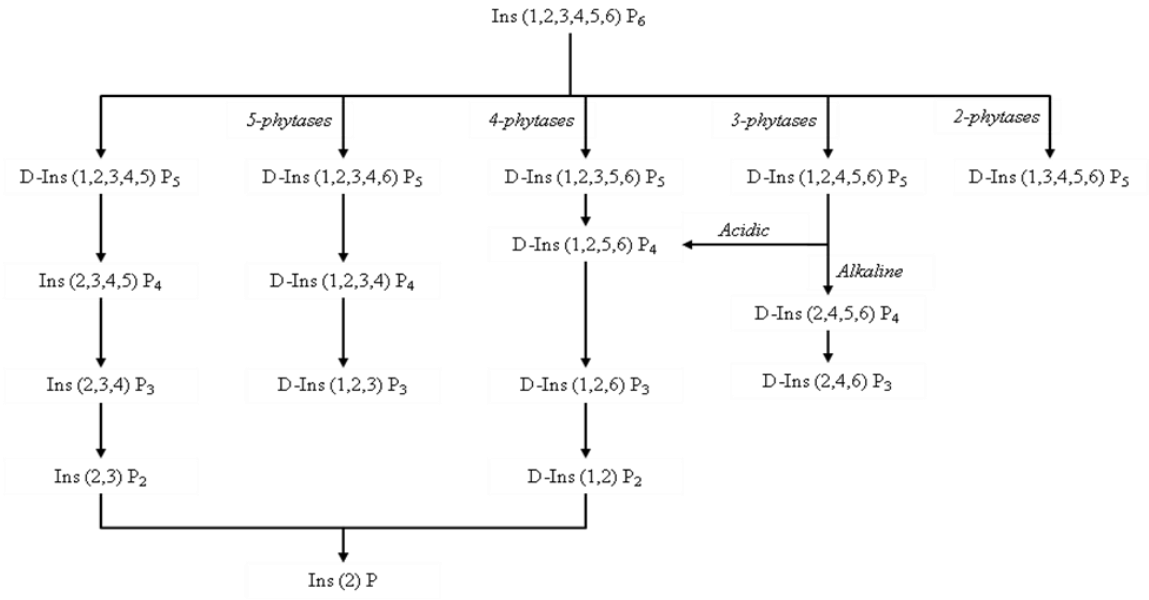


Figure 1.5 Major phytate degradation pathways for the four classes of phytase (Adapted from Greiner and Konietzny, 2011)

The use of microbial phytase in poultry nutrition particularly in broiler chickens has been extensively reviewed by Selle and Ravindran (2007). pH and digesta retention time (RT) were recognized as important physiological factors that contribute in determining the efficacy of phytase mainly on phytate degradation and P

utilization in the digestive tract of poultry. In addition, the mechanism of how phytate interacts with nutrients along the GIT of chickens that leads to the reduction of nutrient utilization has been described by Adeola and Cowieson (2011). The authors also proposed that degradation of phytate as quickly as possible in crop, proventriculus and gizzard is required in order to minimize the antinutritional effects of phytate. The role of exogenous phytases, mainly microbial origin, in limiting the passage of phytate esters (up to $InsP_3$) from gizzard into the duodenum rather than degrading phytate into inositol and free phosphate were emphasised.

The main sites for phytate degradation by microbial phytases are in the crop and gizzard of the chicken. Yu *et al*. (2004) and Onyango *et al.* (2005) demonstrated that microbial phytase activity was relatively higher in the crop, gizzard and followed by duodenum, jejunum and ileum. Beside favourable pH conditions and low protease activity, phytase from *P.lycii* with pH optimum between4.0–4.5 (Augspurger *et al*., 2003) and that from *E.coli* with pH optimum of 4.5 (Onyango *et al*., 2005) would be more active in the crop than in the proventriculus and gizzard of the chicken. In addition, with high solubility of phytate at pH 4 and below, more phytate would be degraded by the time digesta reached the proventriculus and gizzard (Zeller *et al*., 2015a). Phytases with high stability toward proteolytic activity of the gastric region and broader range of optimal pH (2.5 to 6.0) may be able to continue degrading more phytate in the proventriculus and gizzard (Walk *et al*., 2014), and perhaps beyond the upper part of the GIT. Higher phytase activity was also detected in duodenum compared to in the ileum (Onyango *et al*., 2005), therefore higher degradation of residual phytate from gizzard in duodenum would be expected than in ileum.

Despite very low phytase activity was detected in the diet and in each section of GIT of broilers, P digestibility was considerably high in broilers fed on low P and Ca diet without phytase supplementation (Onyango *et al.,* 2005, Tamim *et al*., 2004). Contribution of intestinal phytase, phosphatases and intrinsic plant phytase to the utilization of phytate-P has demonstrated by Morgan *et al*. (2015). Higher capacity of P utilization via intestinal phytase was further induced by low dietary P (Abudabos, 2012). Degradation of undigested phytate was also observed beyond ileo-caecal junction indicating the contribution of gut microbiota in phytate degradation. In addition, Zyla *et al*. (2004) demonstrated that further phytate hydrolysis on the myo-inositol rings is achievable by adding nonspecific phosphatases to diet containing

exogenous phytases at 500 FTU/kg or higher. Nevertheless, due to short RT and small range of optimum pH in each part of GIT, further improvement of exogenous phytase efficacy on phytate degradation and P utilization is considered challenging.

1.1.6. *In vitro* phytate hydrolysis

The increase of *in vitro* phytate hydrolysis by addition of phytase into diets has been reported by several authors. *In vitro* phytate-P hydrolysis was affected by type of phytase, level of added Ca and duration of incubation (Tamim *et al*., 2004). Fungal phytases (mainly 3-phytase) and bacterial phytases (particularly 6-phytase) were shown to have different pH for optimal phytate-P hydrolysis. Although 3-phytases have 2 peaks of activity i.e at pH 3 and pH 5.5, concentration of P released by 6-phytase at pH around 4.5 was significantly higher than the concentration of P released by 3-phytase. Tamim *et al*. (2004) also demonstrated that increasing concentrations of added Ca increased phytate-P hydrolysis by both 3-phytase and 6-phytase at low pH (pH 2.5) but reduced phytate-P hydrolysis at higher pH (pH 6.5) and as low as 0.1 % added Ca negatively influenced phytate-P hydrolysis by both types of phytase. However, in this study, sodium phytate was used as the source of phytate.

Whereas Menezes-Blackburn *et al*. (2015) compared the *in vitro* performance of commercial 6-phytases and 3-phytases using ground wheat that contained inactivated intrinsic phytase, as the source of phytate. In their study, Menezes-Blackburn *et al*. (2015) demonstrated that different phytase products behaved differently and suggested that the *in vitro* degradation system cannot be used to rank phytases based on their bioefficacy as the generated results did not precisely reflect their performance in animals but these systems can be useful in evaluating the potential beneficial of phytases as feed supplement.

The efficacy of phytases on the hydrolysis of phytate and its lower InsPs depends on pH, phytate matrix and phytate origin. (Brejnholt *et al*., 2011). The degree of $InsP_6$ and $InsP_5$ hydrolysis by endogenous and recombinant wheat phytases was the highest at pH 4 but reduced as pH increased beyond or lower than pH 4. Microbial phytase, otherwise, has a broader pH range (pH 3 to 5) in hydrolysing

$InsP_6$ and $InsP_5$. Brejnholt *et al*. (2011) also demonstrated that microbial phytases reduced more than 75% of $InsP_6$ and $InsP_5$ in wheat, corn, barley and rapeseed meal but recombinant wheat phytases showed a variable degree of $InsP_6$ and $InsP_5$ hydrolysis in these feed materials. Brejnholt *et al*., (2011) also suggested that in order to fully compare and determine the efficacy of potential feed phytases dose response feeding trials in animal species of interest would need to be performed.

The efficacy of phytase in feed materials under feed processing conditions was investigated by Denstadli *et al*. (2006). In this study, the effect of moisture level, temperature and incubation time on the *in vitro* hydrolysis of wheat and soybean originated phytate by microbial phytase was investigated. Concentration of $InsP_6$ was reduced by between 76 and 86% when a phytase supplemented feed mixture was moistened to 45% (ml/g DM) during incubation at 45^{o}C for 45 min. However, moisture levels of more than 45% were not studied due to possible complication in extrusion process. Denstadli *et al*. (2006) also suggested that phytase supplementation at 2500 FTU/kg was not sufficient to completely hydrolyse $InsP_6$ in feed materials.

According to Zyla *et al*., (2004), complete dephosphorylation is achieved when each phytate molecule of feed ingredients is degraded and digested and residual concentration of phytate measured after digestion is negligible. In *in vitro* hydrolysis of phytate, the extent of phytate hydrolysis is measured as the percentage of total P removed from the feed and this criterion of measurement is called dephosphorylation level (DL). Another criterion for *in vitro* phytate hydrolysis, suggested by Zyla *et al*. (2004), is the measure of dialysed free P (%) due to phytate degradation over the total P removed from the feed, which is also called the conversion degree (CD). Measure of CD indicates the extent of phytate hydrolysis on the myo-inositol rings, which also relates to changes in free myo-inositol concentration. In their study, Zyla *et al*. (2004) demonstrated that addition of a nonspecific phosphatase to a diet containing 3-phytase or 6-phytase at 500 FTU/kg or higher did not increase DL but increased CD. Zyla *et al*. (2004) also found that without addition of nonspecific phosphatases, the concentration of free myo-inositol was not further enhanced by 3-phytase or 6-phytase.

The efficacy of phytase produced via solid state fermentation (SFF) in *in vitro* digestion in comparison to phytases produced via submerged liquid fermentation (SLF) was reported by Wu *et al*. (2004b). The release of dialysed P in wheat-soy and corn-soy diets was higher with SSF phytase compared to SLF phytases, suggesting the presence of unknown factors in SSF phytase that enhanced phytate-P hydrolysis. Besides phytase, other enzymes such as cellulase, amylase, xylanase, glucanase and other side activities were detected in SSF phytase while in SLF phytase, usually side enzyme activities were undetectable (Sabu *et al*., 2003).

Another approach in determining the *in vitro* efficacy of phytase is by measuring the solubility of P and Ca. At gastric pH (pH 2.75 to 3.5), phytate, P and Ca are soluble and have high solubility value. While at small intestine pH (6.5), phytate, P and Ca are likely to precipitate and low solubility values of P and Ca are expected (Selle *et al*., 2009). The effect of phytase and any factors affecting phytase efficacy, therefore, would change the value of solubility of P and Ca. Walk *et al*. (2012a) found that phytase increased P solubility of corn based and soybean meal based diets, and further increased with the addition of dicalcium phosphate. Addition of phytase together with limestone reduced P solubility. Walk *et al*. (2012b) also demonstrated phytase supplementation increased P and Ca solubility in both diets containing either adequate or lower P and Ca. Solubility of P and Ca were further increased by phytase with smaller particle size diets. Morgan *et al*. (2014b) showed that an increasing phytase level increased P and Ca solubility in soybean meal, rapeseed meal and diets containing high level of soybean meal or rapeseed meal in both gastric and small intestine phase. The effect of phytase on P and Ca solubility in diets was also shown to be affected by pH and Ca to P ratio. Morgan *et al*. (2014b) demonstrated a strong relationship between *in vitro* and *in situ* evaluation of phytase efficacy on P and Ca solubility. Morgan *et al*. (2014b) suggested that *in situ* data on animal response toward supplemental phytase is still required in spite of the successful prediction of phytase efficacy via *in vitro* assays.

1.1.7. Phytate hydrolysis in the chicken's gastrointestinal tract

In contrast to *in vitro* degradation, phytate hydrolysis in the GIT of chickens could be affected not only by supplemental phytase and the intrinsic phytase in feed materials

but also by intestinal mucosa phytase and phytase produced by the intestinal microflora.

Zeller *et al*. (2015a) reported the efficacy of phytases in different sections of the digestive tract of broilers. Supplementation of either 3- or 6- phytase at 500 FTU/kg increased phytate hydrolysis in the crop but did not significantly affect phytate hydrolysis in the duodenum/ jejunum or ileum. However, the presence of $InsP_5$ and $InsP_4$ isomers at high concentration in the duodenum/jejunum and ileum in the 6-phytase supplemented group was assumed to be due to further activities of the enzymes in lower gut sections. Hydrolysis of phytate by 3-phytase was also reported to be higher compared to 6-phytase in the crop (Zeller et al, 2015a). In the gizzard, phytate hydrolysis was almost complete in chickens fed with low P and Ca diets when phytase supplementation level was increased up to 1500 FTU/kg (Walk *et al*., 2014). The gizzard concentrations of $InsP_3$, $InsP_4$ and $InsP_5$ were also reduced resulting in a higher concentration of inositol.

Considerably high phytate hydrolysis was detected in duodenum/jejunum and ileum, ranged from 55% to 59% and 67% to 74%, respectively, when chickens fed on low Ca and P diets without dietary phytase (Zeller *et al*., 2015a; 2015b). About 91% of caecal phytate hydrolysis was also reported (Zeller *et al*., 2015a). These studies demonstrated the occurrence of phytate hydrolysis in small intestine and caeca was either due to endogenous mucosa phytase, the activity of the intestinal microbiota or combination of both endogenous phytase and intestinal microbiota activities. When the level of Ca and P in the diet was increased, the level of phytate hydrolysis in small intestine was reduced, which may be due precipitation of insoluble and undegradable Ca-phytate at the pH of the small intestine (Zeller *et al*., 2015b). However, the possibility of the decrease level of endogenous or microbiota-related phytase due to higher Ca and P level is undeniable.

At a very high level of supplemental phytase, although it did not result in a complete ileal hydrolysis of $InsP_6$, more complete ileal hydrolysis of $InsP_5$ was observed. In addition, ileal hydrolysis of $InsP_6$ and $InsP_5$ due to phytase supplementation at 12,500 FTU/kg was not affected by increasing level of Ca and P in the diet (Zeller *et al*., 2015b). Therefore, intestinal mucosa phytase and phytase produced by the intestinal microflora did contribute in ileal phytate hydrolysis but it is

negatively affected by level of Ca and P in the diet. Very high level of phytase supplementation, however, could be used to further improve ileal phytate hydrolysis.

1.1.8. Ileal P digestibility

Supplementation of phytase has been shown to improve P digestibility and selected studies on the effect of phytase on ileal P digestibility are listed in Table 1.2. Most of the studies were conducted with corn-soybean meal diets, however, Leytem *et al*. (2008) and Rutherfurd *et al*. (2004) evaluated phytase efficacy on ileal P digestibility in other feed materials beside corn. Leytem *et al*. (2008) reported that ileal P digestibility of corn, wheat, oat and barley based diets containing low P were 56%, 57%, 65% and 64%, respectively. With addition of *A. niger* 3-phytase at 1000 FTU/kg, the percentage increase in ileal P digestibility was small, ranging between 1 and 5%. Rutherfurd *et al*. (2002), on the other hand, reported higher ileal P digestibility in feed materials including corn, soybean meal, wheat, rice bran and rapeseed meal increasing by from 6 to 17% when supplemented with 750 FTU/kg 6-phytase of *P. lycii* expressed in *A. oryzae*. Wu *et al*. (2004) found 22.7% increase in ileal P digestibility after supplementing wheat-soybean-canola diets with 1000 FTU/kg phytase produced via solid state fermentation (SSF). Besides demonstrating the difference in ileal P digestibility in different feed materials in response to phytase supplementation, these studies also showed that the type of phytase and level of P and Ca influenced P digestibility.

The efficacies of 3-phytase and 6-phytase on ileal P digestibility were reported by Tamim *et al*. (2004) and Camden *et al*. (2001). At 500 FTU/kg supplementation level, Tamim *et al*. (2004) found lower ileal P digestibility with 6-phytase compared to 3-phytase. Whereas, Camden *et al*. (2001) reported the opposite finding, which is ileal P digestibility with 6-phytase was higher than those with 3-phytase when supplemented at the same phytase and phytate level in a similar corn-soybean meal diet to that used by Tamim *et al*. (2004). The obvious difference between the two studies was the level of Ca. The concentration of Ca in the diet used by Tamim *et al*. (2004) was very low, i.e. 1.7 g/kg compared to 8.0g/kg in the work of Camden *et al*. (2001). Thus, Ca is another contributing factor in determining the extent of ileal P digestibility due to phytase supplementation.

An increase in dietary Ca from 4.5 g/kg to 9.0 g/kg in a corn- soybean meal diet supplemented with 500 FTU/kg significantly reduced the ileal P digestibility from 54.6% to 40.2 % (Walk *et al*., 2012c). Similarly, Amerah *et al*. (2014) found a higher percentage increase in ileal P digestibility with lower Ca content (5.1 g/kg) compared to that with 13.0 g/kg Ca when 1000 FTU/kg phytase was added to corn- soybean meal diet. In contrast, Walk *et al*. (2012d) found a reduction in ileal P digestibility in 16 d old broilers fed on phytase supplemented diets containing 6.1 g/kg total P and either 6.4 or 10.3 g/kg Ca. Tamim *et al*. (2004), on the other hand, demonstrated otherwise where by increasing dietary Ca from 1.7 to 6.5 g/kg, ileal P digestibility in 24 d old broilers was increased. In other word, extremely low Ca content in diets may promote the hydrolysis of phytate by intestinal mucosa phytase resulting in high ileal P digestibility and then with phytase supplementation, the ileal P digestibility was further improved. High Ca content in the diet may promote precipitation of phytate which hinders the activity of intestinal phytase but dietary phytase enhanced phytate hydrolysis and ileal P digestibility although it was not as high as achieved with the low Ca diet.

The content of P in the diet was also shown to influence ileal P digestibility (Ravindran *et al*., 2000). By increasing the non-phytate-P from 2.3 to 4.5g/kg in wheat based diets, addition of phytase increased ileal P digestibility from 39.9% to 70.2% and 46.8%, respectively. The increase in ileal P digestibility was greater in the diet with lower P content compared to those with adequate non phytate-P. Ravindran *et al*. (2000) also showed that increasing the concentration of dietary phytic acid in a phytase supplemented diet did not affect ileal P digestibility.

Increasing the supplementation level of phytase increased ileal P digestibility as demonstrated by several authors. Camden *et al*. (2001) reported increasing ileal P digestibility of 54.7, 59.7, 61.5 and 66.2% when graded level of phytase i.e at 0, 250, 500 and 1000 FTU/kg were added to corn-soybean meal diets. Similarly, Kiarie *et al*. (2015) found ileal P digestibility ranged from 39.5 to 69.1% when phytate doses from 0 to 1000 FTU/kg were added. On the other hand, Rutherfurd *et al*. (2004) showed the increase of ileal P digestibility was limited as the phytase level was increased from 500 FTU/kg to 750 FTU/kg. Differences in the effects of phytase on ileal P digestibility in these studies were due to different Ca: total P i.e. 2.3:1 (Kiarie *et al*., 2015) versus 1.2 (Rutherfurd *et al*., 2004) and different source of phytase i.e

fungal origin (Camden *et al*., 2001) and bacterial origin (Rutherfurd *et al*., 2004). Chung *et al*. (2013) also demonstrated the effect of fungal phytase was greater in increasing ileal total P absorption at supplemental level higher than 1000 FTU/kg when compared to bacterial phytases.

Table 1.2 Results on the effect of supplemental phytase on ileal P digestibility by several authors

Phytase type	Phytase inclusion (FTU/kg)	PP,P, Ca (g/kg)	Ileal P digestibility		%* above control	Diet	Gender/ Age	References
			Without phytase	With phytase				
6-phytase *E. coli*	500	-, 5.2, 7.5	57	64	7.0	Corn-SBM	mixed, 25 d	Zeller *et al*. (2015a)
6-phytase *E. coli*	12,500	-, 4.4, 6.0	52	71	19.0	Corn-SBM	mixed, 24 d	Zeller *et al*. (2015b)
6-phytase *Buttiauxella* in *T. reesei*	500	-. 4.8, 7.9	39.5	64.9	25.4	Corn SBM	Male, 22 d	Kiarie *et al*. (2015)
	2,000	-. 4.8, 7.9	39.5	68.2	28.7			
6-phytase *Buttiauxella* in *T. reesei*	1,000	3.2, 5.1, 5.1	55.1	71.9	16.8	Corn SBM	Male, 21 d	Amerah *et al*. (2014)
6-phytase *T. reesei*	500	-, 4.2, 6.0	40.6	51.7	11.1	Corn SBM	Male, 34 d	Kuhn *et al*. (2012)
6-phytase *T. reesei*	500	-, 6.0, 4.5	40.8	54.6	13.8	Corn SBM	Male, 22 d	Walk *et al*. (2012c)
	2,500	-, 6.0, 4.5	40.8	67.5	26.7			
	500	-, 6.0, 9.0	28.8	40.2	11.4			
	2,500	-, 6.0, 9.0	28.8	52.7	23.9			
3-phytase *A. niger*	1,000	-, 10.6, 9.5	35.81	45.87	10.06	Corn-SBM	Male, 35 d	Nourmohammadi *et al*. (2012)
6-phytase *Peniiphora lycii*	1,000	3.2, 5.6, 8.9	53.3	59.9	6.6	Corn-SBM	Male, 22 d	Rutherfurd *et al*. (2012)
	2,000	3.2, 5.6, 8.9	53.3	61.9	8.6			
3-phytase *A. niger*	1,000	2.5, 5.7, 8.4	56	59	3.0	Corn-SBM	Male, 21 d	Leytem *et al*. (2008a)
	1,000	3.0, 5.9, 8.1	57	62	5.0	Wheat-SBM	Male, 21 d	Leytem *et al*. (2008a)

Continue…

Table 1.2 Results on the effect of supplemental phytase on ileal P digestibility by several authors

Phytase type	Phytase inclusion (FTU/kg)	PP,P, Ca (g/kg)	Ileal P digestibility		%* above control	Diet	Gender/ Age	References
			Without phytase	With phytase				
….continue								
3-phytase *A. niger*	500	3.1, 4.0, 1.7	67.9	75.1	7.2	Corn-SBM	Male, 24 d	Tamim *et al.*, (2004)
	500	3.1, 4.0, 6.5	29.4	50.4	21.0			
6-phytase *P. lycii*	500	3.1, 4.0, 1.7	67.9	71.0	3.1			
E. coli 6-phytase	1,000	2.4, 4.3, 7.7	69.3	73.9	4.6	Corn-SBM	Male, 22 d	Onyango *et al*. (2005)
6-phytase *P. lycii*	500	-,6.5, 7.8	53.14	63.04	9.9	Corn-SBM	Male, 28 d	Rutherfurd *et al*. (2004)
phytase *A. niger* (solid state fermentation)	500	2.9, 5.7, 8.3	43.3 (35.4)	60.9 (58.8)	17.6 (23.1)	Wheat SBM, Canola	Male (Female), 42 d	Wu *et al*. (2004)
	1,000	2.9, 5.7, 8.3	43.3 (35.4)	66 (60)	22.7 (24.6)			
	1,500	2.9, 5.7, 8.3	43.3 (35.4)	67.7 (68.8)	24.4 (33.4)			
	2,000	2.9, 5.7, 8.3	43.3 (35.4)	73.5 (67.4)	30.2 (32.0)			
6-phytase *P. lycii* in *A. oryzae*	750	15.6, 15, 0.7	29	46	17.0	Rice bran	Male, 35 d	Rutherfund *et al*. (2002)
3-phytase *Bacillus*	500	3.1, 5.9, 8.0	54.7	61.5	6.8	Corn-SBM	Male 21 d	Camden *et al*. (2001)
		3.1, 5.9, 8.0	54.7	63.1	8.4			
6-phytase *A. ficuum* in *A. niger*	400	3.7, 6.3, 10.4-15.7	42.2	58.2	16.0	Wheat	Male, 25 d	Ravindran *et al*. (2000)
	800	7, 6.3, 10.4-15.7	42.2	58.4	16.2			

*% above control – significant at $P<0.05$

Based on the concept and the kinetics of complete dephosphorylation of phytate proposed by Zyla *et al*. (2004), more effective phytate degradation may occur within the GIT of the chicken when adding phytase at a dose higher than the dose recommended by the manufacturers, which is normally at 500 FTU/kg for broilers (Cowieson *et al*., 2006b). At similar ratio of Ca: total P, an increase of

between 8.6 to 12.5 % in ileal P digestibility was observed when 2000 to 2500 FTU/kg phytase was added in corn-soybean meal diet compared to non-phytase supplemented diet. (Rutherfurd *et al*., 2012; Walk *et al*., 2012c). A high increase (30%) in ileal P digestibility was observed with the addition of 2000 FTU/kg SSF phytase into a wheat-soybean meal-canola based diet (Wu *et al*., 2004b). Kiarie *et al*. (2015) also observed a high increase (28.7%) in ileal P digestibility when 2000 FTU/kg phytase was added to a corn-soybean diet with higher Ca: total P ratio (2.3:1). Zeller *et al*. (2015b) also reported a higher percentage of ileal P net absorption at extremely high phytase dosage (12,500 FTU/kg) compared to manufacturer's recommended dose (500 FTU/kg).

1.1.9. Growth performance and bone mineralization

Besides ileal P digestibility, growth performance and bone ash are the most commonly used evaluations of phytase efficacy in chickens. According to Selle and Ravindran (2007), hundreds of investigations on the microbial phytase evaluation on growth performance have been reported. Phytase supplementation of diets with inadequate P have been shown to improve growth performance (Selle and Ravindran, 2007) and sometimes bone ash particularly tibia ash was reported together with the growth parameters due to its greatest sensitivity to changes in mineralization (Angle *et al*., 2006). A comprehensive review of phytase supplementation effects on growth performance, bone characteristics and bone mineralization has been reported by Khan *et al*. (2013). They summarized that addition of microbial phytases enhanced the performance of growing broilers and at an increased level of supplementation further improved feed efficiency, nutrient utilization, bone growth and mineral retention.

Supplementation of phytases beyond 500 FTU/kg in broiler diets has been gaining interest, particularly from the commercial sector, due to two main reasons. Firstly, the use of unconventionally high phytase doses apparently improved growth performance due to elimination of anti-nutritional effects of phytate up to below $InsP_3$ in the diets and the generation of myo-inositol as a potential growth promotant in broilers (Cowieson *et al*., 2011). By taking advantages of nutrient release by phytase particularly amino acid and energy, more effective feed could be formulated

using nutrient matrix values for phytase which leads to further reduction of feed costs (Selle, 2008).

Shirley and Edward (2003) demonstrated the benefits of supplementing superdoses of phytase, as high as 12,000 FTU/kg, on growth performance of broilers and numerous similar investigations have been reported since. According to Cowieson *et al*. (2011), phytase supplementation higher than 2500 FTU/kg was considered as superdosing level. Later, the term of 'superdosing phytases' was redefined as supplementation of phytase at 1500 FTU/kg or more either with or without the application of the phytase nutrient matrix (Cowieson *et al*., 2013). Selected studies on the effect of superdosing on growth performance and bone ash of broilers are listed in Table 1.3. It can be summarised that supplemental phytase from 1500 FTU/kg to as high as 40,000 FTU/kg benefited growth performance particularly weight gain and feed efficiency in growing broilers as early as 14 d old. Walk *et al*. (2012c; 2012d) reported a non-significant effect of phytase supplementation at 2500 and 5000 FTU/kg on growth performance but tibia ash was significantly increased indicating phytase benefited broilers via bone mineral retention.

The impacts of phytase on protein/amino acid availability and energy utilization have been extensively reviewed by Selle and Ravindran (2007) and Selle *et al*. (2010). In general, phytase supplementation improved both total and individual ileal amino acid digestibility in broilers and the effect of phytase on amino acid digestibility was more pronounced in wheat based compared to corn based diets. Selle and Ravindran (2007) also deduced that increases in fat, protein and starch digestibility accumulatively contributed to the positive impact of phytase supplementation on energy utilization in broilers.

1.1.10. Approaches for enhancing phytase efficacy

The amount of phytate in the diets, amount of phytases added, the type of phytases used and the gut pH are several factors that influence the efficacy of phytase in poultry. The phytate content varies among commonly used feed ingredients (Table 1.3) and the level of phytate degradation also varies in different feed ingredients as

demonstrated by Leske and Coon (1999). Whereas, Graham *et al*. (2009) reported that at different supplemental levels of Quantum phytase, ranging from 250 FTU/kg to 1000FTU/kg, the release of P has significantly increased by 30%. It was also reported that the release of P by 500 FTU/kg using different type of phytases (i.e. derived from *P. lycii*, *A. niger* and *E. coli*) are significantly different, with 0.67 and 1.30 kg/t, respectively. The efficacy of phytases in hydrolyzing phytate-P could also be affected by dietary level of inorganic P and calcium, dietary endogenous phytase activity and non-starch polysaccharides (NSPs) (Woyengo and Nyachoti, 2011). NSPs are poorly digested by poultry particularly young ones and the utilization of other nutrients is also low due to encapsulation of nutrients including phytate in the plant cell walls. The soluble NSPs, on the other hand, is capable of increasing the viscosity of the digesta, consequently reduce nutrient digestibility and absorption (Bedford 2000, Bedford and Schulze, 1998). When the insoluble NSPs is hydrolyzed, phytate and other nutrients are released from the cell wall and become accessible to and digested by phytase and other digestive enzymes. Breakdown of soluble NSPs partially reduces digesta viscosity and consequently increases the absorption of nutrient that liberated by phytase.

In order to improve the efficacy of phytase in broiler chickens, the above mentioned factors were considered and further studied by several authors (Deepa *et al*., 2011; Jozefiak *et al*., 2010; Manangi and Coon, 2008) and reviewed by Selle *et al*. (2010). However, based on two broiler studies (Camden *et al*., 2001; Tamim *et al*., 2004) and one layer study (van der Klis *et al*., 1997), Selle *et al*. (2010) suggested that the efficacy of phytase in broiler chickens could be further improved by increasing the digesta retention time in crop. By delaying the intestinal retention rate of food/digesta, this may facilitate phytate dephosphorylation through extending the time of exposure of substrates to phytases and phytate-P absorptive sites thus improve the nutrient utilization efficiency in chicken (Mateos and Sell, 1980).

Table 1.3 Effect of superdosing on growth performance and bone ash of broilers

Phytase type	Phytase inclusion (FTU/kg)	PP,TP, Ca (g/kg)	Performance[1] Without phytase	Performance[1] With phytase	Sig[2]	Bone ash (%)[3]	Diet	Age (day)	References
6-phytase *E.coli* in *T. reesei*	1500	-, 6.2, 9.7	1.24; 0.92; 1.36	1.25; 0.95; 1.32	- - -	-	Corn/SBM	21	Walk *et al*. (2013)
	1500	-, 6.2, 9.7	2.97; 4.80; 1.68	3.01; 4.90; 1.65	- - -	Tibia* 51.7 vs 52.6	Corn/SBM	42	Walk *et al*. (2013)
6-phytase *E.coli* in *T. reesei*	1600	-, -, 0.8	1.30; 1.70; 1.37	1.76; 2.02; 1.32	* * *	-	Corn/SBM	28	Campasino *et al*. (2014)
	1600	-, -, 0.8	2.90; 4.70; 1.62	3.26; 5.40; 1.58	* * *	-	Corn/SBM	42	Campasino *et al*. (2014)
6-phytase *Gireobacterbrakii* in *A.oryzae*	2000	3.2, 5.6, 8.9	20.40; 43.10; 1.40	32.40; 45.30; 1.40	* - -	Toe* 11.4 vs 12.8	Corn/SBM	21	Rutherfurd *et al*. (2012)
6-phytase *E.coli* in *T. reesei*	2500	-, 6.1, 5.8	0.62; 0.82; 1.34	0.63; 0.82; 1.31	- - -	Tibia* 46.6 vs 49.4	Corn/SBM	21	Walk *et al*. (2012c)
6-phytase *E.coli*	2500	-, 6.8, 14.8	36.00; 47.00; 0.76	41.10; 54.00; 0.75	* * -	-	Corn/SBM	28	Pirgozliev *et al*. (2008)
3-phytase *A. ficuum*in*A.niger*	4000	-, 5.5, 9.0	52.30; 103.40; 1.97	62.10; 104.70; 1.69	* - *	Tibia* 34.3 vs 46.9	Corn/SBM/canola	42	Taheri and Taherkhani (2015)
6-phytase *E.coli* in *Schizosaccharomyces pombe*	5000	2.7, 4.0, 7.0	0.18; 0.32; 1.84	0.27; 0.41; 1.55	* * *	-	Corn/SBM	25	Manangi and Coon (2008)

Continue….

Table 1.3 Effect of superdosing on growth performance and bone ash of broilers

Phytase type	Phytase inclusion (FTU/kg)	PP,TP, Ca (g/kg)	Performance[1] Without phytase	Performance[1] With phytase	Sig[2]	Bone ash (%)[3]	Diet	Age (day)	References
….continue									
6-phytase *E.coli* in *Pichia pastoris*	5000	-, 6.4,8.2	0.47; 0.63; 1.35	0.47; 0.62; 1.31	- - -	-	Corn/SBM	16	Walk *et al*. (2012d)
3-phytase *A. ficuum* in *A niger*	10,000	-, 3.8, 7.5	0.25; 0.62; -	0.34; 0.70; -	* - -	Tibia* 29.7 vs 44.6	Corn/SBM	21	Augspurger and Baker (2004)
6-phytase *E.coli*	10,000	-, 3.8,7.5	0.25; 0.62; -	0.34; 0.70; -	* * -	Tibia* 29.7 vs 45.7			
3-phytase *A.niger*	12,000	2.7, 4.6,8.8	0.29; 0.38; 0.75	0.52; 0.60; 0.86	* * *	Tibia* 26.0 vs 40.7	Corn/SBM	16	Shirly and Edward (2002)
6-phytase *E.coli* in *T. reesei*	12,500	-, 5.2, 8.9	23.0; 33.0; 1.40	33.0; 44.0; 1.30	* * -	Tibia* 40.0 vs 47.0	Corn/SBM	21	Zeller *et al*. (2015b)
6-phytase *E.coli*	24,000	2.41, 5.67, 10.1	11.3; 29.4; 1.88	17.5; 27.7; 1.60	* * *	Toe * 12.5 vs 15.6	Corn/SBM	14	Cowieson *et al*. (2006)
6-phytase in *A.oryzae*	40,000	-, 5, 6	1.7; -; 1.91	1.94; -; 1.80	* - *	-	Corn SBM	35	Aureli *et al*. (2011)

[1]Weight gain (kg) or average daily gain (g); feed intake (kg) or average daily intake (g); FCR. [2]The effect is (*) significant at P< 0.05; (-) not significant. [3]without versus with supplemental phytases

Table 1.4. Typical concentration of calcium, total P, phytate-P, proportion of phytate-P to total P, and phytate in key feed ingredients (Adapted from Selle *et al*., 2009)

Feed ingredient	Ca (g/kg)[a]	Total P (g/kg)[b]	Phytate-P (g/kg)[b]	Phytate-P/ total P (%)[b]	Phytate (g/kg)[c]
Barley	0.30	3.21	1.96	60	7.0
Corn	0.20	2.62	1.88	72	6.7
Sorghum	0.40	3.01	2.18	73	7.7
Wheat	0.50	3.07	2.19	73	7.8
Canola meal	6.80	9.72	6.45	66	22.9
Cottonseed meal	1.50	10.02	7.72	77	27.4
Soybean meal	2.70	6.49	3.88	60	13.8
Rice bran	0.50	17.82	14.17	80	50.3
Wheat bran	1.40	10.96	8.36	76	29.6

[a] NRC (1994);
[b]Selle and Revindran (2007);
[c] Calculated on the basis that phytate contains 282 g/kg

Intestinal RT is affected by numerous dietary and husbandry factors. Dietary fat is one of the factors that affected intestinal RT, beside particle sizes and types of fibre and carbohydrate. Mateos *et al.* (1982) showed that the intestinal RT increased as inclusion level of yellow grease increased from 193 min (0% fat) to 270 min (30% fat) in chickens fed with corn-based diet. For rye-based diet, the intestinal RT was higher with addition of 100g/kg soya oil (499 min) compared to beef tallow (414 min) at the same level of inclusion (Danicke *et al.*, 1999). However, they did not report on the effect of fat inclusion level on intestinal RT. Conversely, Golian and Maurice (1992) found the intestinal RT was not affected by addition of poultry fat but it increased as the age of chickens increased from 170 min (1 week old) to 211 min (6 week old). Although there was only one report in the literature evaluating the effect phytase on intestinal RT (Watson *et al*., 2006), the effect of fat addition, type and inclusion level of fat in the presence of phytate on intestinal RT was not reported.

In addition to ambient temperature, lighting schedule was reported to affect the intestinal RT. Chickens reared under a 14L: 10D lighting schedule had significantly longer intestinal RT during the scotoperiod (dark) than during the photoperiod (Buyse *et al*, 1993). The effect of shorter scotoperiod on growth performance and intestinal RT was described by Duve *et al.* (2011). With continuous 8 h scotoperiod (16L:8D) in a day, the mean intestinal RT in chickens fed on a wheat-based diet was 475 min and it was longer than the RT for intermittent 8 h scotoperiod with two

equally spaced 4 h dark (351min). Nevertheless, the RT for these light schedules were much higher than those under continuous lighting program as reported by Hughes (2004) and Watson *et al*. (2006) with mean RT of 206 min (wheat-based diet) and 112 min (corn-based diet), respectively. Amerah *et al.* (2008) also found shorter intestinal RT with the average of 139 min for young chickens fed on both corn-based and wheat-based diets under continuous lighting program. In the case of phytase supplementation, Bedford *et al.* (2007) found that longer lighting time reduced weight gain of chickens fed on phytase supplemented diet but the intestinal RT was not reported. Meanwhile, Watson *et al.* (2006) found that by phytase supplementation, the intestinal RT of chickens fed on corn-based diets was reduced. Since the lighting program was not mentioned, it is assumed the chickens were subjected to continuous lighting program.

Supplementation of other enzymes such as xylanase and glucanase is expected to reduce intestinal RT, shorter than those in non-enzyme supplemented diet. The enzymes hydrolyse non-starch polysaccharides in the diet into oligo and monosaccharides, reduce viscosity of intestinal content (Choct, 1997) and reduce time for passage of digesta through the gastrointestinal tract (Danicke *et al.*, 1997; 1999; 2000). Lázaro *et al.*(2003) and Almirall and Esteve-Gracia (1994) concluded that supplementation of xylanase and glucanase to rye-based and barley-based diets reduced intestinal viscosity and RT.

1.1.11. Indigestible markers for evaluating nutrient digestibility

In determining mineral digestibility due to phytase supplementation in broilers, several indigestible markers including chromic oxide (Cr_2O_3), titanium dioxide (TiO_2) and acid insoluble ash (AIA) are added in the diets. These external markers are used in estimating nutrient uptake at specific site along the gut of chickens. Ideally, the markers should be indigestible, inert and easy to determine. Sales and Junssens (2003) and Selle et al. (2006) reviewed the choice of markers in determining metabolisable energy (ME) and amino acid digestibility (AAD). The amino acid digestibility responses to phytase are more pronounced with TiO_2 or AIA compared to Cr_2O_3. The use of markers in ME determination might lead to greater precision

than the total collection method. However, Cr_2O_3 is still unfavourable compared to TiO_2 or AIA due to poor repeatability and variability of its analytical methods, incomplete excreta recovery and its potential carcinogenicity. Internal markers are alternative to external markers which are naturally occurred in feeds and feed ingredients. Natural AIA is the most widely internal marker used in avian metabolizability and digestibility studies. Normally the natural AIA content in poultry feed is considerably very low to be measured accurately by the gravimetric method. Addition of dietary AIA such as celite, sand or silica helps to increase the total AIA content in test feed, resulting in AIA used as external marker.

1.1.12. Passage of digesta, transit time or retention time?

Besides used in evaluating nutrient digestibility, dietary marker is also used for estimation of digesta passage along the GIT. The passage of digesta at certain sites in the GIT is important in regulating the rate at which digesta come in contact with digestive enzymes and absorptive surfaces. Longer transit of digesta in GIT may increase nutrient utilization. $Cr2O_3$ and TiO_2 are commonly used markers for the determination of digesta passage rate.

The passage of digesta is usually expressed as transit time (TT) and retention time (RT) and can be assessed by several methods. Sibbald (1979), Mateos et al. (1982) and Watson et al. (2006) measured digesta transit time, which is based on time until visual first appearance of a marker substance in the excreta, or first appearance of excreta following a fasting period. Calculation of mean retention time (MRT) using cumulative marker excretion curves is another approach to determine the time required to excrete a specified proportion of ingested marker, such as 1% (T1) or 50% (T50). MRT also This method has been demonstrated as appropriate measurement for intestinal TT because it is more precise and less dependent on visual first appearance (Vergara et al., 1989; Almirall and Esteve-Garcia, 1994, Svihus et al., 2002). Another method to estimate the MRT is by expressing the amount the marker present in the different segments of the GIT as a percentage of the daily marker intake (Van der Klis et al., 1990). The following equation is used with the assumption that the amount of marker in each segment of GIT is constant or in steady state.

MRT = 1440 x marker in segment (mg) / marker intake (mg/day) (1)

Where MRT is mean retention time (min) and 1440 is minutes per day (24 h).

In this thesis, the MRT was determined according to Method 1 by Van der Klis et al. (1990) using titanium (Ti) as the marker and with slight modification in method of calculation. Basically, on day 21, after the weekly weighing of chickens and feed residues, chickens were fed with fresh pre-weighed diet and the time of feeding is recorded as "Start Time of Feeding". The time of slaughter is recorded as "End Time" for feeding period and feed residues are weighed. After the abdomen is opened, each segment of the GIT including crop, gizzard, duodenum-jejunum and ileum is removed and the full and the empty weight of each segment is recorded for determining the total weight of digesta. The digesta from all birds in the same pen are pooled and stored at -20^{o}C prior to analysis. Both feed and digesta samples are analysed for dry matter (DM) and (Ti) content. The MRT is calculated as follows;

Feed intake (FI) in g = weight of pre-weighed diet - weight of feed residue (2)

Total weight of digesta (g) = the full weight - the empty weight of each segment (3)

Ti in segment (mg) = Total DM in segment (g) x Ti concentration (mg/g digesta) (4)

$$\text{Ti Intake (mg/min)} = \frac{\text{FI (g) x Ti concentration (mg/g diet)}}{\text{(End time - start time of feeding x 1440)}} \quad (5)$$

$$\text{MRT (min)} = \frac{\text{Ti in segment (mg)}}{\text{Ti intake (mg/min)}} \quad (6)$$

1.1.13. Tibia bone for quantifying bone mineralization

In evaluating the efficacy of a phytase in low P diets, the approach is to study its effect on P digestibility. Kuhn (2012) reported that supplementation of two E. coli derived phytases (Quantum QP and Quantum Blue at 500 FTU/kg improved ileal P

digestibility in chickens. As the phytase dosage increased to 700 FTU/kg, no improvement in ileal P digestibility was observed but growth performance and bone mineralization clearly showed improvement. This study demonstrated that P digestibility has limitation on evaluating *in vivo* phytase efficacy and it should be evaluated along with growth performance and bone mineralization. Bone ash was reported to be more sensitive in quantifying bone mineralization in chicks compared to other response criteria such as weight gain, feed intake, feed efficiency, plasma P concentration (Sands et al., 2003; Adedokun et al.,2004; Onyango et al., 2005). In studies related to *in vivo* phytase evaluation in chickens, tibia was the most popular criterion in quantifying bone mineralization (Augspurger et al., 2004; Tang et al., 2012; Rousseau et al., 2012; Walk et al, 2012; Walk e al., 2014, Zeller et al., 2012c). The tibia is the fastest growing bone in the body and very sensitive to Ca and P deficiencies. In this thesis, tibia bone was used as the mean of evaluating bone mineralization as recommended by the AOAC (1990).The amount of tibia ash was presented as tibia ash concentration (mass/volume, mg/cm^3) instead of tibia ash as percentage of dry matter due to better measurement in reflecting bone status in chicken (Zhang and Coon, 1997).

1.1.14. Conclusion

Phytate is the primary storage form of both phosphate and inositol in plant-based feed ingredients. It forms insoluble complexes with dietary minerals, especially calcium, magnesium, iron and zinc, and reduces the bioavailability of P, Ca, Zn and other metabolically important minerals in the gut. Phytate also interferes with the digestibility of energy, protein and fat, impediments that have significant nutritional and health consequences. It is worth noting that limitations of intestinal RT and pH within the GIT of chickens do not allow complete degradation of phytate to myo-inositol and inorganic P. Clearly, there are huge possibilities in developing strategies to increase the degradation of phytate-P and P utilization in poultry, particularly broilers. Phytase reactivity under different environmental conditions, the interaction between phytate, protein, carbohydrate and minerals in the intestine, and also the appropriate use of phytate mediation strategies such as phytase, pH manipulation, gut motility manipulation and divalent cation intake need further investigations.

1.2. Objectives and Hypotheses

The main questions addressed in this research project were; 1) to assess the effect of methodology on estimation of phytate hydrolysis and P digestibility in young broilers and 2) to investigate whether or not dietary fat and fibre could further improve phytase efficacy in broilers fed diets containing wheat by delaying digesta RT. A series of experiments were conducted in order to answer the above questions.

The third chapter in this thesis describes three preliminary studies that have separate aims and hypotheses.

1. Study 1 was conducted with the aim to investigate whether or not feed withdrawal followed by feed refeeding is necessary to obtain adequate amounts of digesta for subsequent analyses. The specific objective was to determine the effect of feed withdrawal and refeeding period on the contents of GIT of 21-22 days old chickens. It was hypothesized that feed withdrawal and refeeding would results in higher dry matter content in each segment of the GIT compared to those with continuous availability of feed.
2. Study 2 was conducted to investigate whether or not a common sample preparation could minimize the amount of digesta samples used and enable sequential chemical analyses. The specific objective of the study was to compare the content of Ti, P and Ca in diet and digesta samples after being separately prepared using different acids in the sample digestion. The digested samples were also used in comparing the content of P, Ca and Ti as determined by colorimetry and ICP-OES method. It was hypothesised that the content of P, Ca and Ti measured following either HCl or H_2SO_4 digestion would not differ from each other. Similarly, the content of P, Ca and Ti as determined by colorimetry method and ICP-OES would be similar.
3. Study 3 was to evaluate the effect of anticoagulant, sample deproteinization, myo-inositol method, dietary phytase treatment and feeding methods prior to sampling on the concentration of myo-inositol, P and Ca in the blood of broiler chickens. It was hypothesized that myo-inositol, Ca and P will not be affected by deproteinization and feeding method prior to sampling but will change with

different type of anticoagulant. Dietary phytase treatment, on the other hand, increases blood myo-inositol, Ca and P.

The fourth chapter describes a study that was designed to test the hypothesis that different feeding methods after a 6 h scotoperiod would change the measured effectiveness of dietary phytase and that this would be reflected in the hydrolysis of phytate, relative concentrations of InsPs and apparent digestibility of P in the gizzard and ileum of broilers. The primary aim of this study was to investigate the effect of phytase supplementation at 1500 FTU/kg, different feeding methods prior to slaughter and their interaction on InsPs concentrations, phytate hydrolysis and P digestibility in male broilers fed a wheat based diet. In addition, the effects on growth performance, total DM and pH of digesta and digesta mean RT were evaluated.

In fifth chapter, a study was conducted to investigate the effect of different levels of dietary fat on phytase efficacy with regard to growth performance, utilization of phosphorus and calcium, and bone mineralization in broiler chickens. It was hypothesized that diets containing both phytase and high level of fat would improve the growth rate and nutrient intake of chickens over and above that of birds fed diets supplemented with phytase only. Higher fat level and phytase supplementation were also expected to improve both Ca and P digestibility in the gut and retention in the bone.

The final experimental chapter (Chapter 6) describes a study that was conducted to investigate the effect of dietary fat and fibre on the digesta mean RT of broiler chicken fed a phytase supplemented diet. Their effects on gut development, digesta pH and phytate P degradation were also studied. It was hypothesised that MRT would be increased with higher fat supplementation but not affected by addition of dietary fibre, thereby improving phytate P degradation in each section of the GIT.

Chapter 2
Materials and Methods

This chapter provides information on animal management, selection and allocation of birds to treatment and chemical analysis throughout the course of this research. Specific differences between experiments are indicated in the relevant chapters. Experimental design and methods of statistical analysis are described fully within each chapter. All experimental procedures complied with The Animals (Scientific Procedures) ACT 1986, under Animal Ethical Review Committee of The University of Leeds.

2.1. Housing and management

On arrival, each chick was weighed, tagged using either a wing tag or leg band and randomly allocated into a pen. The number of chicks in a pen depended on the size of the pen but did not exceed 33 kg/m^2 (The Welfare of Farm Animals (England) (Amendment) Regulations 2010). Chicks were placed onto a paper within the pen area. Supplementary feeders and drinkers were placed in the vicinity of the paper to enable the chicks to reach feed and water immediately and easily. The chicks were left to settle for one to two hours to become accustomed to their new environment prior to allocation to treatment pens. By 6 to 8 h and 24 h after arrival on the farm, the crop of chicks were checked whether or not it was filled with feed. If the crop is full or half full means they had found feed.

Prior to the arrival of day old chicks, the room was pre-heated up to 30 ± 2^oC a day. Passive ventilation was applied via adjustable air vents to ensure that fresh air was supplied to the chicks for the first few days. Fan assisted ventilation was used to control room temperature and air exchange after 7 d. The temperature of the room was reduced by 1^oC every 3 d to reach 22^oC at d 21 and maintained at 22^oC until the experiment ended. The temperature in the room, outside the room, bedding temperature and temperature at the top of the pens were monitored and recorded. Relative humidity of the room was also monitored and recorded, which should be 60

to 70%. Continuous ventilation, continuous lighting with light intensity of 40 lux from d 1 to d 3 and 18 h light and 6 h dark (18L: 6D) from d 3 to d 23 were applied. The intensity of the light was reduced to 10 lux after d 7. Feed and water were provided *ad libitum* at all times.

Individual body weights and pen feed intake were recorded weekly while health conditions and mortality were recorded daily. The condition and pattern of movement of the chickens were monitored daily by giving conditions and gait scores according to Table 2.1 and 2.2. Chick's daily count (alive), dead, off trial due to lameness, sick or splay legs were also recorded. The chick's body weight and residual feed for the pen were recorded in the occurrence of any chicks removal from a pen..

Table 2.1 Simplified gait score according to Garner *et al*. (2002)

Gait Score	Degree of impairment
0	Normally walking bird
1	Detectable abnormality
2	Complete lameness

Table 2.2 Chicken's condition score according to Aviagen (2014)

Condition score	Description of chicken's condition
1	Chicks make no noise, pant, head & wings droop, widely spread in the pen (temp. too high)
2	Chicks evenly spread, noise level signifies contentment (temperature correct)
3	Chicks crowd together, noisy (temperature too low)
4	Chicks crowd together at one side/corner of the pen (draught, uneven light distribution, external noise)

2.2. Selection and allocation to treatment

The selection of chicks and allocation to treatment were conducted 2 h after arrival. Any chicks that appeared poor or lame were discarded from the allocation. Small or extremely large chicks were excluded where possible. The remaining chicks were allocated to experimental pens on the basis of live weight. Each pen had the same number of chicks, had similar mean chick weight, a similar live weight range (within

10 g) and a coefficient of variation of less than 5%. Treatments were allocated within a room, taking pen into account in order to remove variation due to the pen.

2.3. Experimental diets

All experimental diets were formulated to meet or exceed the specification for As-Hatched Broilers Grown to <1.9kg (4.2lb) live weight for Ross 308 Broiler (Aviagen, 2007) except with lower content of available P (0.25 - 0.35%) and Ca (0.86 – 0.90%) and contained 0.5% titanium dioxide (TiO_2) except diets in Chapter 5. In Chapter 5, internal acid insoluble ash (AIA) was used as a marker but the concentrations of internal AIA in digesta samples were very low and large amount of digesta samples were used for the AIA analysis. Therefore, TiO_2 was chosen as a marker to replace internal AIA in subsequent experiments. All diets were wheat-corn-soybean meal based diets that contained either 0 or 1500 FTU/kg of phytase (Quantum Blue 5G, AB Vista Feed Ingredients, Marlborough, UK) , 10 or 50 g/kg of dietary fat (soya oil) and 0 or 40 g/kg of dietary fibre (Vitacel®), depending on the experimental design. The diets were presented in crumble form except in Chapter 1(Study1) and Chapter 6 where the diets were in mash form. This was due to unavoidable circumstances at feedmill that prevented pelletization of experimental diets.

2.4. Sample Collection and Measurements

Generally, on the day of sampling, following 1 h of feed withdrawal and 1 h of refeeding, a number of birds per treatment (e.g. 3 birds per pen) were randomly selected and weighed. The birds were killed by cervical dislocation and dissected. In order to avoid post mortem digesta movement, crop, proventriculus-gizzard, duodenum-jejunum and ileum were clamped. The contents of each part of the gastrointestinal tract from each bird were collected, pooled within a pen and then pH values of the digesta were measured immediately. The digesta samples were freeze-dried, ground and sieved to pass through 1.0 mm screen prior to chemical analyses. The left legs were removed from the body and each of bone samples were individually packed in polyethylene bags and stored at -20^{o}C prior to bone

preparation. After thawing, tibia bones were obtained by cutting the tibiometatarsal joint, the joints between femur and tibia/fibula and the joint between tibia and fibula. Tibiae were cleaned of the adhering tissues and dried in the oven at 100^{o}C for 24 h. The bone parameters including dry weight, length, volume and density were determined according to Zhang and Choon (1997). Dried tibiae were ashed in a muffle furnace (Carbolite 1100) at 600^{o}C for 24 h to determine the contents of tibia ash and tibia minerals. About 200g of each of the experimental diets were taken from representative feed bags weekly and stored at -20^{o}C prior to further analysis. Table 2.3 shows the list of samples taken for each experiment chapters.

Table 2.3 List of samples taken for each experiment chapters

Chapter	Samples
3 (Study 1)	• Digesta – pooled sample of proventriculus and gizzard, pooled sample of duodenum – jejunum, ileum • The full and empty weights of crops, proventriculus-gizzard, duodenum-jejunum and ileum • Feed samples
3 (Study 2)	• Digesta from gizzard and terminal i leum • Feed samples
3 (Study 3)	Blood samples - collected into 3 different tubes; without anticoagulant (serum), Li-heparin and K_2EDTA (plasma)
4	• Digesta from gizzard and terminal i leum • The full and empty weights of gizzard and terminal ileum • Blood samples (plasma). • Feed samples
5	• Digesta from crop, proventriculus-gizzard, duodenum-jejunum and ileum • Tibia bones • Feed samples
6	• Digesta from crop, proventriculus-gizzard, duodenum-jejunum and ileum • The full and empty weights of crops, proventriculus-gizzard, duodenum-jejunum, ileum and pancreas • Feed samples

2.5. Chemical analysis

Dry matter content was determined using the standard procedure according to AOAC Official Method 930.15. Samples were weighed into pre-weighed squat clear glass vials and placed into a drying oven overnight at 105^{o}C. The dry weights were recorded after cooling in a desiccator. The dried samples were then ignited at 600^{o}C overnight, cooled in a desiccator before the weights were recorded for ash content determination. In the same glass vials, sample ash were digested with 5 M hydrochloric acid for 30 min at boiling temperature and for samples containing titanium dioxide, sample ash were digested in 7.4 M sulphuric acid for 1 to 1.5 h at boiling temperature. The digested samples were filtered through ashless Whatman No 541 filter paper and diluted to 100 ml with ultra-pure water ready for analysis of minerals.

Phosphorus contents in digested samples were determined according to standard procedures (Method 965.17, AOAC 2000). Briefly, molybdovanadate reagent was prepared dissolving 25 g ammonium molybdate and 1.25 g ammonium vanadate separately using ultrapure water. Molybdate solution was gradually added into vanadate solution with stirring on a hot plate and the mixture was then diluted to 500 ml. Prior to the analysis, 20 ml molybdovanadate reagent and 20 ml 5 M HCl were added to 120 ml ultrapure water and mixed well. Forty µl of digested samples were pipetted into 96-wells flat bottom microplates before adding 160 µl of diluted molybdovanadate solution. A pale yellow colour was allowed to develop for 10 min before being measured by UV spectrophotometer at the wavelength of 405 nm.

Calcium contents were determined by Arsenazo III according to the method of Attin *et al*. (2005). Arsenazo III reagent was prepared by separately dissolving 19.4 mg of Arsenazo III and 3.4 g Imidazole with ultrapure water. Arsenazo solution was added to Imizadole buffer solution with stirring and the pH of the mixture was adjusted to 6.5 with 3.0 M NaOH or 3.0 HCl dropwise before being diluted to 500 ml. Arsenazo III buffered reagent should contain 0.05 mM Arsenazo III and 0.1 M Imidazole buffer. The content of Ca was determined by mixing each sample with Arsenazo III reagent at the ratio of 1 to 50. Four µl of digested samples were pipetted into 96-wells flat bottom microplates before adding 200 µl of Arsenazo III

buffered reagent. A bluish purple colour was allowed to develop for 10 min before being measured by UV spectrophotometer at the wavelength of 650 nm.

Titanium contents were determined according to AOAC Official Method 973.36. Briefly, 3 ml of H_2SO_4 digested samples were diluted with 2 ml 7.5 M H_2SO_4 before adding 0.2 ml 30% hydrogen peroxide. An instantaneously developed yellow colour solution was measured by UV spectrophotometer at 405 nm.

For the inductively coupled plasma optical emission spectrometry (ICP-OES) assay, acid digested samples were diluted with ultrapure water 10 times for diet and digesta samples and 100 times for bone ash samples. The samples were assayed according to AOAC Official Method 985.01 using ICP-OES spectroscopy (Optima 2100 DV ICP-OES, model PQ Excell VG Elemental; Perkin-Elmer, Shelton, CT, USA), set to detect Ti, P, Ca, Zn, Mg, Mn, Na, and Cu at wavelength 334.9, 214.9, 317.9, 213.8, 279.5, 257.6, 598.6 and 324.7 nm, respectively, with less than 5 % of uncertainty at 95% confidence level.

Myo-inositol contents in blood samples were determined by either colorimetry or high performance liquid chromatography (HPLC) analysis. Whole blood was collected into Li-heparin tubes and plasma was obtained after centrifugation at 1,800 g for 15 min at 4°C. Proteins were precipitated from the plasma by addition of 2 volumes of either 1 M perchloric acid or acetonitrile and removed by centrifugation at 19,000 x g for 10 min before before being analysed using Megazyme (K-INOXL 02/14) assay kits and HPLC analysis, respectively.

In the HPLC assay, inositol was separated by HPLC on a Dionex DX600 HPLC system and detected by pulsed amperometry on a gold electrode. Inositol phosphates (InsPs) were determined using high-performance ion chromatography (HPIC). Briefly, about 0.1 g samples were extracted for 60 min room temperature in 5mL of 100mM NaF, 20mM Na_2EDTA, adjusted to pH 10 with NaOH and filtered through a 0.45 µM polypropylene filter. The chromatograph equipped with a PA-200 (3 mm i.d. x 50 mm.) guard column (Dionex Corp., Sunnyvale, CA) and an HPIC CarboPac PA-200 (4 mm i.d. x 200 mm) analytical column (Dionex Corp.). The column was eluted at a flow rate of 0.4 ml/min with a gradient of methane sulfonic acid delivered from buffer reservoirs containing: A, water; B, 600 mM methane sulfonic acid according to the following schedule: time (minutes), % B; 0,0; 25,100;

38,100; 39,0; 49,0. The column eluate was mixed using a mixing tee with a solution of 0.1% w/v ferric nitrate in 2% w/v perchloric acid delivered at a flow rate of 0.2 ml/min, before passage through a knitted reaction coil (200ul). The inositol phosphates were detected after post-column reaction using a Jasco UV-2077 Plus UV detector at 290 nm.

Chapter 3
Preliminary studies on feeding method prior to sampling, sample preparation and chemical analyses of feed, digesta and blood of broiler chickens

3. Introduction

In this chapter, three preliminary studies are reported. The main aim of these studies was to compare several technical procedures and chemical assays which would later be used in the main experiments (Chapters 4, 5 and 6). This chapter is divided into 3 sections each one reporting on the individual study performed. The specific objectives of each study are specified in their respective section.

Study 1: Effect of feed withdrawal and refeeding period on gut contents of young broilers

Study 2: Comparison of sample preparation methods and chemical assays for determination of titanium (Ti), phosphorus (P), and calcium (Ca) in broiler diets and digesta

Study 3: Effects of anticoagulant, sample deproteinization, methods for determining myo-inositol, dietary phytase treatment and feeding method on the concentration of myo-inositol, P and Ca in broiler chickens Effects of anticoagulant, sample deproteinization, methods for determining myo-inositol, dietary phytase treatment and feeding method on the concentration of myo-inositol, P and Ca in broiler chickens

3.1. Study 1: Effect of feed withdrawal and refeeding period on gut contents of young broilers

3.1.1. Abstract

The aim of this study was to investigate whether or not feed withdrawal (FW) and feed refeeding (FR) is necessary to obtain adequate amounts of digesta sample for subsequent analyses. It was hypothesized that FW and FR would result in higher dry matter content in each segment of the gastrointestinal tract (GIT) compared to those without FW and FR (continuous feeding). In this experiment, 21 d old broilers were used. Prior to euthanization and digesta collection, the chickens were randomly assigned to one of 6 treatment groups based on the duration of FW and FR. In Treatments 1 and 2, chickens were killed without FW and FR with sampling done in the morning and afternoon, respectively. In Treatments 3 and 4, feeding with 1 h FW followed by 1 h FR was applied prior to morning and afternoon sampling, respectively. In Treatments 5 and 6, feeding with 4 h FW followed by 4 and 6 h FR, respectively, was applied. In comparison to continuous feeding, feeding with 1 h FW followed by 1 h FR increased crop content but reduced ileal content, whereas feeding with 4 h FW followed by either 4 h reduced proventriculus-gizzard content. FW and FR did not affect the weight of duodenum-jejunal content. Sampling time (morning versus afternoon) significantly affected the relative weight of crop and ileal digesta. Higher amount of crop content was found in the afternoon sampling for both with or without FW and FR. but lower ileal content in the afternoon sampling for feeding with FW and FR. It was suggested that continuous feeding could be applied to obtain adequate amounts of digesta for subsequent analyses.

3.1.2. Introduction

In evaluating phytate degradation and nutrient digestibility in different segments of GIT, several aspects should be considered before and during digesta collection including feeding regime, feed structure, time and method of killing and sampling site. Sampling time between 2 h to 6 h following the start of feeding influenced the amount of digesta collected, with the most ileal digesta dry matter (DM) collected at 4 h (Kadim and Moughan, 1997a). However, 24 h fasting was applied in that study prior to 1 h free access to the diet or forced fed via intubation tube followed by killing and sampling. Kadim and Moughan (1997b) also showed that total DM of ileal digesta of broilers that continuously fed was not different from those fasted either 12 h or 24 h prior to 1 h FR. Furthermore, the amount of crop digesta was higher at 1 h compared to 4 h sampling time following 24 h fasting and 1 h FR (Yap *et al*., 1997). Duve *et al* (2011), on the other hand, demonstrated that light program also affects the amount of digesta collected from broilers with continuous feeding. The amount of digesta from each segment of the GIT was higher at 4 h sampling time compared to those collected immediately after the light period started.

There is considerable variation among phytase studies in the aspect of feeding method prior to euthanization and digesta sampling. For standardization of feed intake and feed retention time in the GIT, Rutherfurd *et al.* (2002) applied 16 h FW and 2 h FR while Zeller *et al*. (2015a; 2015b) applied 1 h FW and 1 h FR. Whereas, others did not mention the feeding method prior to sampling except the lighting program such as 14L:10D (Walk *et al.*, 2014), 23L:1D (Amerah *et al*., 2014; Leytem *et al.*, 2008b), 12L:12D (Leslie, 2006) and continuous lighting (Leslie, 2006; Ravindran *et al*., 2000). More experiments are required to evaluate how these technical aspects of digesta sampling including duration for FW and FR and lighting schedule may affect the results and to allow standardization on procedure of digesta collection besides ensuring adequate amount of samples are collected from each part of the GIT for further analysis. The aim of this study was to investigate whether or not FW followed by FR is necessary to obtain adequate amounts of digesta for subsequent analyses. The specific objective was to determine the effect of FW and FR period on the contents of GIT of 21-22 days old chickens. It was hypothesized

that FW and FR would results in higher DM content in each segment of the GIT compared to those with continuous availability of feed.

3.1.3. Materials and methods

All experimental procedures were conducted in accordance with The Animals (Scientific Procedures) ACT 1986, under Animal Ethical Review Committee of University of Leeds

3.1.3.1. Animals and diets

One-day -old Ross, male broiler chickens were obtained from a commercial hatchery (P. D. Hook (Hatcheries) Ltd, Bampton, Oxfordshire). On arrival, chicks were weighed individually and randomly allocated to 6 floor pens. The chickens received continuous ventilation, continuous lighting from d 1 to d 3 and nearly continuous lighting (20L: 4D) from d 4 to d 23. The temperature of the room was initially set at $32^{o}C \pm 2^{o}C$ and gradually reduced to $22^{o}C$ at d 21. Feed and water were provided *ad libitum* at all times except during FW period. The chickens were fed with the same mash diet, which nutrient specification was up to commercial standard and manufactured by commercial feedmiller. The main ingredients in the diet were maize, soybean meal, soybean oil, dicalcium phosphate, calcium carbonate, DL-methionine, vitamins and minerals (Table 3.0).

3.1.3.2. Experimental procedures

Birds were grown until 21 days of age. Individual body weight and pen feed intake were recorded on d 7, 14 and 21. Mortality and health conditions of the birds were recorded daily. A week before the termination of the trial, all pens were randomly assigned to one of the 6 treatment groups based on the duration of FW and FR. In Treatment 1, chickens were killed without FW and FR and sampling was done in the morning and Treatment 2 was the same as Treatment 1 but killing and sampling were done in the afternoon. In Treatments 3 and 4, chickens were killed after 1 h FW followed by 1 h FR and sampling was done in the morning and the afternoon, respectively. Whereas, in Treatments 5 and 6, chickens were killed after 4 h FW

followed by 4 and 6 h FR, respectively. Each of the treatments had 8 replicates of one bird.

Table 3.0 Composition of starter diet (%) for young broiler chickens

Ingredients (%)		Starter diet
Wheat		41.1
Corn		23.8
Soya extract (48%)		19.7
Potato protein (79%)		6.1
Corn gluten meal (62%)		1.4
Soybean oil		2.8
Lysine HCl		0.36
Methionine		0.15
Threonine		0.07
Dicalcium phosphate, anhyd., 18% P		1.45
Limestone		0.73
Salt (NaCl)		0.12
Sodium carbonate		0.17
Vitamin & mineral premix[1]		0.40
Titanium dioxide (TiO_2)		0.50
Binder (Ligno Bond DD)		1.25
Total		100.00
Calculated nutrients	*Recommended level*[2]	
AME Chick MJ/kg	12.65	12.67
Crude Protein	22-25	22.03
Energy: protein	0.575	0.575
Lysine	1.43	1.43
Methionine	0.51	0.51
Threonine	0.94	0.94
Ca	1.05	0.86
P		0.62
aP	0.50	0.35
Ca:aP	2.10	2.46
Phytate-P		0.24
Sodium	0.16	0.13
Chloride	0.16-0.23	0.16
Fibre		2.10
Fat		4.78

[1]Premixes provided the following (per kg of diet): vitamin A, 13,200 IU; vitamin D3, 4,000 IU; vitamin E, 66 IU; vitamin B12, 39.6 µg; riboflavin, 13.2 mg; niacin, 110 mg; Dpantothenate, 22 mg; menadione (K3), 4 mg; folic acid, 2.2 mg; thiamine, 4 mg; pyridoxine, 8 mg; D-biotin, 252 µg; selenium (as $_{Na2SeO3}$), 0.30 mg; manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; cobalt, 1.0 mg; choline chloride, 1,200 mg; coccidiostat, 500 mg.
[2]The requirement was according to The Specification for As-Hatched Broilers Grown to <1.9kg (4.2lb) live weight for Ross 308 Broiler (Aviagen, 2007).

Samplings of the digesta were done over 2 days. On both d 21 and d 22, feeders were taken out from the pens at 6.00 am to start the FW period. The feeders were returned to the pens to stop the FW period (1 or 4 h) and start FR at several

time points (Figure 3.1). After the appropriate FR duration either 1, 4 or 6 h, the chickens were randomly selected, individually weighed and killed via cervical dislocation followed by digesta collection.

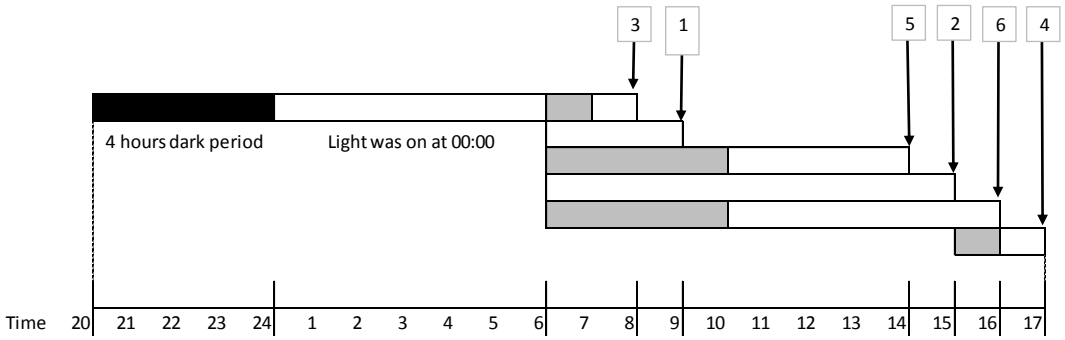


Figure 3.1 Schematic presentation of periods for feed withdrawal and feed refeeding. The black bar represents the daily 4 h dark period. White bars represent the period when the birds had access to feed and the grey bars represent the feed withdrawal period. Arrows indicate times for sampling intestinal tract contents and numbers in boxes indicate the treatments (see text for details).

The abdomen was opened and the GIT were exposed. The skin over the crop was incised and the upper and lower junctions of the crop were carefully clamped. The upper junction of proventriculus, the lower junction of ventriculus (gizzard), duodenum and ileum, defined as Meckel's diverticulum to ileoceccal junction were also clamped before the digesta were collected. The samples from proventriculus and gizzard were pooled. Similarly, digesta from duodenum and jejunum were pooled. The full and empty weights of crops, proventriculus-gizzard, duodenum-jejunum and ileum were obtained separately. All collected digesta were immediately stored in closed containers at -20^{o}C until determination of DM.

3.1.3.3. Statistical analysis

The data were analysed by 1- way analysis of variance (ANOVA) to compare the differences among treatments by using the GLM procedure of Minitab 16 Statistical Software (Minitab Inc, 2010). Significant differences among the treatments were identified at 95% confidence level by Multiple Comparison of Means using Tukey's method. Test for normality was done by performing an Anderson-Darling Test

3.1.4. Results

Mortality during the trial was low (1.6%; SD = 0.55) and the death were not related to dietary treatment. Final live body weights were not significantly different between treatment groups.

Different treatments significantly ($p<0.01$) affected the relative weight of digesta in proventriculus-gizzard and ileum but did not affect the relative weight of crop and duodenum-jejunal digesta (Table 3.1). In proventriculus-gizzard, 1 h FW followed by 1 h FR resulted in the highest weight of digesta and significantly higher than those with 4 h FW followed by 4 h FR but similar to those in other treatments. While in the ileum, the relative weight of digesta was lower with 1 h FW followed by 1 h FR collected in the afternoon compared to other treatments. The relative weight of digesta from each segment of the GIT was similar after 4 h FW followed by either 4 h or 6 h FR.

When the treatments were grouped according to the duration of FW, i.e. continuous feeding (without FW), 1 h and 4 h FW, the relative weight of digesta in crop was significantly affected by duration of FW but the relative weight of duodenum-jejunal digesta remained unaffected by the treatments (Table 3.2). Without FW, the amount of crop digesta collected was the lower compared to those with 4 h FW but did not statistically different from the amount collected with 1 h FW. The weights of digesta in the proventriculus–gizzard without FW was also similar to those with 1 h FW but the weight of ileal digesta was similar to those with 4 h FW. The highest weights of digesta in the crop and proventriculus-gizzard were with 4 h and 1 h FW, respectively.

Relative weights of crop contents were higher when sampling was done in the afternoon (at 3 p.m. and 5 p.m.) compared to in the morning (at 8 a.m. and 9 a.m.) with the highest amount of crop digesta obtained from treatment with 1 h FW and 1 h FR in the afternoon sampling (Figure 2A). While, the relative weight of ileal digesta similar when sampling was done in morning and in the afternoon except for those collected after 1 h FW and 1 FR in the afternoon (Figure 2B).

Table 3.1 Relative weight (g/kg empty body weight) of digesta from different sections of gastrointestinal tract of Ross 308 male broiler chickens at 3 weeks of age.

Treatment	Weight[1] of digesta (g DM/kg EBW[2])			
	Crop	Proventriculus -gizzard	Duodenum-jejunum	Ileum
Without FW & FR, a.m. sampling	2.38±0.61	6.53±0.34^{ab}	5.12±0.31	3.09±0.19^{a}
Without FW & FR, p.m. sampling	3.51±0.65	5.57±0.32^{ab}	5.63±0.53	3.95±0.14^{a}
1 h FW & 1 h FR, a.m. sampling	3.64±0.97	7.36±0,51^{a}	4.72±0.57	3.12±0.26^{a}
1 h FW & 1 h FR, p.m. sampling	5.41±0.64	7.01±0.81^{ab}	4.68±0.31	1.97±0.30^{b}
4 h FW & 4 h FR	5.78±1.13	5.20±0,37^{b}	4.88±0.25	3.78±0.25^{a}
4 h FW & 6 h FR	5.25±1.04	5.37±0.36^{ab}	4.97±0.43	3.54±0.28^{a}
SEM	0.84	0.45	0.40	0.24
P-value	0.053	0.008	0.636	<0.001

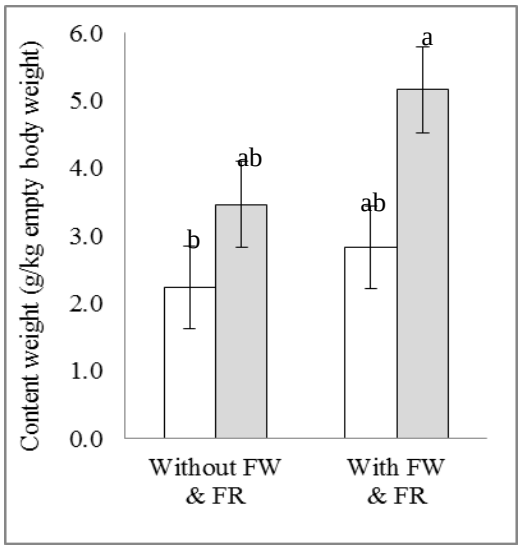
[1] Mean ± SE , n=8; [a, b] Means in the same column with different superscripts differ ($p<0.05$).
[2] EBW – live body weight minus content of whole gastrointestinal tract
FW – feed withdrawal; FR – Feed refeeding

Table 3.2 Effect of feed withdrawal duration on the relative weight (g/kg empty body weight) of digesta from different sections of gastrointestinal tract.

Treatment	Weight[1] of digesta (g DM/kg EBW[2])			
	Crop	Proventriculus -gizzard	Duodenum-jejunum	Ileum
Without FW & FR	2.95±0.46^{b}	6.05±0.26^{ab}	5.37±0.30	3.52±0.16^{a}
1 h FW & 1 h FR	4.52±0.61^{ab}	7.19±0.47^{a}	4.70±0.32	2.54±0.24^{b}
4 h FW & 4 h FR /6 h FR	5.51±0.75^{a}	5.28±0.25^{b}	4.92±0.24	3.66±0.29^{a}
SEM	0.60	0.32	0.29	0.20
P-value	0.018	0.001	0.256	<0.001

[1] Mean ± SE , n=8; [a, b] Means in the same column with different superscripts differ ($p<0.05$).
[2] EBW – live body weight minus content of whole gastrointestinal tract
FW – feed withdrawal; FR – Feed refeeding

A. Crop content



B. Ileal digesta

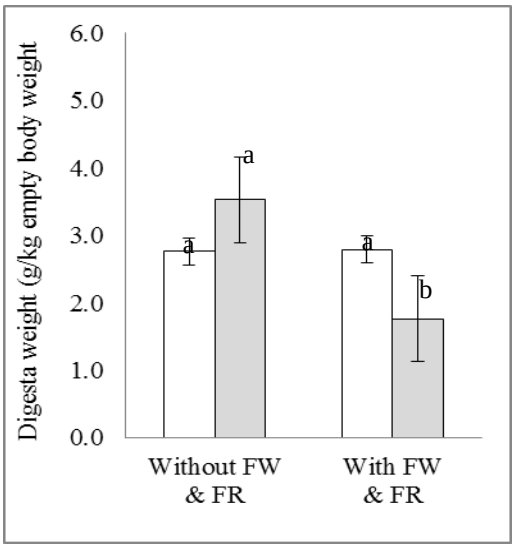


Figure 3.2 Relative weight of A) crop and B) ileal digesta (g/kg empty body weight; mean ± SE) for morning (white bar) and afternoon (grey bar) samplings for treatments without and with 1 h FW and 1 h FR. Significant at $P< 0.05$. FW – feed withdrawal; FR – Feed refeeding

3.1.5. Discussion and Conclusion

The birds with continuous feeding had access to the feed at all times tended to by-pass the crop (Scanes *et al.*, 1987, Svihus, 2014) and filled up the proventriculus-gizzard which indicated by higher proventriculus-gizzard content compared to crop content. Feeding with 1 h FW and 1 h FR, on the other hand, had higher crop content than those with continuous feeding because after feed deprivation, chickens tended to eat more and stored the ingested feed in the crop (Duve *et al.*, 2011, Svihus, 2014). If the gizzard empty, the feed is directly passed to the proventriculus-gizzard and fills to its maximum capacity and the crop will be the storage for the excess feed intake (Svihus, 2014).

According to Danicke *et al*. (1999), the time taken for ingested feed to fill up the empty GIT of a broiler chicken from crop to ileum was 394 minutes. The crop was filled up in 1 h, followed by gizzard within another 1 h. This could explain the higher content of crop when 1 h FW followed by 1 h FR and 4 h FW followed by either 4 h or 6 h FR were applied. Ileal digesta was significantly lower when 1 h FW and 1 h RF was applied. This maybe due to the slow movement of digesta along the small intestine which took about 6 h to fill up the ileum (Danicke *e al.*, 1999).

According to Summers and Leeson (1979), when feed was withdrawn for 4 h, 60% of the digesta was in the gizzard and the rest was in the duodenum while crop, jejunum, ileum and large intestine were empty. In addition, Jackson and Duke (1995) demonstrated that after 10 h without feed, ileum was rapidly filled up soon after the start of FR. In the present study, as the feed was reintroduced after 4 h FW, more feed was consumed which indicated by higher crop content compared to 1 h FW and continuous feeding. The ingested food may be by-passed proventriculus-gizzard (Svihus *et al*., 2002), directly enters and fills up the empty ileum to its maximum, which may explain lower content of proventriculus-gizzard and similar ileal content, respectively, as those observed in birds with continuous feeding.

In this study, sampling time (morning versus afternoon) significantly affected the relative weight of crop and ileal contents. Higher amounts of crop content were observed from the afternoon sampling which were done at about 2 h prior to the start of dark period for both continuous feeding and feeding with FW and FR compared to those observed in morning sampling. These results were in agreement with observation reported by Scanes *et al.* (1987), who found large quantities of feed present in the crop at the start of dark period. The chickens probably already had the habit of filling up their crop by increasing their feed intake as they prepared for darkness. On the other hand, sampling time only affected ileal contents from chickens with FW and FR, with lower ileal content from afternoon sampling. In the light of this evidence, it is expected that at the point of sampling, the degree of phytate degradation in the crop and ileal phytate digestibility are higher at afternoon sampling compared to at the morning sampling. The storage of feed in the crop prior to dark period and the slower food passage during the dark period provide more exposure of phytate to phytase and longer contact time of digesta to the absorptive surface of intestine.

In conclusion, it is suggested that besides 1 FW followed by 1 h FR, continuous feeding could also be applied in order to obtain adequate amount of digesta in each segment of GIT for subsequent analyses due to higher intestinal content. Higher crop could be obtained by sampling in the afternoon. Increasing the number of chickens and sample pooling could be an alternative to obtain higher amount of digesta sample as described by Morgan *et al.* (2013).

3.2. Study 2: Comparison of sample preparation methods and chemical assays for determination of titanium, phosphorus and calcium in broiler diets and digesta

3.2.1. Abstract

In understanding the effectiveness of phytase in improving nutrient digestibility, digesta samples from each segment of the gastrointestinal tract (GIT) need to be analysed for several different nutrients which require large amount of dry matter. The aim of this study was to develop a common sample preparation that could minimize the amount of digesta samples required and enable sequential chemical analyses. The specific objectives were to compare the content of titanium (Ti), phosphorus (P) and calcium (Ca) in diet and digesta samples after being separately prepared using different acid digestion and analysed by different chemical methods. In this study, gizzard and terminal ileal digesta were collected from 20 d old Ross 308 male broilers fed with wheat/corn diets either with or without supplemental phytase at 1500 FTU/kg. Diets and digesta samples were digested in hydrochloric acid (HCl) and sulphuric acid (H_2SO_4) and the contents of Ti, P and Ca were determined by colorimetry and ICP-OES methods. In the present study, it was shown that H_2SO_4 can be used to replace HCl in sample digestion prior to mineral analysis. Furthermore, digestion of diet and digesta using H_2SO_4 enabled sequential analysis of Ti, P and other minerals.

3.2.2. Introduction

Efficacy of supplemental phytase on performance and nutrient digestibility in broilers fed wheat based diet are now well documented (Cabahug *et al.*, 1999, Svihus *et al.*, 2013). Most studies on nutrient digestibility in relation to phytate degradation have focused on the ileal digesta (Ravindran *et al.*, 2000, Rutherfurd *et al.*, 2002, Leytem *et al.*, 2008a). In further understanding the effectiveness of supplemental phytase in elevating the adverse influence of wheat phytate on nutrient digestibility, digesta samples from each segment of the GIT were collected and analysed for several different nutrients (Rutherfurd *et al.*, 2002, Walk *et al.*, 2012d, Zeller *et al.*, 2015a).

The analyses may include proximate analysis, amino acids, polysaccharides, minerals, dietary markers, phytate and inositol-6-phosphate esters. Most of the analyses require separate sample preparation and large amount of sample dry matter which is the main constraint concerning the amount of digesta obtained from each segment of GIT. By having a common sample preparation for digesta samples which later could be used in several analyses could minimize the amount of sample required.

Morgan *et al* (2014a) has successfully shown that an inductively coupled plasma optical emission spectrophotometer (ICP-OES) assay is able to replace the UV-spectroscopy assay for rapid analysis of TiO_2 in broiler feed and ileal digesta samples and incorporate the measurement of TiO_2 into the analysis of other minerals. The ICP-OES assay has been shown to be more sensitive at quantitative analysis with improved detection limits, less time-consuming and enables simultaneous measurement of several elements. However, this rapid and efficient technique required high technology and specialized instrument and UV-spectroscopy assays probably be the most doable methods for determining Ti (Short *et al.*,1996) , P and Ca (AOAC, 2000) in a basic chemistry laboratory. Therefore, in order to have a common sample preparation for diet and digesta samples prior to Ti, P and Ca assays, this study was conducted to determine the concentration of Ti, P and Ca of diet and digesta samples from two different acid digestions (HCl versus H_2SO_4) using colorimetric methods. The use of H_2SO_4in sample digestion for P and Ca assays was also evaluated using ICP-OES assay as the reference method in order to replace HCl in sample digestion and enable simultaneous measurement of Ti. It was hypothesised that the content of P and Ca measured following either HCl or H_2SO_4 digestion would not differ from each other. Similarly, the content of P, Ca and Ti in H_2SO_4as determined by colorimetry and ICP-OES methods would be similar.

3.2.3. Material and methods

All experimental procedures were complied with the The Animals (Scientific Procedures) ACT 1986, under Animal Ethical Review Committee of University of Leeds.

3.2.3.1. Experimental diets and husbandry

Ross 308 male broilers fed on wheat/corn diets either with or without supplemental phytase at 1500 FTU/kg were used in this study. The diet was formulated to meet the specification for Ross 308 Broiler (Aviagen, 2007) except with lower content of available P (0.35%) and Ca (0.86%) and contained 0.5% titanium dioxide (TiO_2). The crumble diets were given *ad libitum* to chickens from day old to 23 d, which were randomly allocated in 16 pens with 5 chicks in a pen for each diet. Initial temperature (32 ± 2^oC) of the chicken shed was gradually reduced to 21^oC on d 23. Continuous lighting with light intensity of 40 lux was applied at start of the trial, followed with 18 h light and 6 h dark (18L:6D) lighting program on d 4 and light intensity reduction to 10 lux after d 7. Water was available all the time. On d 20, 3 birds were randomly selected, individually weighed, killed by intravenous injections of pentobarbital sodium followed with cervical dislocation. The digesta samples were collected from the gizzard and terminal ileum. Terminal ileum was defined as the segment between a distal two third of ileum away from Meckel's diverticulum to about 2 cm from ileoceccal junction. The samples were stored at -20^oC prior further processing and analysis.

3.2.3.2. Experimental design and chemical analysis

The overall flow of work is shown in Figure 3.3. Part 1 of this study, 2 different acid digestions were done prior to the determination of Ti, P, and Ca in all diets and ileal digesta samples. The sample preparation methods were; (1) HCl digestion according to AOAC Official Method 965.17 for determination of P in animal feed and pet food and (2) H_2SO_4 digestion as in method by Short *et al.* (1996) for determination of TiO_2 in chicken digesta, both with slight modification. Briefly, for HCl digestion, 0.1 g of sample was ashed at 600^oC overnight and cooled before being digested with 10 ml of 5M HCl at boiling point for 30 min. For H_2SO_4 digestion, 0.1 g sample was ashed at 600^oC overnight and cooled before being digested with 10 ml of 7.4M H_2SO_4 at boiling point for 1 h. After cooling, the digested sample solutions were poured through Whatman No.541 filter paper into 100 ml volumetric flask, diluted to 100 ml and analysed for Ti, P and Ca following ICP-OES method of AOAC Official Method 985.01 (AOAC, 2000).

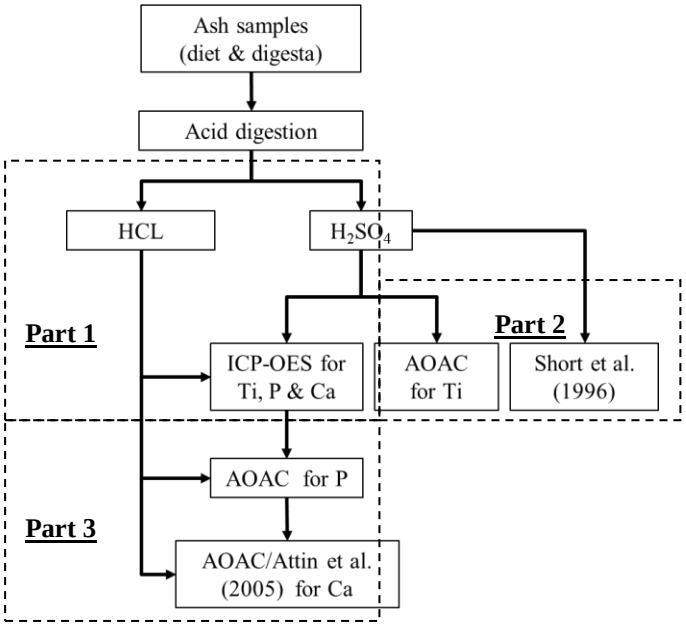


Figure 3.3. The flow of work for chemical analysis after acid digestion. Detailed procedures are described in the materials and methods (Section 2.3).

For Part 2, in order to compare 2 colorimetric methods for determining Ti content in H_2SO_4 digestion samples, digested samples from Part 1 was analysed by AOAC Official Method 973.36 Titanium in cheese, whereas a separate set of samples were prepared and analysed following as the method of Short *et al*. (1996). For Part 3, digested samples from Part 1 were analysed for P according to method of AOAC Official Method 965.17 and Ca according to method of AOAC Official Method 927.02 and Attin *et al*. (2005) with slight modifications. The detailed procedure is described in Chapter 2 Materials and Methods.

All the data were analysed using Minitab 17 Statistical Software (Minitab Inc, 2014). Diet and digesta content of Ti, P and Ca were first subjected to normality test and the non-normal data were transformed by the best estimate λ value prior to the analysis of variance (ANOVA) using general linear model (GLM). Significant differences between means were identified at $P \leq 0.05$ by Multiple Comparison of Means Tukey's Method. The Pearson's correlation coefficients of Ti and P contents from chemical assays following from two digestion methods were also calculated before a fitted regression line was plotted to illustrate the relationship between 2 sets of data resulted from the chemical assays.

3.2.4. Results and Discussion

3.2.4.1. Effect of different acid digestion on Ti, P and Ca contents

There were no significant differences observed between P content of the diets measured by ICP-OES assay following HCl or H_2SO_4 digestion (Table 3.3). Different acid digestion significantly affected ($P<0.05$) ileal P content and the content of Ca and Ti in both diet and ileal digesta. Contents of P and Ca were lower in HCl compared to H_2SO_4 and the differences in P and Ca content between the two acid digestions were larger in digesta compared to those in diets. This is probably due to different boiling times (Morgan *et al*., 2014a) and the high concentration of TiO_2 in digesta samples. Based on our observation, greyish white opaque solution was produced when acid was added to ash containing TiO_2 and H_2SO_4 solution became clear after 60 min of boiling, indicating TiO_2 was completely dissolved. For digesta sample with higher concentration of TiO_2, boiling time was about 90 min before the acid solution became clear, while HCl solution remained cloudy after boiling for 60 min. Very low Ti was detected in HCl digested samples which was unexpected and unexplainable since Ti is insoluble in HCl (FAO JECFA Monographs 13, 2012).

3.2.4.2. Effect of different colorimetric assays on Ti contents

In the present study, content of Ti measured in H_2SO_4 digested diet samples by ICP-OES method was lower than the expected value i.e. 0.5%, therefore the samples were analysed further using colorimetric methods. The content of Ti measured according to the method of Short *et al*. (1996) and AOAC official Method 973.36 Ti in cheese are presented in Table 3.4. The content of Ti in diets and ileal digesta were significantly affected by different Ti assays. Contents of Ti in diets measured by AOAC method was higher than those measured by the method of Shorts *et al.* (1996) but both measured values were close to the amount of Ti added to the diets. Ileal Ti measured by AOAC method was higher than those measured by Short *et al*. (1996) method. It was also observed that the difference in Ti contents between the two methods was larger in ileal digesta compared in diets (Table 3.4).

Table 3.3 Total phosphorus (P), calcium (Ca) and titanium (Ti) in diets and ileal digesta of broilers as prepared by different acid digestion methods.

Acid digestion	Diet			Ileal digesta		
	P (%)	Ca (%)	Ti (%)	P (%)	Ca (%)	Ti (%)
HCl	0.63±0.03	0.83±0.04[b]	0.03±0.04[b]	0.76±0.05[b]	1.43±0.12[b]	1.10±0.14[b]
H_2SO_4	0.68±0.01	0.94±0.04[a]	2.98±0.15[a]	0.98±0.02[a]	1.90±0.07[a]	10.78±0.38[a]
SEM	0.02	0.03	0.35	0.04	0.09	1.27
P-value	0.158	0.023	<0.001	<0.001	<0.001	<0.001

[a,b]Means ± SE with a different letter within a column were significantly different, $P < 0.05$

Table 3.4 Titanium (Ti) in diets, gizzard and ileal digesta of broilers as determined by Short *et al*. (1996) and AOAC Method 973.36 (Ti in cheese).

Ti assays	Diet (%)	Gizzard digesta (%)	Ileal digesta (%)
Short *et al*. (1996)	0.46±0.01[b]	0.44±0.02	1.61±0.02[b]
AOAC 973.36	0.50±0.02[a]	0.49±0.03	1.80±0.04[a]
SEM	0.02	0.02	0.03
P-value	<0.001	<0.001	<0.001

[a,b]Means ± SE with a different letter within a column were significantly different, $P < 0.05$

3.2.4.3. Effect of different assays on P and Ca contents

There was no significant effect of different assays on P content in H_2SO_4 digested samples (Table 3.5). On the other hand, concentration of P in samples digested with HCl was significantly lower ($P < 0.05$) when measured by molybdovanadate method in comparison to ICP-OES method. H_2SO_4 digestion resulted in more consistent results in both assays of P compared to HCl digestion, particularly for ileal digesta. These results suggested that either molybdovanate method or ICP-OES method used in this study was equally efficient in determining P content in diets and digesta of broilers hydrolysed by H_2SO_4.

Table 3.5 Phosphorus (P) in diets, gizzard and ileal digesta of broilers as determined by methods of molybdovanadate[1] and ICP-OES[2].

Sample type	P assays	P (%)	
		H_2SO_4 hydrolysis	HCl hydrolysis
Diet	Molybdovanadate	0.58±0.13[ab]	0.54±0.03[b]
Diet	ICP-OES	0.65±0.03[ab]	0.62±0.04[ab]
Gizzard digesta	Molybdovanadate	0.45±0.03[b]	0.28±0.05[c]
Gizzard digesta	ICP-OES	0.34±0.02[c]	0.27±0.02[c]
Ileal digesta	Molybdovanadate	0.80±0.06[a]	0.56±0.03[b]
Ileal digesta	ICP-OES	0.83±0.06[a]	0.78±0.06[a]
Main effects			
P assays			
Molybdovanadate		0.58±0.05	0.44±0.03[b]
ICP-OES		0.55±0.06	0.51±0.05[a]
Sample type			
Diet		0.61±0.05[a]	0.58±0.03[a]
Gizzard digesta		0.39±0.02[b]	0.28±0.03[b]
Ileal digesta		0.82±0.04[a]	0.66±0.05[a]
P-value			
P assays		0.447	0.032
Sample type		<0.001	<0.001
P assays x sample type		0.015	0.086

[a,b,c] Means ± SE with a different letter within a column were significantly different, $P< 0.05$;
[1]AOAC Official Method 965.17 Phosphorus in Animal Feed and Pet Food,
[2]AOAC Official Method 985.01 Metals and other elements in plants and pet foods (ICP-OES).

The advantages and disadvantages of ICP-OES method over the colorimetric method was reported by Morgan *et al*. (2014a). In the present study, colorimetric methods were shown to be as efficient as ICP-OES in analysing Ti and P in H_2SO_4 digested samples although the ICP-OES method is unquestionably more sensitive and capable of analysing multi-elements simultaneously. Beside P, colorimetric methods could be used for determining other minerals, for example Arsenazo III method (AOAC Official Method 927.02, Attin *et al*., 2005) could be used in analysing Ca content in H_2SO_4 digested samples. In the present study, Ca content in digested samples prepared in Part 1 was determined by Arsenazo III method and the content of Ca in HCl digested samples was highly correlated with those determined by ICP-OES method (Figure 3.4). Conversely, Arsenazo III method was not successful in

analysing Ca content in H_2SO_4 digested samples although H_2SO_4 solution itself did not interfere with the analysis as various levels of Ca in H_2SO_4 solution were effectively determined by Arsenazo III for the construction of Ca standard curve (data not shown). The concentration of Ti could be one of the interfering factors for Ca determination by Arsenazo III in H_2SO_4 digested sample as TiO_2 was highly dissolved in H_2SO_4 compared to in HCl (Table 3.2) and this warrants further investigation.

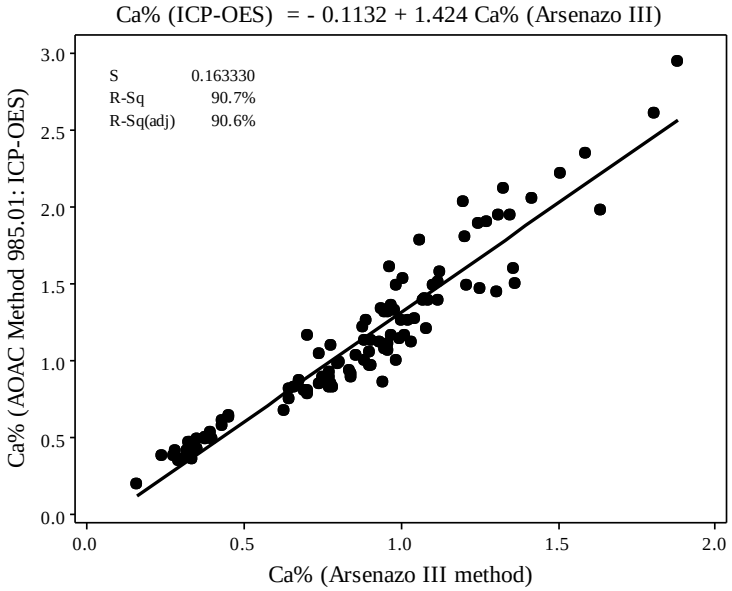


Figure 3.4 Relationship between Ca content of diet and digesta as determined by Arsenazo III methodand AOAC Official Method 985.01 (ICP-OES). HCl digestion (Pearson correlation = 0.952; P-value <0.001).

In common practice, samples are prepared separately for different analyses, for example P content in a diet containing TiO_2 is analysed using the molybdovanadate method that involves HCl digestion but TiO_2 does not fully dissolve in HCl. Therefore, Ti content is determined separately by method of Short *et al.*(1996) and consequently no sample will be left for any other analysis as total volume of sample prepared is used in this method. On the other hand, it was reported that sequential analysis of several nutrients was able to minimize the unavoidable samples losses when the analyses were performed in a single container instead of using several containers (de Coca-Sinova *et al.*, 2011).

Based on the findings of the study, therefore, the sequential analysis is proposed for determining of DM, total ash, Ti and other minerals for diet and digesta. Briefly, a 20 ml borosilicate glass vial is used to determine DM and ash content of a sample and followed by acid digestion. Total content of the vial is filtered through a filter paper into a 100 ml volumetric flask and after diluted to 100 ml with ultra-pure water, the sample is ready for analysis of minerals using either colorimetric or ICP-OES methods. The filtered residue can be further ashed for AIA determination if required.

In conclusion, it is suggested that H_2SO_4 could be used in replacing HCl in sample digestion prior to P and Ca analysis. Digestion of diet and digesta using H_2SO_4enabled sequential analysis of Ti, P and other minerals.

3.3. Study 3: Effects of anticoagulant and sample deproteinization on the concentration of myo-inositol, Ca and P in the blood of broiler chickens

3.3.1. Abstract

The objective of this study was to evaluate the effect of anticoagulant, sample deproteinization, methods for determining myo-inositol, dietary phytase treatment and feeding method on the concentration of myo-inositol, Ca and P in the blood of broiler chickens. Blood samples were collected from 28 d old broilers fed on a low P and Ca diet supplemented with phytase using tubes either with or without anticoagulants. The blood samples were deproteinized with either perchloric acid or trichloroacetic acid. All blood samples were analysed for myo-inositol, P and Ca. The results showed that circulating myo-inositol, Ca and P in chickens can be determined from plasma or serum with similar results. Either heparin or EDTA can be used as an anticoagulant in preparing plasma samples for myo-inositol, P and Ca determination except EDTA for Ca analysis. Sample deproteinization reduced myo-inositol in blood samples.

3.3.2. Introduction

Blood biochemical analysis is commonly conducted using either serum or plasma but in avian medicine, plasma is preferred. However, the choice of samples is not universal and choice of anticoagulant used depends on the analysis to be conducted (Hochleithner, 1994). Mineral concentrations especially Ca and P are usually used as indicators of animal health (Brue, 1994). Meanwhile myo-inositol is believed to have significant roles in regulating metabolism and promoting growth thus it is becoming important in the research of nutrition (Cowieson *et al*., 2011). This study was conducted to evaluate the effect of anticoagulant, sample deproteinization, myo-inositol method, dietary phytase treatment and feeding method on the concentration of myo-inositol, Ca and P in broiler chickens. It was hypothesized that myo-inositol, Ca and P will not be affected by deproteinization but will change with different type of anticoagulant.

3.3.3. Material and methods

All experimental procedures complied with The Animals (Scientific Procedures) ACT 1986, under Animal Ethical Review Committee of The University of Leeds. Twenty eight-day-old Ross, male broiler chickens fed on a wheat-corn based diet supplemented with phytase at 1500 FTU/kg were used in this study. The diet was formulated to meet the specification for Ross 308 Broiler (Aviagen, 2007). On d 28, 5 birds were individually weighed, killed by intravenous injections of pentobarbital sodium followed by exsanguinations. Blood samples were collected into 3 different tubes; without anticoagulant, Li-heparin and K_2EDTA. Serum was allowed to clot at room temperature for 1 h and centrifuged at 2300 g for 15 min. Blood in both heparin and EDTA tubes were immediately centrifuged at 2300 g, 4^{o}C for 15 min. Deproteinization was done by adding equal volumes of ice-cold either 1 M perchloric acid or 3 M trichloroacetic acid (TCA) to the samples, mixing vigorously and centrifuging at 1500 g for 10 min. The pH of supernatants was adjusted to pH range of 7 to 8 with 4 M KOH. The blood samples were analysed for myo-inositol using myo-inositol assay kits by Megazyme (K-INOSL 02/14). Plasma P and Ca were analysed using methods of molybdovanadate according to AOAC Official Method

965.17 and Arsenazo III according to AOAC Official Method 927.02 and Attin *et al*.(2005), respectively.

All the data were analysed using Minitab 17 Statistical Software (Minitab Inc, 2014). Plasma concentration of myo-inositol, P and Ca were first subjected to normality test and the non-normal data were transformed by the best estimate λ value prior to the analysis of variance (ANOVA) using general linear model (GLM). Significant differences between treatment means were identified at $P < 0.05$ by Multiple Comparison of Means Tukey's Method. The Pearson's correlation coefficient of myo-inositol concentrations from different methods were calculated before a fitted regression line was plotted to illustrate the relationship between myo-inositol concentrations resulted from colorimetry and HPLC methods.

3.3.4. Results and Discussion

The concentration of myo-inositol, Ca and P in serum were not significantly different from those in plasma, except for Ca from tubes containing K_2EDTA ($P < 0.001$) as shown in Table 3.6. The very low concentration of Ca in EDTA treated plasma was as expected, as EDTA binds Ca in preventing the formation of blood clots (Banfi *et al.*, 2007). Concentrations of plasma P and Ca observed in the present study were in contrast to those reported by Hrubec *et al*. (2002), who demonstrated that P and Ca concentrations differed between serum and plasma samples and suggested that the differences were due to the prolonged contact of serum to red blood cells.

Myo-inositol concentration in TCA deproteinized blood samples was significantly lower ($P < 0.045$) than those in non-deproteinized samples. The concentration of myo-inositol in perchloric acid deproteinized samples, on the other hand, was not significantly different from those in TCA deproteinized and non-deproteinized blood samples The differences in myo-inositol concentrations observed between deproteinized and non- deproteinized samples could probably be due to the interference of blood proteins toward chemical reactions in the assay consequently leading to overestimating myo-inositol concentration. In untreated blood samples, the concentration of myo-inositol was the highest and appeared to have the highest amount of protein among all 3 samples because in perchloric acid and TCA treated samples, most of the proteins were precipitated and removed. According to Blanchard (1981) in his evaluation of various techniques for deproteinizing plasma

found that the percentage of plasma proteins precipitated was very low with perchloric acid (35.4%) compared to TCA (99.7%) when the same amount of each acid was added to plasma samples. These results were confirmed by Rajalingam *et al*. (2009) who demonstrated that TCA precipitated proteins have very high intensity of Commassie blue stain on SDS-PAGE gel, indicating high amounts of protein in comparison to those precipitated by perchloric acid. These findings may support the suggestion that there is a linear relationship between protein concentration in blood samples and the concentration of myo-inositol as measured by colorimetric myo-inositol assay kits (Megazyme, K-INOSL 02/14), which involve the reaction between nicotinamide-adenine dinucleotide (NADH) and light sensitive iodonitrotetrazolium chloride (INT). Protein concentrations may also be the reason for numerically lower myo-inositol concentration in serum compared to in plasma as coagulant proteins were removed in serum.

Table 3.6 Effects of anticoagulants and deproteinization on the concentration of circulating myo-inositol, Ca and P in male broiler chickens fed phytase supplemented wheat-maize-soya diet

Main effects	Myo-inositol (mg/l)	Ca (mg/dl)	P (mg/dl)
Anticoagulant			
No anticoagulant	56.41±3.89	5.63±0.5[a]	5.86±0.5
Li-heparin	65.80±3.64	6.69±0.5[a]	4.68±0.4
K_2EDTA	62.50±3.51	0.02±0.0[b]	5.56±0.5
Deproteinization			
Untreated	67.38±3.66[a]	4.49±0.4	ND
Perchloric acid	63.11±3.64[ab]	3.56±0.4	ND
TCA	54.27±3.75[b]	ND	ND
P-value			
Anticoagulants	0.211	<0.001	0.179
Deproteinization	0.045	0.115	-
Anticoagulants x Deproteinization	0.457	0.975	-

[a,b]Means ± SE with different letters within a column were significantly different, P < 0.05; Deproteinization was done in triplicates; (ND) not determined

In the present study, two different methods i.e. colorimetry and HPLC were used for determination of plasma myo-inositol and the results were compared as in Table 3.7. The concentration of myo-inositol in plasma as determined by the colorimetric method was significantly higher (P = 0.025) than that determined by

HPLC. Despite the differences, there was a strong relationship between the concentrations of myo-inositol determined by both methods with Pearson's correlation coefficient of 0.601 (Figure 3.5). This result indicates that both methods can be used indistinctly for determining blood my-inositol provided a great attention is given to avoid errors when conducting the colorimetry method since reagents involved are light sensitive.

Table 3.7 Effects of analysis methods on concentration of plasma myo-inositol

Assay method	Myo-inositol (mg/l)	P-value
Colorimetry	79.46±3.64[a]	0.025
HPLC	67.61±3.45[b]	

[a,b]Means ± SE with different letters within a column were significantly different, P < 0.05.

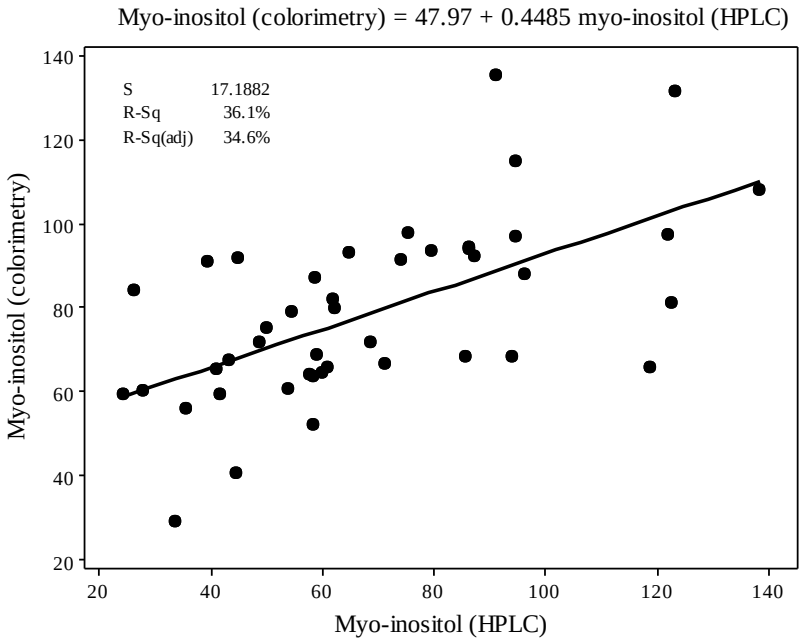


Figure 3.5 Relationship between concentrations of plasma myo-inositol as determined by Myo-inositol Assay kits (colorimetry) and HPLC. Pearson's correlation coefficient = 0.601; P < 0.001).

In conclusion, circulating myo-inositol, Ca and P in chickens can be determined from plasma or serum with similar results. Either heparin or EDTA can be used as an anticoagulant except EDTA cannot be used for Ca determination. Sample deproteinization reduced the concentration of myo-inositol in blood samples.

Chapter 4

Effect of phytase and different feeding methods prior to sampling on inositol phosphate concentrations, phytate hydrolysis and phosphorus digestibility in the gizzard and ileum of broilers

4.1. Abstract

The objective of this study was to investigate the effect of phytase supplementation, feeding methods before slaughter and their interactions on inositol phosphates (InsPs) concentrations, phytate hydrolysis and phosphorus (P) digestibility of male broilers fed a wheat based diet. It was hypothesised that different feeding methods before slaughter would change the measured effectiveness of dietary phytase and that this would be reflected in the hydrolysis of phytate, relative concentrations of InsPs and digestibility of P in the gizzard and ileum of broilers. This trial was conducted as a 2 x 3 arrangement, treatments included low P and calcium (Ca) diets either with or without phytase and 3 different feeding methods either 1 h feeding (1h), 1 h feeding followed by 1 h feed withdrawal (FW) and 1 h feed refeeding (FR) (1+1+1 h) or 5 h feeding (5 h) before slaughter and sample collection. Addition of dietary phytase at 1500 FTU/kg reduced ($P < 0.05$) the concentrations of InsPs in the gizzard and $InsP_{5-6}$ in the ileum, improved ($P < 0.05$) digestibility of P and hydrolysis of phytate and $InsP_{5-6}$ for all feeding methods. Feeding method after 6 h darkness increased ($P < 0.05$) the concentrations of InsPs, digestibility of P and hydrolysis of $InsP_{5-6}$ in both the gizzard and the ileum of broilers. However, the effects of feeding method were more prominent in the ileum compared to in the gizzard. Phytase supplementation also increased the mean retention time (MRT) in both the gizzard and the ileum but feeding method only influenced the ileal MRT. Sampling shortly after the start of light period results in higher phytate degradation especially for diets fed without phytase which might overestimate the phytate degradation for the majority of the feed digested over the daytime. Therefore, it is suggested that the sampling of digesta should be done at least 3 hours from the start of light period without 1 h FW and 1 h FR.

4.2. Introduction

The efficacy of phytase in improving P utilization in broiler chickens fed wheat based diets has been demonstrated in many studies (Cabahug *et al*., 1999; Selle *et al*., 2003; Singh *et al*., 2003; Wu *et al*., 2004a; Kühn *et al*., 2011). The magnitude of growth response has not been consistent in these studies and factors contributing to the variability include levels of dietary phosphorus (P), calcium (Ca) and phytase supplementation (Zeller *et al*., 2015a; Beesons *et al*., 2014; Walk *et al*., 2014). The concentration of inositol phosphates ($InsP_{3-6}$), the values of phytate hydrolysis and digestibility in gastrointestinal tract (GIT) have also varied depending on the presence of intrinsic phytase of plant origin or endogenous phytase (Morgan *et al*., 2015; Zeller *et al*., 2015c; Shastak *et al*., 2014), pH conditions (Menezes-Blackburn *et al*., 2015) and enzyme-digesta contact time in each segment of the GIT as demonstrated by different incubation time in *in vitro* studies (Walk *et al*., 2012; Denstadli *et al*., 2006). The main sites of phytate degradation by supplemental phytases in poultry are the crop, proventriculus and gizzard and little phytate degradation takes place in the small intestines (Selle and Ravindran, 2007; Dersjant-Li *et al*., 2014). Leslie (2006) found that phytase supplementation in diets with adequate levels of available P (aP) significantly increased phytate degradation in the crop and proventriculus-gizzard but very little phytate degradation occurred in duodenum-jejunum and ileum. Similar findings were reported by Zeller *et al*. (2015b) when they characterized the degradation of phytate and the formation of InsPs isomers in different GIT segments of broilers fed on low P and Ca diets. Zeller *et al*. (2015b) also demonstrated further activity of supplemental phytase in duodenum-jejunum and to some extent in the ileum as indicated by the increase of InsP5 and InsP4 isomers concentrations.

In evaluating phytate degradation and nutrient digestibility in different segments of GIT, several aspects should be considered before and during digesta collection including feeding regime, feed structure, time and method of killing and sampling site. Kadim and Moughan (1997a) demonstrated that sampling time between 2 h to 6 h following the start of feeding influenced the amount of digesta collected, with the greatest dry matter (DM) content collected at 4 h when ileal digesta was sampled from the final two third of the ileum. However, in that study, the chickens were fasted for 24 h and then either given free access to the diet or forced

fed via intubation tube before being sacrificed. Rodehutscord *et al*. (2012) suggested the same site for collecting ileal digesta but the chickens were fed *ad libitum* without mentioning details of the feeding method before slaughter. Lighting program and time of sampling have also been shown to influence the degree of dephosphorylation of phytate molecules (Leslie, 2006). Degradation of phytate and InsPs in the ileum and excreta of broilers fed diets with phytase supplementation were improved with a 12 h lighting program due to slower feed passage compared to continuous light. Leslie (2006) also suggested that timing of dissection may be responsible for the lack of effect of supplemental phytase on phytate degradation in the crop and proventriculus-gizzard as chickens rapidly consumed the diet as soon as scotoperiod ended. Our previous study with 21 day old broiler chickens fed on mash corn based diets (Chapter 3: Study 1) showed that FW and FR were not necessary to obtain adequate amounts of digesta from different segment of the GIT when feed was withdrawn for either 1 or 4 h followed by FR period of 1, 4 or 6 h. However, phytate degradation was not investigated in this study. In several digestibility studies, overnight FW is to allow clearance of the GIT and to stimulate the intake of test diets before collection of digesta samples. Alternatively, providing at least 6 h of darkness could be used to prevent feed intake consequently empty the GIT and with chickens eating feed as soon as light returns (Duve *et al*., 2011).

Thus, the present study was designed to test the hypothesis that different feeding methods after a 6 h scotoperiod would change the measured effectiveness of dietary phytase and that this would be reflected in the hydrolysis of phytate, relative concentrations of InsPs and apparent digestibility of P in the gizzard and ileum of broilers. The primary aim of this study was to investigate the effect of phytase supplementation at 1500 FTU/kg, different feeding methods prior to slaughter and their interaction on InsPs concentrations, phytate hydrolysis and P digestibility in male broilers fed a wheat based diet. In addition, the effects on growth performance, total DM and pH of digesta and digesta MRT were evaluated.

4.3. Materials and Methods

All experimental procedures complied with The Animals (Scientific Procedures) ACT 1986, under Animal Ethical Review Committee of The University of Leeds.

4.3.1. Experimental animal and diets

One-day-old Ross, male broiler chickens were obtained from a commercial hatchery (P. D. Hook (Hatcheries) Ltd, Bampton, Oxfordshire) and transported to the research farm, the University of Leeds. These birds were vaccinated with Infectious Bronchitis (IBH) and Marek's disease vaccines at the hatchery. Upon arrival, the chicks were individually weighed, given a leg band for identification and randomly allocated into 16 pens (15 or 16 chicks per pen). Each pen had a floor space of 82 cm x 75 cm, equipped with 6 nipple drinkers and 3 feeders. On day 14, 96 birds from each diet group were weighed and randomly re-allocated into 16 smaller pens with 6 birds per pen (a floor space of 82 cm x 50 cm). The floor of each pen was covered with AstroTurf with a layer of wood shavings. The pens complied with DEFRA recommendations and met the Welfare of Farmed Animals (England) Regulations 2007 and Code of Practice for the housing and care of animals used in scientific procedures (1989). The temperature of the broiler shed was initially set at 32^{o}C ± 2^{o}C and gradually reduced to 24^{o}C on d 14 and to 21^{o}C on d 23. Relative humidity (RH) was recorded on a daily basis. Continuous ventilation, continuous lighting with light intensity of 40 lux from d 1 to d 3 and 18 h light and 6 h dark (18L: 6D) from d 3 to d 23 were applied. The intensity of the light was reduced to 10 lux after d 7. Feed and water were provided *ad libitum* at all times except during FW period.

Wheat-corn soybean meal based diets were formulated to meet or exceed the specification for As-Hatched Broilers Grown to <1.9kg (4.2lb) live weight for Ross 308 Broiler (Aviagen, 2007) except for the level of available P (aP) and Ca, which were reduced to 0.35 % and 0.86 %, respectively. The diets were; (1) negative control (Diet NC) with reduced aP and Ca and 2) NC plus phytase (Quantum Blue 5G, AB Vista, UK) at 1500 FTU/kg (300 g/t) (Diet P). The NC diet was not expected to reduce growth performance by more than 20% when compared to diet P which met all nutrient requirements when taking the matrix values for aP and Ca delivered by the phytase into consideration. The experimental diets also contained titanium dioxide (5 g TiO_2/kg) as the indigestible marker as a reference for nutrient digestibility. All diets were formulated to be isonitrogenous and isoenergetic (Table 4.1) and fed in crumble form.

Table 4.1 Composition, calculated and analysed nutrient contents of the experimental diets (%) for young broiler chickens

Ingredients (%)		Negative control (NC)	NC + Phytase (P)[1]
Wheat		41.1	41.1
Corn		23.8	23.8
Soya extract (48%)		19.7	19.7
Potato protein (79%)		6.1	6.1
Corn gluten meal (62%)		1.4	1.4
Soybean oil		2.8	2.8
Lysine HCl		0.36	0.36
Methionine		0.15	0.15
Threonine		0.07	0.07
Dicalcium phosphate, anhyd., 18% P		1.45	1.45
Limestone		0.73	0.73
Salt (NaCl)		0.12	0.12
Sodium carbonate		0.17	0.17
Vitamin & mineral premix[2]		0.40	0.40
Titanium dioxide (TiO_2)		0.50	0.50
Binder (Ligno Bond DD)		1.25	1.25
Phytase		0.00	0.03
Total		100.00	100.03
Calculated nutrients	*Recommended level*		
AME Chick MJ/kg	12.65	12.67	12.67
Crude Protein	22-25	22.03	22.03
Energy: protein	0.575	0.575	0.575
Lysine	1.43	1.43	1.43
Methionine	0.51	0.51	0.51
Threonine	0.94	0.94	0.94
Ca	1.05	0.86	0.86
P		0.62	0.62
aP	0.50	0.35	0.35
Ca:aP	2.10	2.46	2.46
Phytate-P		0.24	0.24
Sodium	0.16	0.13	0.13
Chloride	0.16-0.23	0.16	0.16
Fibre		2.10	2.10
Fat		4.78	4.78
Analysed nutrients			
Total Ca		0.89	1.02
Total P		0.59	0.60
Phytate-P		0.27	0.27

[1]Diet P has the same compositions as Diet NC but supplemented with phytase Quantum Blue 5G at 300g/t to achieve 1500 FTU/kg diet.
[2]Premixes provided the following (per kg of diet): vitamin A, 13,200 IU; vitamin D3, 4,000 IU; vitamin E, 66 IU; vitamin B12, 39.6 µg; riboflavin, 13.2 mg; niacin, 110 mg; Dpantothenate, 22 mg; menadione (K3), 4 mg; folic acid, 2.2 mg; thiamine, 4 mg; pyridoxine, 8 mg; D-biotin, 252 µg; selenium (as Na2SeO3), 0.30 mg; manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; cobalt, 1.0 mg; choline chloride, 1,200 mg; coccidiostat, 500 mg.

4.3.2. Experimental procedures and sampling

This trial was conducted as a 2 x 3 arrangement of treatments with 2 diets, either with or without phytase and 3 different feeding methods either 1 h feeding (1 h), 1h feeding followed by 1 h FW and 1 h RF (1+1+1 h) or 5 h feeding (5 h) following a 6 h dark period. On d 1, both diets (NC and P) were randomly assigned to the 16 larger pens (8 pens fed the NC diet and 8 pens fed the P diet). On d 14, the birds in each pen were randomly re-allocated into the smaller pens and remained on their designated diet. Chickens were grown until 23 d of age. Individual body weight and pen feed intake were recorded on d 7, 14, 19 and at each sampling time. Mortality and chicken health were recorded daily. Chickens from each diet group were killed and gut contents were sampled at the end of the trial at one of 3 different feeding methods (Figure 4.1) creating 6 treatments (Table 4.2). The "feeding method" treatment was assigned to the trial pens based on randomized block design according to the arrangement of treatments in Table 4.3.

Table 4.2 Treatments, phytase dosage and added phytase activity of the diets

Treatments	Diet	Feeding method[1]	Added activity (FTU/kg diet)	Measured activity (FTU/kg diet)
T1	- phytase	1 h	-	273
T2	- phytase	1+1+1 h[2]	-	254
T3	- phytase	5 h	-	235
T4	+ phytase	1 h	1500	1760
T5	+ phytase	1+1+1 h	1500	1850
T6	+ phytase	5 h	1500	1860

(-) without phytase; (+) with phytase; [1]after 6 h dark period and before slaughter; [2]1 h feeding followed by 1 h FW and 1 h RF

Sampling took place over a 4 d period (d 20 to 23). A day before each sampling point, feed residues in each pen was weighed and the time of weighing is recorded as "Start Time" of feeding. At each sampling point, 3 chickens were randomly selected from each of 2 pens (6 chickens in total), individually weighed and killed by cervical dislocation and followed by exsanguination. Blood samples were collected into Li-heparin tubes. The time of slaughter was recorded as "End Time" for feeding period and feed residues were re-weighed and recorded. The abdomen was opened and the GIT exposed. The oesophagus just before proventriculus and the lower part of gizzard were carefully clamped. The terminal

ileum which was defined as the two third of the ileum away from Meckel's diverticulum to about 2 cm from ileocecal junction (Rodehutscord *et al*., 2012) was also clamped before both sections were cut out and put into separate pre-weighed clean containers. The weight of both proventriculus-gizzard and terminal ileum before digesta collection and the empty weight of the guts were recorded. The digesta from all 3 birds representing that treatment were pooled and mixed before pH measurement. All collected digesta samples were stored at -20°C until further analysis.

Table 4.3 Allocation of treatments to experimental pens

Sampling day	Pen[1]	Diets	Feeding method[2]	1 h feeding		1+1+1 h[3] or 5 h feeding	Treatment	Rep
				Treatment	Rep			
Day 1	1	(-)	1+ (1+1+1)	T1	1	1+1+1 h	T2	1
	2	(-)	(1+1+1)			1+1+1 h	T2	2
	3	(-)	1+5	T1	2	5 h	T3	2
	4	(-)	5			5 h	T3	1
	5	(+)	1+(1+1+1)	T4	1	1+1+1 h	T5	1
	6	(+)	(1+1+1)			1+1+1 h	T5	2
	7	(+)	1+5	T4	2	5 h	T6	2
	8	(+)	5			5 h	T6	1
Day 2	9	(-)	1+(1+1+1)	T1	3	1+1+1 h	T2	3
	10	(-)	(1+1+1)			1+1+1 h	T2	4
	11	(-)	1+5	T1	4	5 h	T3	4
	12	(-)	5			5 h	T3	3
	13	(+)	1+(1+1+1)	T4	3	1+1+1 h	T5	3
	14	(+)	(1+1+1)			1+1+1 h	T5	4
	15	(+)	1+5	T4	4	5 h	T6	4
	16	(+)	5			5 h	T6	3
Day 3	17	(-)	1+(1+1+1)	T1	5	1+1+1 h	T2	5
	18	(-)	(1+1+1)			1+1+1 h	T2	6
	19	(-)	1+5	T1	6	5 h	T3	6
	20	(-)	5			5 h	T3	5
	21	(+)	1+(1+1+1)	T4	5	1+1+1 h	T5	5
	22	(+)	(1+1+1)			1+1+1 h	T5	6
	23	(+)	1+5	T4	6	5 h	T6	6
	24	(+)	5			5 h	T6	5
Day 4	25	(-)	1+(1+1+1)	T1	7	1+1+1 h	T2	7
	26	(-)	(1+1+1)			1+1+1 h	T2	8
	27	(-)	1+5	T1	8	5 h	T3	8
	28	(-)	5			5 h	T3	7
	29	(+)	1+(1+1+1)	T4	7	1+1+1 h	T5	7
	30	(+)	(1+1+1)			1+1+1 h	T5	8
	31	(+)	1+5	T4	8	5 h	T6	8
	32	(+)	5			5 h	T6	7

[1]During the trial, diet (-) without and (+) with phytase were randomly assigned to the pens; [2]Birds from the same pen were dissected at 2 sampling times; "1+(1+1+1)h" and "1+5" means 3 birds were dissected after 1 h feeding and another 3 birds were dissected after 1+1+1 h or 5 h feeding; [3]1 h feeding followed by 1 h feed withdrawal and 1 h refeeding.

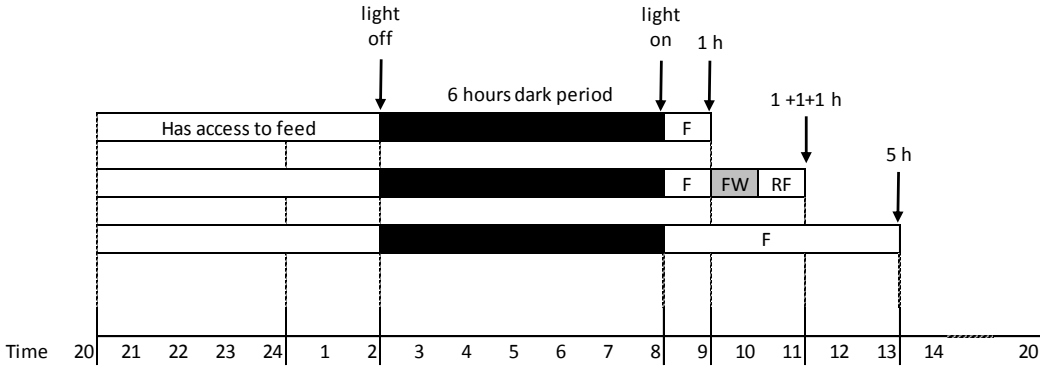


Figure 4.1 Schematic diagram of feed access before slaughter and sampling method, feeding (F), feed withdrawal (FW) and refeeding (RF). Black bars represent the daily 6 h dark period. White bars represent a period when the birds have access to feed under 10 lux light intensity and grey bar represents 1 h FW. Arrows indicate times for dissection and sampling of gut contents

4.3.3. Chemical assays, Calculations and Statistical analysis

Prior to analysis, digesta samples were freeze dried and ground through a 1.0 mm sieve. The content of P in the diets, gizzard and ileal digesta were determined in accordance with AOAC (2000) with slight modification (Chapter 3: Study 2). Phytase activity in feed samples was determined by Enzyme Services and Consultancy Ltd, Wales, according to the internal, validated methods of the producer (Quantum method). Feed samples were a stored at -18°C until InsPs analysis. Titanium (Ti) was measured on a UV spectrophotometer following the method of AOAC Official Method 973.36 (AOAC, 2000). Concentrations of $InsP_{3-6}$ and inositol in the diets and digesta samples were analysed by high-pressure ion chromatography (HPIC) and high-performance liquid chromatography (HPLC), respectively. Digestibility of DM, P and phytate-P and hydrolysis of $InsP_6$ and $\sum InsP_{5-6}$ in the gizzard and terminal ileum (Y) were calculated as follows:

$$Y\ (\%) = \frac{(NT/Ti)_{diet} - (NT/Ti)_{digesta}}{(NT/Ti)_{diet}} \times 100$$

where $(NT/Ti)_{diet}$ = ratio of nutrient and Ti in diet and $(NT/Ti)_{digesta}$ = ratio of nutrient and Ti in digesta.

The digesta MRT in each part of the digestive tract was calculated according to Method 1 by Van Der Klis *et al.* (1990) using the following equations where 1440 is minutes per day (24 h);

Feed intake (FI) in g = weight of feed residue at "Start Time" of feeding - weight of feed residue at "End Time" of feeding

Total weight of digesta (g) = full weight of a segment - empty weight of a segment

Ti in segment (mg) = Total DM in a segment (g) x Ti concentration (mg/g digesta)

$$\text{Ti Intake (mg/min)} = \frac{\text{FI (g) x Ti concentration (mg/g diet)}}{\text{(End time - start time of feeding x 1440)}}$$

$$\text{Digesta MRT (min)} = \frac{\text{Ti in segment (mg)}}{\text{Ti intake (mg/min)}}$$

Data were analysed using general linear model (GLM) to assess the effects of phytase supplementation, feeding method and their interactions using Minitab 17 Statistical Software (Minitab Inc, 2014). Significant differences between the treatments were identified at the 95% confidence level by Multiple Comparison of Means using Tukey's Method.

4.4. Results

The analysed dietary Ca and P in the diets were similar to formulated values and within acceptable ranges (Table 4.1). Measured phytase activity in the experimental diets are presented in Table 4.2. Phytase activity was as expected and in agreement with the experimental design.

4.4.1. Broiler performance

Growth performance of male broilers from d 0 to d 19 is presented in Table 4.4. Supplementation of phytase at 1500 FTU/kg improved (P < 0.05) body weight gain (BWG) throughout the trial. Phytase increased (P < 0.05) feed intake (FI) to d 14. Feed conversion ratio (FCR) between 0-7 d and 0 - 14 d were not affected by phytase, however FCR was significantly improved (P < 0.05) between 0-19 d by phytase supplementation.

Table 4.4 Effect of phytase on growth performances of male broiler chickens fed a wheat/corn/soy-based diet

Phytase, FTU/kg	Day 0 -7	Day 0-14	Day 0 -19
	Body weight gain (g/bird)		
0	123.97(2.32)[b]	448.08(6.72)[b]	836.35(7.87)[b]
1500	136.89(1.38)[a]	493.98(5.55)[a]	890.51(6.21)[a]
SEM	2.12	7.27	6.92
P-value	0.003	0.002	<0.001
	Feed intake (g/bird)		
0	147.72(2.23)	550.44(7.83)[b]	1035.96(8.41)
1500	149.56(1.56)	574.33(4.70)[a]	1039.08(15.1)
SEM	1.33	5.38	8.50
P-value	0.247	0.004	0.813
	Feed conversion ratio (FCR)		
0	0.88(0)	1.12(0)	1.18(0.01)[a]
1500	0.83(0.01)	1.07(0)	1.11(0.02)[b]
SEM	0.01	0.01	0.01
P-value	0.076	0.312	0.014

[a,b]Means(±SE) without a different letter within a column were not significantly different, $P < 0.05$

4.4.2. Total dry matter and pH of digesta

Phytase and feeding method had no effect on total DM or pH of gizzard contents. Phytase also had no effect on total DM and pH of ileal digesta. However, feeding method increased total DM (P < 0.01) and pH value (P < 0.05) of ileal digesta (Table 4.5).

4.4.3. Concentrations of phosphorus, inositol phosphates and inositol

Concentrations of total P, phytate-P, non phytate-P, InsPs and inositol in the experimental diets are presented in Table 4.6. Both diets contained similar concentrations of all forms of P, InsPs and inositol. Concentrations of total P and non phytate-P were close to the calculated values in the formulation (Table 4.1). Concentrations of phytate-P as presented by the sum of all P in the InsPs in Table 4.6 were higher than the calculated values and were confirmed by additional phytate-P analysis in mg/g by scanning on Foss NIR spectrometer with the phytate-P levels predicted using AUNIR calibration standards. The concentration of non phytate-P in nmol/g was not significantly different (diet without phytase, $P = 0.232$; diet with phytase, $P = 0.301$) from total P but the concentration of non phytate-P in mg/g were significantly lower ($P < 0.001$ for both diets) than total P. $InsP_6$ and $InsP_5$ were the main InsPs detected in the diets, accounting for between 86 to 90% of total InsPs. The ratio of $InsP_6$ to $InsP_5$ was about 1:1. Low concentrations of $InsP_4$, $InsP_3$ and inositol were also detected. Due to the different results obtained when non phytate-P was compared with total P in 2 different concentration units (nmol/g versus mg/g), only 1 unit concentration i.e. nmol/g was used throughout the rest of this report.

Supplementation of phytase significantly affected ($P < 0.005$) the concentration of phytate-P, InsPs and inositol in both the gizzard and ileum (Table 4.7 and Table 4.8). In the gizzard, supplementation with phytase reduced ($P \leq 0.005$) concentrations of $InsP_6$, $InsP_5$, $InsP_4$ and $InsP_3$ but increased inositol concentration compared to concentrations in the gizzard of non-supplemented control birds ($P < 0.005$). In the ileum, phytase supplementation reduced the concentration of total P ($P < 0.01$), phytate-P, $InsP_6$ and $InsP_5$ ($P < 0.001$) and increased the concentration of $InsP_4$, $InsP_3$ and inositol ($P < 0.001$) compared to control.

Table 4.5 The effects of phytase and feeding method on total dry matter weight and pH of gizzard contents and terminal ileal digesta in male broilers fed a wheat/corn/soy-based diet.

Item	Feeding method[1]	Gizzard content		Terminal ileal digesta	
		Total DM (g/bird)	pH	Total DM (g/bird)	pH
Phytase, FTU/kg					
0		3.28(0.41)	2.52(0.09)	1.05(0.05)	7.14(0.06)
1,500		3.75(0.90)	2.73(0.12)	0.99(0.05)	7.05(0.10)
SEM		0.47	0.15	0.08	0.12
Feeding method, h					
1		3.07(0.27)	2.44(0.14)	0.86(0.07)[b]	6.87(0.09)[b]
1+1+1[2]		3.76(0.53)	2.77(0.09)	1.10(0.04)[a]	7.16(0.10)[ab]
5		3.72(0.39)	2.67(0.15)	1.10(0.07)[a]	7.26(0.09)[a]
SEM		0.71	0.22	0.10	0.16
Phytase, FTU/kg					
0	1	2.77(0.36)	2.49(0.17)	0.88(0.11)	6.86(0.08)
0	1+1+1	2.90(0.41)	2.61(0.11)	1.16(0.08)	7.22(0.09)
0	5	4.17(0.42)	2.47(0.18)	1.12(0.07)	7.33(0.06)
1,500	1	3.36(0.41)	2.39(0.22)	0.84(0.09)	6.87(0.17)
1,500	1+1+1	4.61(0.90)	2.94(0.12)	1.04(0.04)	7.11(0.18)
1,500	5	3.27(0.65)	2.87(0.23)	1.08(0.11)	7.19(0.16)
SEM		1.37	0.44	0.21	0.33
P-value					
Phytase		0.314	0.146	0.351	0.452
Feeding method		0.389	0.170	0.009	0.014
Phytase x feeding method		0.076	0.322	0.875	0.844

[a,b]Means (±SE) with a different letter within a column were significantly different, $P < 0.05$;

[1]Duration from the end of 6 hours dark to time of sampling; [2]1 h feeding followed by 1 h FW and 1 h RF

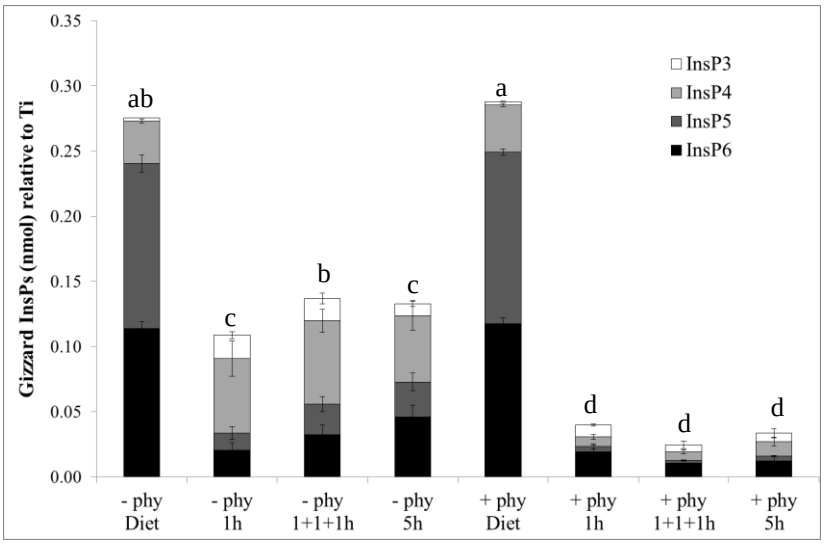
Table 4.6 Concentrations of total phosphorus, non phytate-P, phytate-P, inositol phosphates and inositol in experimental diets

Phytase, FTU/kg	0	1,500	SEM	*P*-value
	nmol/g			
Total P	193,427(2145)[a]	198,396(2656)[a]	2,401	0.219
Non phytate-P	189,074(2223)[a]	193,854(2756)[a]	2,490	0.248
Phytate-P	4,352(198)[c]	4,542(106)[d]	152	0.445
$InsP_6$	6,417(295)[b]	6,627(295)[c]	295	0.640
$InsP_5$	7,156(291)[b]	7,420(291)[b]	291	0.557
$InsP_4$	1,839(80)[d]	2,055(80)[e]	80	0.128
$InsP_3$	146(7)[e]	140(7)[f]	7	0.613
Inositol	211(16)[e]	193(16)[f]	16	0.459
P-value	<0.001	<0.001		
	mg/g			
Total P	6.00(0.07)[a]	6.15(0.08)[a]	0.074	0.219
Non phytate-P	3.45(0.16)[c]	3.50(0.14)[c]	0.150	0.832
Phytate-P	2.64(0.06)[d]	2.65(0.06)[d]	0.064	0.926
$InsP_6$	4.24(0.21)[b]	4.37(0.18)[b]	0.194	0.640
$InsP_5$	4.15(0.23)[b]	4.30(0.08)[b]	0.152	0.557
$InsP_4$	0.92(0.04)[e]	1.03(0.04)[e]	0.040	0.128
$InsP_3$	0.06(0.00)[f]	0.06(0.00)[f]	0.003	0.613
Inositol	0.04(0.00)[f]	0.03(0.00)[f]	0.003	0.097
P-value	<0.001	<0.001		

[a,b,c,d,e,f] Means with a different letter within a column were significantly different, $P < 0.001$

Feeding method only had a significant effect on total P and non phytate-P ($P < 0.05$) in the gizzard. The concentrations of total P and non phytate-P was increased by feeding method. The concentrations of $InsP_6$ and $InsP_5$ also had a tendency to be higher at 1+1+1 h and 5 h than the 1 h feeding method. In the ileum, the concentrations of all forms of P and InsPs were higher at 1+1+1 h and 5 h compared to the 1 h feeding method. However, the concentrations of P and InsPs at 1+1+1 h feeding method were not different from those detected at 5 h. Inositol concentration was not affected ($P = 0.088$) by feeding method. There were no interactions between phytase supplementation and feeding method for any of the P compounds studied. In contrary, the concentration of marker related $InsP_6$ in ileal digesta for birds fed non-phytase supplemented diets were significantly higher at 1+1+1 h and 5 h sampling compared to the initial concentration of marker related $InsP_6$ in the diet. (Figure 4.2).

A



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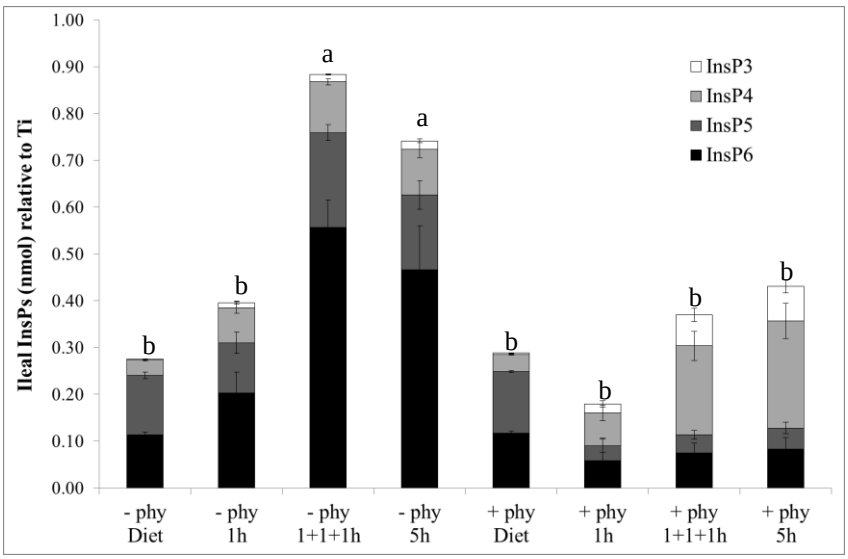

Figure 4.2 The concentrations of InsPs in digesta of (A) gizzard and (B) ileum, expressed as ratio of marker, Ti. -phy, diet without phytase; +phy, diet containing phytase. 1 h, 1+1+1+ h and 5 h are the feeding method. [a,b,c,d] Means (± SE) of the sum of InsPs with different letters were significantly different ($P < 0.05$).

Table 4.7 Concentrations of total P, phytate-P, non-phytate-P, inositol phosphates and inositol in the gizzard

Item	Feeding method[1]	Total P	Phytate-P	Non phytate-P	$InsP_6$	$InsP_5$	$InsP_4$	$InsP_3$	Inositol
Phytase, FTU/kg		nmol/g							
0		134,272(1954)	2,149(168)^{a}	132,122(1916)	2,365(207)^{a}	1,676(156)^{a}	3,900(288)^{a}	876(93)^{a}	620(104)^{b}
1,500		128,921(3279)	522(59)^{b}	128,399(3290)	997(281)^{b}	195(212)^{b}	613(391)^{b}	391(126)^{b}	1,120(142)^{a}
SEM		2,617	113	2,603	244	184	339	110	123
Feeding method, h									
1		124,641(3034)^{b}	1,280(223)	123,361(2898)^{b}	1,259(319)	658(240)	2,376(443)	784(133)	810(161)
1+1+1[2]		136,188(3752)^{a}	1,347(261)	134,841(3757)^{a}	1,492(290)	891(218)	2,073(402)	585(121)	723(146)
5		133,961(2581)^{ab}	1,380(296)	132,580(2564)^{ab}	2,292(298)	1,258(225)	2,321(414)	533(125)	1,077(150)
SEM		3,122	260	3,073	302	228	420	136	152
Phytase, FTU/kg									
0	1	130,483(4,329)	1,927(277)^{a}	128,556(4124)	1,464(349)^{b}	1,101(263)^{abc}	4,230(485)^{a}	1,090(157)^{a}	701(176)^{ab}
0	1+1+1	136,092(2758)	2,285(197)^{a}	133,807(2655)	2,325(349)^{ab}	1,653(263)^{ab}	3,633(485)^{a}	944(157)^{ab}	548(176)^{b}
0	5	136,241(2867)	2,236(391)^{a}	134,005(3063)	3,305(377)^{a}	2,274(284)^{a}	3,837(524)^{a}	595(170)^{ab}	609(190)^{b}
1,500	1	118,799(3312)	633(129)^{b}	118,166(3356)	1,053(534)^{b}	215(402)^{bc}	521(741)^{b}	477(240)^{ab}	918(269)^{ab}
1,500	1+1+1	136,284(7261)	409(409)^{b}	135,875(7290)	660(462)^{b}	129(48)^{c}	514(642)^{b}	225(208)^{b}	898(233)^{ab}
1,500	5	131,680(4342)	525(525)^{b}	131,156(4268)	1,278(462)^{b}	243(348)^{c}	804(642)^{b}	471(208)^{ab}	1,545(233)^{a}
SEM		4,145	191	4,127	422	318	587	190	213
P-value									
Phytase		0.145	<0.001	0.306	0.001	<0.001	<0.001	0.005	0.009
Feeding method		0.029	0.901	0.030	0.059	0.201	0.861	0.422	0.236
Phytase x feeding method		0.408	0.413	0.370	0.177	0.239	0.836	0.263	0.228

^{a,b}Means (±SE) with a different letter within a column were significantly different, $P < 0.05$; [1]Duration from the end of 6 hours dark to time of sampling; [2]1 h feeding followed by 1 h FW and 1 h RF

Table 4.8 Concentrations of total P, phytate-P, non-phytate-P, inositol phosphates and inositol in ileal digesta

Item	Feeding method[1]	Total P	Phytate-P	Non phytate-P	$InsP_6$	$InsP_5$	$InsP_4$	$InsP_3$	Inositol
Phytase, FTU/kg		nmol/g							
0		316,563(12621)^{a}	12,010(1146)^{a}	304,553(11864)	28,329(1279)^{a}	10,804(467)^{a}	6,202(784)^{b}	952(289)^{b}	2,085(548)^{b}
1,500		286,373(13527)^{b}	5,708(617)^{b}	280,665(13133)	4,764(1315)^{b}	2,459(480)^{b}	10,148(806)^{a}	3,294(297)^{a}	6,180(563)^{a}
SEM		13,074	883	12,499	1,297	473	795	293	556
Feeding method, h									
1		238,242(16078)^{b}	5,314(860)^{b}	232,928(15491)^{b}	8,496(1665)^{b}	4,628(608)^{b}	5,112(1020)^{b}	1,200(376)^{b}	3,719(713)
1+1+1[2]		318,647(8887)^{a}	10,228(1276)^{a}	308,419(8294)^{a}	18,704(1496)^{a}	7,026(546)^{a}	8,812(917)^{a}	2,511(338)^{a}	4,144(641)
5		347,515(8233)^{a}	11,036(8233)^{a}	336,479(7600)^{a}	22,438(1600)^{a}	8,240(584)^{a}	10,599(980)^{a}	2,657(361)^{a}	4,535(685)
SEM		11,066	1,212	10,462	1,587	579	973	358	680
Phytase, FTU/kg									
0	1	254,498(22809)^{bc}	7,002(1194)^{b}	247,496(21915)^{bc}	14,068(2262)^{b}	7,523(826)^{b}	5,114(1386)^{c}	843(511)^{b}	2,966(969)^{bc}
0	1+1+1	329,758(9049)^{a}	14,288(1249)^{a}	315,470(8173)^{a}	32,440(2116)^{a}	11,615(772)^{a}	6,194(1297)^{bc}	881(478)^{b}	1,571(907)^{c}
0	5	365,433(7183)^{a}	14,740(2152)^{a}	350,693(7351)^{a}	38,478(2262)^{a}	13,274(826)^{a}	7,296(1386)^{bc}	1,130(511)^{b}	1,719(969)^{c}
1,500	1	221,986(22629)^{c}	3,625(963)^{b}	218,360(22080)^{c}	2,925(2443)^{c}	1,733(892)^{c}	5,110(1498)^{c}	1,557(552)^{b}	4,473(1047)^{ab} ^{c}
1,500	1+1+1	307,536(14876)^{ab}	6,167(840)^{b}	301,368(14622)^{ab}	4,968(2116)^{bc}	2,436(772)^{c}	11,430(1297)^{ab}	4,141(478)^{a}	6,718(907)^{ab}
1,500	5	329,597(12132)^{a}	7,331(1046)^{b}	322,266(11653)^{a}	6,398(2262(^{bc}	3,206(826)^{c}	13,903(1386)^{a}	4,185(511)^{a}	7,351(969)^{c}
SEM		14,780	1,241	14,299	2,244	819	1,375	507	961
P-value									
Phytase		0.026	<0.001	0.066	<0.001	<0.001	0.001	<0.001	<0.001
Feeding method		<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.015	0.714
Phytase x feeding method		0.906	0.163	0.861	<0.001	0.038	0.060	0.033	0.088

^{a,b}Means (±SE) with a different letter within a column were significantly different, $P < 0.05$; [1]Duration from the end of 6 hours dark to time of sampling; [2]1 h feeding followed by 1 h FW and 1 h RF

4.4.4. Apparent Digestibility of dry matter, phytate-P and hydrolysis of $InsP_6$, $InsP_5$ and $\sum InsP_{5-6}$

Apparent digestibility of DM, total P, phytate-P and hydrolysis of $InsP_6$ as well as the sum of $InsP_{5-6}$ ($\sum InsP_{5-6}$) in the gizzard and lower ileum are presented in Tables 4.9 and 4.10, respectively. Supplementation of phytase had no influence on the digestibility of DM and total P but significantly increased phytate-P disappearance up to the gizzard. In the ileum, phytase increased P digestibility ($P=0.05$) and phytate-P digestibility ($P<0.01$) but did not affect DM digestibility. Phytase supplementation also resulted in a significant increase ($P<0.001$) in $InsP_6$ and the sum of $InsP_{5-6}$ hydrolysis in both the gizzard and the ileum. However, negative $InsP_6$ hydrolysis was calculated for broilers fed the non-phytase supplemented diets.

In the gizzard, feeding method only affected P disappearance ($P<0.001$) which was reduced from 40.95% at 1 h to 33.24% at 3 h and 34.31% at 5 h but had no other effects on InsPs hydrolysis, although a significant interaction between phytase supplementation and feeding method was observed for $InsP_6$ hydrolysis. In contrast, ileal nutrient digestibility and ileal $InsP_{5-6}$ hydrolysis were significantly affected by feeding method. Digestibility of DM was increased but digestibility of total P and phytate-P were reduced in 1+1+1 h and 5 h compared to the 1 h feeding method. Hydrolysis of ileal InsP6, InsP5 and the sum of $InsP_{5-6}$ were also reduced with FW and longer feeding duration. Significant interactions between phytase supplementation and feeding method on apparent phytate-P digestibility and hydrolysis of $InsP_6$ and $\sum InsP_{5-6}$ were also observed. Without supplemental phytase, phytate-P degradation and $\sum InsP_{5-6}$ hydrolysis was reduced with different feeding methods prior o sampling but phytase supplementation evened out t he P digestibility and $\sum InsP_{5-6}$ hydrolysis over 3 different feeding methods.

Table 4.9 Disappearance of dry matter, phosphorus, phytate-P and hydrolysis of inositol phosphates, $InsP_6$ and $InsP_5$ and the sum of $InsP_{5-6}$ ($\sum InsP_{5-6}$) in the gizzard

Item	Feeding method[1]	Disappearance, %			Hydrolysis %[3]		
		DM	P	Phytate-P	$\sum InsP_{5-6}$	$InsP_6$	$InsP_5$
Phytase, FTU/kg							
0		5.69(1.61)	35.22(1.56)	50.69(2.79)^{b}	75.34(3.08)^{b}	69.01(3.94)^{b}	81.02(2.59)^{b}
1,500		6.17(1.65)	37.12(1.77)	89.20(1.04)^{a}	91.07(1.43)^{a}	85.93(2.18)^{a}	96.96(0.63)^{a}
SEM		1.63	1.67	1.92	2.26	3.06	1.61
Feeding method							
1		8.41(1.98)	40.95(1.96)^{a}	72.25(5.25)	86.65(2.38)	80.92(3.39)	91.77(2.18)
1+1+1[2]		5.01(1.98)	33.24(2.00)^{b}	69.86(6.08)	81.16 (4.61)	78.56 (4.61)	86.25(4.09)
5		4.37(2.04)	34.31(1.78)^{b}	67.72(5.92)	77.86 (4.18)	72.94 (5.10)	85.51(3.66)
SEM		2.00	1.88	5.75	3.72	4.37	3.31
Phytase, FTU/kg							
0	1	9.60(2.80)	39.70(2.48)	55.68(4.95)^{b}	84.29(3.77)^{abc}	80.71(5.14)^{abc}	87.50(3.64)^{ab}
0	1+1+1	0.56(2.80)	30.94(1.89)	48.18(4.84)^{b}	73.16(5.88)^{bc}	66.97(6.93)^{bc}	78.70(5.04)^{b}
0	5	6.90(2.80)	35.02(2.98)	48.56(4.84)^{b}	68.58(5.13)^{c}	59.36(6.73)^{c}	76.85(4.28)^{b}
1,500	1	7.22(2.80)	42.20(3.14)	88.83(1.86)^{a}	89.01(2.92)^{ab}	81.13(4.79)^{abc}	96.03(1.41)^{a}
1,500	1+1+1	9.45(2.80)	35.55(3.47)	91.54(0.81)^{a}	93.96(1.51)^{a}	90.15(2.15)^{a}	98.32(0.24)^{a}
1,500	5	1.84(2.99)	33.60(1.77)	86.88(2.36)^{a}	90.22(1.78)^{ab}	86.51(3.65)^{ab}	97.05(0.45)^{a}
SEM		2.83	2.62	3.29	3.50	4.90	2.51
P-value							
Phytase		0.684	0.394	<0.001	<0.001	<0.001	<0.001
Feeding method		0.394	0.013	0.490	0.239	0.295	0.374
Phytase x feeding method		0.061	0.530	0.384	0.088	0.028	0.179

[a,b] Means (±SE) with a different letter within a column were significantly different, $P < 0.05$; [1]Duration from the end of 6 hours dark to time of sampling; [2]1 h feeding followed by 1 h FW and 1 h RF**;** [3]Determined as InsPs relative to Ti in diet minus relative to Ti in gizzard divided by InsPs relative to Ti in diet (unit: nmol/g)

Table 4.10 Digestibility of dry matter, phosphorus, phytate-P and hydrolysis of inositol phosphates, $InsP_6$ and $InsP_5$ and the sum of $InsP_{5-6}$ ($\sum InsP_{5-6}$) in the ileum

Item	Feeding method[1]	Digestibility, %			Hydrolysis %[3]		
		DM	P	Phytate-P	$\sum InsP_{5-6}$	$InsP_6$	$InsP_5$
Phytase, FTU/kg							
0		73.02(0.42)	57.13(1.62)^{b}	23.62(6.24)^{b}	26.63(6.68)^{b}	-7.70(11.7)^{b}	58.88(3.40)^{b}
1,500		73.56(0.40)	60.83(1.70)^{a}	70.07(2.72)^{a}	84.65(2.69)^{a}	78.00(4.24)^{a}	89.43(1.60)^{a}
SEM		0.41	1.66	4.48	4.69	7.97	2.55
Feeding method							
1		72.08(0.50)^{b}	66.47(2.01)^{a}	68.88(5.22)^{a}	73.04(5,63)^{a}	62.18(8.25)^{a}	80.72 (3.89)
1+1+1[2]		73.07(0.50)^{b}	55.65(1.58)^{b}	39.29(8.35)^{b}	48.80(10.2)^{b}	21.20(16.3)^{b}	70.38 (5.36)
5		74.73(0.52)^{a}	54.83(1.10)^{b}	32.37(8.96)^{b}	46.30(11.2)^{b}	22.00(17.7)^{b}	71.36 (5.69)
SEM		0.51	1.56	7.51	9.01	14.12	4.98
Phytase, FTU/kg							
0	1	71.94(0.73)	64.74(3.03)^{ab}	56.90(7.14)^{a}	60.94(7.12)^{a}	46.20(10.0)^{ab}	71.12(5.23)^{bc}
0	1+1+1	72.60(0.68)	53.83(1.68)^{c}	11.52(6.91)^{b}	12.50(8.59)^{b}	-36.40(12.8)^{b}	51.22(3.45)^{d}
0	5	74.52(0.79)	52.83(1.19)^{c}	2.45(5.00)^{b}	3.57(6.44)^{b}	-33.00(21.4)^{b}	54.31(6.86)^{cd}
1,500	1	72.21(0.68)	68.19(2.69)^{a}	80.87(2.11)^{a}	85.13(6.59)^{a}	78.10(10.8)^{a}	90.33(3.20)^{a}
1,500	1+1+1	73.54(0.73)	57.46(2.62)^{bc}	67.06(3.28)^{a}	85.08(3.59)^{a}	78.80(5.33)^{a}	89.54(2.55)^{a}
1,500	5	74.94(0.68)	56.84(1.61)^{bc}	62.29(4.99)^{a}	83.73(3.85)^{a}	77.07(5.54)^{a}	88.41(2.89)^{ab}
SEM		0.72	2.14	4.91	5.87	10.98	4.05
P-value							
Phytase		0.485	0.050	<0.001	<0.001	<0.001	<0.001
Feeding method		0.001	<0.001	<0.001	<0.001	0.002	0.040
Phytase x feeding method		0.739	0.992	0.006	<0.001	0.002	0.079

[a,b] Means (±SE) with a different letter within a column were significantly different, $P < 0.05$; [1]Duration from the end of 6 hours dark to time of sampling; [2]1 h feeding followed by 1 h FW and 1 h RF**;** [3]Determined as InsPs relative to Ti in diet minus relative to Ti in gizzard divided by InsPs relative to Ti in diet (unit: nmol/g)

4.4.5. Plasma myo-inositol, P and Ca

The effect of supplemental phytase in P and Ca limited diets and different feeding methods on plasma myo-inositol, P and Ca are shown in Table 4.11. Phytase supplementation significantly increased (P = 0.01) the concentration of plasma myo-inositol but did not affect the concentration of P and Ca. Different feeding methods prior to blood sampling, however, did not affect the concentration of myo-inositol, P and Ca in plasma samples.

Table 4.11 Effects of dietary treatment and feeding method on the concentration of circulating myo-inositol, Ca and P in male broiler chickens fed on wheat-corn diets either with or without phytase supplementation[1]

	Feeding method	Myo-inositol (mg/l)	P (mg/dl)	Ca (mg/dl)
Dietary treatment				
NC	1 h	53.99(6.72)	3.01(0.08)	20.35(0.74)
NC	1+1+1h	62.55(6.99)	3.21(0.13)	20.63(0.65)
NC	5 h	51.10(9.95)	2.95(0.07)	17.55(1.04)
Phytase	1 h	76.44(8.17)	2.97(0.20)	18.55(1.22)
Phytase	1+1+1h	75.21(13.2)	3.17(0.15)	19.18(1.06)
Phytase	5 h	82.21(12.2)	3.22(0.11)	19.78(0.98)
SEM[2]		9.54	0.24	0.95
Main effects				
Dietary treatment				
NC		55.88(4.34)[b]	3.07(0.06)	19.51(0.54)
Phytase		77.96(6.33)[a]	3.14(0.08)	19.17(0.60)
SEM		5.34	0.15	0.57
Feeding method				
1 h		65.21(5.87)	2.99(0.10)	19.51(0.71)
1+1+1h		68.88(7.40)	3.19(0.09)	19.91(0.63)
5 h		66.66(8.99)	3.10(0.08)	18.66(0.75)
SEM		7.42	0.18	0.69
P-value				
Dietary treatment		0.010	0.433	0.665
Feeding method		0.930	0.309	0.420
Dietary treatment x feeding method		0.660	0.334	0.076

[a,b]Means (±SE) with a different letter within a column were significantly different, P < 0.05;

[1]Phytase supplementation at 1500 FTU/kg; [2]Standard error of means (8 replicates per treatment)

4.4.6. Digesta mean retention time (MRT) in the gizzard and the ileum

Phytase supplementation significantly increased ($P < 0.05$) the MRT of digesta in both the gizzard and the ileum (Table 4.12). In contrast, feeding method had no effect on digesta MRT in the gizzard. Feeding duration did delay the passage rate of digesta through the ileum as it increased from 1 h to 5 h. However, digesta MRT for FW and FR procedure was not significantly different from digesta MRT at 5 h feeding.

Table 4.12 Effects of phytase and feeding method on digesta transit time in the gizzard and ileum of 3 week old male broiler chickens

Item	Feeding method[1]	Digesta TT (min)	
		Gizzard	Ileum
Phytase, FTU/kg			
0		37(3)[b]	43(2)[b]
1,500		54(6)[a]	53(3)[a]
SEM		5	3
Feeding method			
1		42(5)	39(3)[b]
1+1+1[2]		52(9)	51(3)[a]
5		43(4)	54(4)[a]
SEM		6	3
Phytase, FTU/kg			
0	1	31(3)	37(3)[c]
0	1+1+1[2]	40(8)	44(4)[abc]
0	5	41(6)	47(5)[abc]
1,500	1	53(7)	40(5)[bc]
1,500	1+1+1[2]	63(17)	58(4)[ab]
1,500	5	45(7)	60(6)[a]
SEM		8	4
P-value			
Phytase		0.032	0.009
Feeding method		0.518	0.004
Phytase x feeding method		0.521	0.423

[a,b,c] Means (±SE) with a different letter within a column were significantly different, $P < 0.05$; [1]Duration from the end of 6 hours dark to time of sampling; [2]1 h feeding followed by 1 h FW and 1 h RF.

4.5. Discussion

Weight gain and feed efficiency of broilers fed on wheat based diets with reduced non-phytate-P, total P and Ca were improved by phytase supplementation in this study, as has been reported previously (e.g. Cabahug *et al*., 1999; Selle *et al*., 2003; Singh *et al*., 2003; Wu *et al*., 2004; Kühn *et al*., 2011). However, feed intake in the current study was not significantly different from those without phytase supplementation, which may indicate non phytate-P at 3.2 g/kg was not severely limiting as the greatest responses usually occurs in lower non phytate-P diets compared to higher or adequate non phytate-P diets (Cabahug *et al*., 1999).

Dietary phytase inclusion of 500 FTU/kg (Akyurek *et al*., 2011, de Sousa *et al*., 2015) and 1000 FTU/kg (Lehman *et al*., 2011, Paiva *et a*l., 2013) in corn/soy diets did not affect the pH of gizzard or ileum contents of 21 d old broilers.. In contrast, Amerah *et al.* (2014) reported that phytase supplementation at 1000 FTU/kg in corn/soy based diets with 2.8 g aP/kg and 6.0 to 8.0 g Ca/kg resulted in a higher pH in the gizzard but lower pH in the ileum than those without phytase supplementation. At higher phytase inclusion, up to 5000 FTU/kg, pH of the digesta in both the gizzard and ileum in 16-d-old broilers were significantly increased (Walk *et al*., 2012d). Similar findings were reported when microbial phytase was added to wheat based diets at 2500 FTU/kg (Svihus *et al.*, 2013) and 5000 FTU/kg (Ptak *et al.*, 2015). In the current study, dietary phytase inclusion of 1500 FTU/kg to a wheat based diet had no influence on the pH of either gizzard or ileal digesta in 21-d-old broilers. The reason for insignificant results may be that the level of phytase used in the current study was not high enough to liberate more Ca from Ca-phytase complexes or/and to improve the acidogenic effect of phytate in the GIT (Pereira Gonçalves, 2014, Walk *et al.*, 2012d). Although in the current study pH tended to increase in the gizzard and tended to reduce in the ileum as found by Amerah *et al.* (2014), the less pronounced effect of phytase observed was perhaps due to higher levels of aP and Ca used in the current study. Different feeding methods had an effect on ileal pH. Ileal pH increased as feeding duration increased from 1 h after 6 h scotoperiod to 1+1+1 h and 5 h. A tendency for gizzard pH to increase was also observed.

Increasing time from the dark period until sampling also resulted in higher DM of ileal digesta and tended to result in higher gizzard DM. These results are in accordance with the findings of Duve *et al.* (2011), who sampled intestinal contents from different segments of GITs of broilers fed on wheat based diet at several sampling points (5 min, 30 min, 4 h, 8 h and 16 h) after 4-8 h scotoperiod. Scanes *et al.* (1987) described a model of daily changes of ingesta/digesta in the GIT of laying hens. At the start of the photoperiod, food partly bypassed the crop and moved straight to the gizzard and on to the small intestine, As feed intake continued, the crop started to fill up.

During scotoperiod, feed in the crop and gizzard gradually decreased and the DM of the crop and gizzard digesta with DM levels being lowest at the end of the scotoperiod. Despite little apparent variation through the day or night, DM digesta of the small intestine increased toward the end of photoperiod and decreased during scotoperiod (Scanes *et al.,* 1987), which explains the lower ileal DM when birds were fed with the 1 h feeding method compared to the 1+1+1 h and 5 h feeding method.

Similarly, when feed is eaten, proventriculus become distended and promotes the release of stimulants (acetylcholine, gastrin, histamine) to induce HCl secretion in proventriculus thus reduce the pH of gizzard content (Hersey and Sachs, 1995), which explains lower pH at 1 h feeding method (Table 4.5). As more feed enter proventriculus and gizzard, pH of gizzard digesta slightly increased probably either due to the gastric acidity control mechanism between acid secretion and gastrin release (Hersey and Sachs, 1995) or due to buffering capacity of the diet (Svihus, 2011b). When the acidic gastric content enters duodenum, secretin produced by duodenum stimulates pancreatic secretion of bicarbonate (Hogan *et al*., 1994). This resulted in neutralization of HCl which increases digesta pH in duodenum and the pH neutralization continues in jejunum and ileum. As more feed is consumed, more bicarbonate-rich pancreatic secretion is released (Pandol, 2010) and leads to higher digesta pH in the ileal as observed in chickens with 1+1+1 h and 5 h feeding method compared to the 1 h feeding method (Table 4.5).

The analysis of P and InsPs reported in Table 4.6 indicated, that, both experimental diets used in the current study were identical except for phytase inclusion level. The results of the analysis were presented in two different units

(nmol/g and mg/g) to demonstrate the importance of consideration when choosing unit selection for expressing concentrations of substances. In the current study, the amount of non phytate-P in nmol/g was not different from total P but it was significantly lower than total P when it was expressed in mg/g. Similarly, 1 mol of $InsP_6$ will be degraded into 1 mol of $InsP_5$ but 1 mg of $InsP_6$ will not be degraded into 1 mg of Ins_5. This is due to the difference of molecular mass in $InsP_6$ (MW= 660.04 g/mol) and $InsP_5$ (MW=580.06 g/mol).

The amount of $InsP_6$ in the diets of the current study was 40 to 43% lower and $InsP_5$ was correspondingly higher than those reported in other studies (Zeller *et al.*, 2015a; Zeller *et al.*, 2015b). Zeller *et al.* (2015a) reported the $InsP_6$ and $InsP_5$ level of 15,900 to 16,200 nmol/g and 800 to 900 nmol/g dietary DM, respectively. While in another trial of Zeller *et al.*, (2015b) slightly lower $InsP_6$ (14,600-15,400 nmol/g feed DM) and higher $InsP_5$ (1,800-2,000 nmol/g feed DM) were analysed. In the current study either $InsP_6$ was degraded into $InsP_5$ during feed production or $InsP_6$ in feed ingredients, particularly wheat, was already degraded as demonstrated by Blaabjerg *et al.* (2010a), where a part of the $InsP_6$ in the feed ingredients were readily degraded prior to phytase supplementation.

It is well established that phytase enhances hydrolysis of $InsP_6$ into lower InsPs at recommended inclusion levels of 500 FTU/kg, or higher, for broilers (Selle and Ravindran, 2007). Phytase was reported to be more effective in the anterior of digestive tract compared to posterior segment whereas differences were seen between phytases (Zeller *et al.*, 2015a), possibly due to the different protease stability. Extremely low concentrations of $InsP_6$ were observed in the gizzard compared to those in the ileum while the concentrations of lower InsPs were inconsistent (Walk *et al.*, 2014; Zeller *et al.*, 2015a; Zeller *et al.*, 2015b). Zeller *et al.* (2015b) reported that inclusion levels of phytase at 500 FTU/kg increased the concentration of $InsP_5$ in the gizzard as a result of $InsP_6$ hydrolysis. Contrarily, at the same phytase level, Walk *et al.* (2014) reported a reduction in both $InsP_6$ and $InsP_5$ concentration. However, higher concentrations of $InsP_4$, $InsP_3$ and inositol were observed in both studies. Reduction of gizzard concentration of $InsP_5$ by phytase and higher concentrations of inositol were also observed in chickens fed diets either adequate in aP (Beesons *et al.*, 2014) or reduced to 0.3% aP (Walk *et al.*, 2014).

Nonetheless, these studies were conducted on chickens fed corn based diets and similar reports on wheat based diets is scarcely available.

In the present study, phytase inclusion of 1500 FTU/kg effectively hydrolysed $InsP_6$ and lower InsPs ($InsP_{3-5}$) and increased inositol concentration in the gizzard contents (Table 4.7) while in the ileum, further hydrolysis of $InsP_6$ and $InsP_5$ has increased resulting in and increase in inositol but also an increase in the concentration of $InsP_4$, $InsP_3$ (Table 4.8). These results are in line with Zeller *et al.* (2015b) and Beeson *et al*. (2014) who suggested more complete hydrolysis of InsPs when higher dose of the same phytase (such as 1500 FTU/kg) were used. In the present study, gizzard and ileal digesta were collected 1 h, 1+1+1 h and 5 h after 6 h darkness. Low concentrations of $InsP_6$ and lower InsPs after 1 h feeding indicate greater hydrolysis of InsPs when birds start feeding following a period of darkness. As feeding method changed, the hydrolysis of InsPs was reduced, indicated by higher concentration of $InsP_5$ and $InsP_6$. The effects of feeding method on the concentrations of P and InsPs were more profound in the ileum compared to the gizzard. These results suggest rapid hydrolysis or disappearance of InsPs occurred in the gizzard resulting in similar $InsP_5$ and $InsP_6$ across feeding methods. This finding conformed to Zeller *et al*. (2015b) who suggested better hydrolysis of $InsP_6$ in the upper part of digestive tract due to better $InsP_6$ solubility and pH condition than in lower segments of the digestive tract. The increased concentration of InsPs up to the terminal ileum suggests hydrolysis of InsPs is less efficient with longer time for feed intake.

Low concentrations of InsPs in both gizzard and ileum at 1 h feeding time may also be due to the slower passage of digesta during the 6 h scotoperiod resulting in more time for phytate degradation. This finding was in agreement with Leslie (2006), who showed a 12 h dark period reduced food passage rate and lowered gizzard concentration of $InsP_6$ compared to continuous light. Phytate hydrolysis was further increased with phytase supplementation with 12 h darkness (Leslie, 2006). Duve *et al.* (2011) demonstrated that prior to an uninterrupted 8 h darkness period, chickens filled up their crop and the ingesta were released into other parts of the digestive tract slowly during the dark period. By using the method of cumulative chromic oxide excretion, longer mean retention time was measured in chickens that were subjected to 8 h of uninterrupted scotoperiod compared to a split

4 h + 4 h scotoperiod (Duve *et al*., 2011). May *et al*. (1990) also found that digesta passage in the gizzard was slower than in crop and even slower during a dark period. In support of this, the *in vitro* hydrolysis of $InsP_6$ by phytase was further enhanced with a longer reaction time as shown by Walk *et al*. (2012b) and Denstadli *et al*. (2006).

Low pH condition may also promote hydrolysis of $InsP_6$ due to high phytate-P solubility (Graynspan and Cheryan, 1983). According to Menezes-Blackburn *et al*. (2015), although phytase has a high affinity towards phytate at pH 5, phytate degradation at pH 3 (represent gizzard pH) and at pH 5.5 (represent crop pH) were similar during *in vitro* digestion of ground wheat. Low concentrations of $InsP_6$ in the ileum of birds at 1 h 'feeding method' could also be due to enzymatic degradation activity by the ileal microbiota. The potential of gut microbiota in hydrolysing $InsP_6$ in broilers fed with Ca and P deficient diets in the absence of supplemental phytase has also been assumed by Zeller *et al*. (2015a, 2015b). Although the majority of the $InsP_6$ were hydrolysed in the anterior part of the digestive tract, further hydrolysis of $InsP_6$ was still ongoing up to the terminal ileum (67%). Ptak *et al*. (2015) reported high concentrations (CFU/g) of ileal bacteria, mainly *Bifidobacterium* sp. and *Lactobacillus* sp. were isolated from broilers fed on wheat based diets low in Ca (0.71%) and P (0.55%). These bacteria have high ability in degrading $InsP_6$ in the chicken intestine (Raghavendran and Hanili, 2009) and human intestine (Haros *et al*., 2009; Markiewicz *et al*., 2013). Morgan *et al*. (2015) demonstrated that intrinsic phytase from cereal and intestinal phytase contributed in $InsP_6$ hydrolysis in the gut but Zeller *et al*. (2015c) assumed the magnitude of contribution of microbiota associated phytase was greater than that of intrinsic plant phytases. Therefore, in the present study, low concentrations of InsPs in both the gizzard and ileum of broilers at 1 h feeding method could be due to slow passage of digesta during the dark period and $InsP_6$ degradation by the microbiota.

In contrast to scotoperiod, the feed that consumed during light period would by-pass the crop (Scanes *et al*., 1987; Buyse *et al*, 1993) resulting in a faster rate of passage (May *et al*. 1990) and thus reduced time for phytase-phytate reaction in the crop. When raising birds under light, the quantity of ileal digesta can increase (May *et al*., 1990) and may result in higher intestinal InsP6 contents (Dersjant-Li *et al*.,

2014). Additionally, under intestinal pH, phytate degradation by phytase can be reduced (Menezes-Blackburn *et al*., 2015) due to reduced solubility of $InsP_6$ especially when the molar ratio of Ca: $InsP_6$ is 4 or more (Graynspan and Cheryan, 1983). In the present study, higher concentrations of InsPs in both the gizzard and ileum of broilers of the 1+1+1 h and 5 h feeding method compared to those of the 1h feeding method (Table 4.8) could be due to the faster passage rate of crop and gizzard digesta, reduced phytase capability in hydrolysing $InsP_6$ in the small intestine up to the ileum and reduced solubility of $InsP_6$ at intestinal pH. These factors may also explain the increasing trend of gizzard and ileal InsPs concentrations in relation to Ti marker particularly in non-phytase supplemented groups as illustrated in Figure 4.2. However, the reason for the magnitude of this increase observed for ileal $InsP_6$ in broilers fed on non-phytase diet at 1+1+1 h and 5 h sampling time is not known.

Crop and gizzard as the main sites for phytate hydrolysis have been reviewed by several authors (Selle and Ravindran, 2007; Dersjant-Li *et al*., 2014). Optimal pH conditions in the crop and the gizzard increased phytate solubility and phytate hydrolysis (Menezes-Blackburn *et al*., 2015). However, published data on phytate hydrolysis and P digestibility in the gizzard is limited. Zeller *et al*. (2015a) and Walk *et al*. (2014) reported that the concentration of $InsP_6$ was very low in the gizzard following phytase supplementation in corn based diet, which indicated high $InsP_6$ hydrolysis. While Morgan *et al*. (2015) and Sooncharernying and Edward (1993) reported that $InsP_6$ hydrolysis in the gizzard of broilers fed on non-phytase supplemented corn based diet was only 39.6% and 45.0%. The present study also showed high hydrolysis of $InsP_6$ and $InsP_{5\text{-}6}$ in broilers fed on wheat based diets following phytase supplementation, which resulted in high phytate-P digestibility. The hydrolysis of $InsP_{5\text{-}6}$ was higher (91.1%) than that of $InsP_6$ (85.93%) but the extent of hydrolysis achieved by supplemental phytase was similar for both $InsP_6$ (16.9%) and $InsP_{5\text{-}6}$ (15.7%). This demonstrates that the phytase used is as efficient in degrading $InsP_5$ as degrading $InsP_6$. $InsP_6$ hydrolysis in the present study was high when taking into consideration that dietary P levels affect the extent of phytate degradation (Shastak *et al*., 2014) and the diets used were not severely reduced in P. This in part may bedue to a better $InsP_6$ solubility even without phytase supplementation in wheat compared to corn diets. High efficacy of intrinsic wheat phytase in crop pH (Shastak *et al*., 2014) could have contributed to the high $InsP_6$

hydrolysis in the gizzard. The disappearance of phytate-P by supplemental phytase in the present study can be explained by the $InsP_6$ degradation mentioned above, whereas the levels found for P digestibility in the gizzard can be explained by the elution of P to lower intestinal segments as no P absorption is assumed up to the gizzard. This is supported by the fact that phytase did not affect (P=0.394) P disappearance in the gizzard.

The improvements of ileal phytate hydrolysis and apparent ileal P digestibility due to phytase supplementation have been reported by numerous researchers (Camden *et. al*., 2001; Tamim *et al*., 2004; Amerah *et al*., 2014). The values of phytate hydrolysis and P digestibility vary depending on the level of P and Ca in the diet. Diets used in those studies were P and Ca deficient, thus, the magnitude of the impact of phytase supplementation on ileal phytate hydrolysis were similar and reflected in the improved ileal digestibility of P and Ca. Although the positive effect of phytase supplementation on ileal phytate hydrolysis and P digestibility were observed, the magnitude of improvements were not significant following phytase supplementation at 600 and 750 FTU/kg (Rutherfurd *et al*., 2002; Afsharmanesh *et al*., 2008). The impact of phytase supplementation on P digestibility in broilers could have more prominent results if higher phytase dosages are used to achieve a more complete hydrolysis of $InsP_6$ reflected by better growth performance (Walk *et al*., 2014). In the present study, phytase supplementation at 1500 FTU/kg improved ileal digestibility of P ($P = 0.05$) and phytate-P ($P < 0.001$) and increased ($P < 0.001$) ileal hydrolysis of $InsP_6$ and $InsP_{5\text{-}6}$, which confirmed the hypothesis by Walk *et al.* (2014). As discussed previously, the effect of feeding method was also prominent on P digestibility and $InsP_{5\text{-}6}$ hydrolysis in the ileum. The interactions between phytase supplementation and feeding method on gizzard $InsP_6$ hydrolysis (Table 4.9), ileal digestibility of phytate-P and ileal hydrolysis of $InsP_6$ and $InsP_{5\text{-}6}$ (Table 4.10) implies that feeding method is critical for the analysed effect of the supplemented phytase. Variation of phytase effects found in the literature partially related to the different feeding methods prior to sampling and the standardisation of the feeding method seems essential.

The effects of feeding method and related interactions on ileal $InsP_6$ hydrolysis seems closely related to the negative values calculated for $InsP_6$ hydrolysis

in broilers fed diet without supplemental phytase (Table 4.10). The negative values are the results from the higher marker related $InsP_6$ concentration in the ileum at all feeding methods compared to those in the diets (Figure 4.2B). The increase of ileal $InsP_6$ at 1+1+1 h and 5 h feeding method is difficult to explain on the basis of current knowledge and at this point neither an interaction of a part of the phytate with the marker nor a *de novo* synthesis of $InsP_6$ in the small intestine as hypothesised by Jongbloed *et al*. (1992) can be excluded. De *novo synthesis* of InsPs so far has been demonstrated in tissues, for example, $InsP_5$ is synthesised in the erythrocytes of chickens by the activity of myo-inositol kinase that phosphorylates inositol synthesis (Isaacks *et al*. ,1982) and *in vivo* synthesis of $InsP_6$ at the expense of $InsP_5$ via the action of $InsP_5$ 2-kinase (Versky *et al*., 2002). Evidence for the existence of any such *de novo* synthesis in the chicken gut to support this hypothesis has not yet been reported.

Phytase supplementation significantly increased (P = 0.01) the concentration of plasma myo-inositol but did not affect the concentration of P and Ca. These results were in agreement with results reported by Cowieson et al. (2015), who suggested that increasing myo-inositol concentration due to supplemental phytase benefited chicken's growth performance through insulin mimetic mechanisms in transportation of nutrient and deposition of protein. Different feeding methods prior to blood sampling, however, did not affect the concentration of myo-inositol, P and Ca in plasma samples.

Digesta mean retention time or MRT in the gizzard and ileum may also contribute in the higher ileal $InsP_6$ levels seen in some treatments. In the present study, the estimated MRT up to the gizzard of broilers fed on a diet without phytase supplementation was shorter than those fed on a phytase supplemented diet (Table 4.12). This indicates diet without supplemental phytase has less retention time in low pH environment which is required for efficient $InsP_6$ hydrolysis in the gizzard. Thus, more undegraded $InsP_6$ was analysed in the gizzard with higher concentrations in feeding method 1+1+1 h and 5 h as illustrated in Figure 4.2A, as well as in the ileum (Figure 4.2B). This may also explain the high concentration of ileal $InsP_6$ and the negative values of $InsP_6$ hydrolysis after 1+1+1 h and 5 h feeding methods for broilers fed the non-phytase supplemented diet. Whereas, with supplementation of

phytase, digesta MRT was increased in both the gizzard ($P < 0.05$) and the ileum ($P < 0.01$) and was further increased ($P<0.01$) with feeding method in the ileum. This indicates that more time was available for phytase-phytate reaction to occur in releasing more phytate-P and more time for P absorption, reflected by the low concentrations of InsPs (Figure 4.2), high total P and phytate-P digestibility and high hydrolysis of $InsP_6$, and $InsP_{5-6}$ (Table 4.9 and Table 4.10). However, the effect of phytase on digesta MRT observed in the present study was contradictory to what was reported by Watson *et al*. (2006) for broilers fed on corn based diet. This discrepancy may be due to the method used in measuring MRT, level of dietary phytase, level of Ca and P and also type of cereal used in the diet.

4.6. Conclusion

It is concluded that feeding method after 6 h darkness did increase the concentrations of InsPs, digestibility of P and hydrolysis of $InsP_{5-6}$ in both the gizzard and the ileum of broilers and the effect of feeding methods was more prominent in the ileum compared to in the gizzard. The addition of dietary phytase reduced concentrations of InsPs in the gizzard and $InsP_{5-6}$ in the ileum, improved P digestibility and phytate hydrolysis for each feeding method. Phytase supplementation also increased the MRT in both the gizzard and the ileum but feeding method only influenced the ileal MRT. Because of the methodological effects on the extent of the phytate degradability, it is necessary to standardise and report in detail the feeding method used prior to slaughter and sample collection. Sampling shortly after the start of light period results in higher phytate degradation especially for diets fed without phytase which might overestimate the phytate degradation for the majority of the feed digested over the daytime. Therefore, it is suggested that the sampling of digesta should be done at least 3 h from the start of light period without 1 h FW and 1 h FR.

Chapter 5
The effect of dietary fat inclusion level on the efficacy of phytase in broiler chickens fed on wheat based diets

5.1. Abstract

The objective of this study was to investigate the effect of different level of dietary fat on phytase efficacy with regard to growth performance, utilization of phosphorus and calcium and bone mineralization in broiler chickens. It was hypothesized that together high fat level and phytase would improve growth performance parameters, calcium (Ca) and phosphorus (P) digestibility in the gut and mineral retention in the bone over and above that of chickens fed diets supplemented with phytase only. This experiment was conducted as a 2 x 2 factorial arrangement of treatments with 2 levels of dietary fat (low fat at 10 g/kg diet and high fat at 50 g/kg diet) and 2 levels of phytase (0 or 1500 FTU/kg diet). Phytase supplementation improved FI and BWG. With addition of higher dietary fat level in phytase supplemented diets, the growth performance of broilers were further improved. Phytate degradation and P digestibility were also improved with fat and phytase supplementation. On the other hand, phytase supplementation reduced duodenum-jejunal and ileal calcium digestibility and it may be related to the formation of insoluble tertiary protein-mineral-phytate complexes. Conversely, addition of dietary fat and phytase separately increased total ash and mineral retention in tibia. However, simultaneous addition of fat and phytase in broiler diet has no beneficial effect on bone mineralization. Adding cellulose as filler has diluted the nutrient concentration in 5% fat diet that eventually led to poorer FCR as compared to those fed 5% fat diet without cellulose addition (high density diet). However, phytase supplementation eliminated the negative effect of nutrient dilution due to cellulose addition by improving the growth performance of chickens as good as those fed on high density diet.

5.2. Introduction

Phosphorus in phytate is largely unavailable to chickens unless they are provided with dietary phytase. Phytate may also reduce the availability of other cations which in turn may depress the digestion of other nutrients through the inhibition of digestive enzymes in the gastrointestinal tract requiring cations for optimal enzyme activity. Phytases liberate P from phytate and reduce its anti-nutritive effects. Supplementing poultry diets with phytase has been shown to improve P utilization, P retention, nutrient digestibility and growth performance. Selle *et al*. (2010) suggested that efficacy of phytase in broiler chickens could be further improved by increasing digesta retention time in the crop. This may facilitate phytate dephosphorylation by extending exposure of substrates to phytases. Dietary fats have been shown to increase intestinal retention time in chickens (Mateos *et al.*, 1982). Mabayo *et al.* (1992) demonstrated lipid, particularly medium chain fatty acids, delayed crop emptying in chickens. However there is limited information on the effect of different levels of fat on phytase efficacy in poultry.

Thus, the objective of this study was to investigate the effect of different levels of dietary fat on phytase efficacy with regard to growth performance, utilization of phosphorus and calcium, and bone mineralization in broiler chickens. It was hypothesized that diets containing both phytase and high level of fat would improve the growth rate and nutrient intake of chickens over and above that of birds fed diets supplemented with phytase only. Higher fat level and phytase supplementation were also expected to improve both Ca and P digestibility in the gut and retention in the bone. There were 2 additional diets having similar compositions as 5% fat diet with and without phytase except for filler (cellulose), designated as high density (HD) diets, were tested along with the 2 x 2 design. These diets were tested in order to investigate the effect of cellulose addition on the performance of broilers fed phytase supplemented diet and hypothesized that cellulose addition does not affect the performance of broilers fed on phytase diet

5.3. Materials and methods

All experimental procedures were conducted in accordance with The Animals (Scientific Procedures) ACT 1986, under Animal Ethical Review Committee of University of Leeds.

5.3.1. Diets and Experimental Design

Two wheat based crumbled diets were formulated according to Aviagen Nutrient Specification for starter phase, having except that levels of available P (aP) and calcium (Ca) were reduced from 5.0 and 10.5 g/kg diet to 2.5 and 9.0 g/kg diet, respectively. The calculated differences in the ME of soybean oil were overcome by adding maize starch or cellulose. The diets were formulated to be isonitrogenous and isoenergetic (Table 5.1). Each of the diets was either supplemented with phytase at or without phytase. This enhanced Escherichia coli-derived liquid phytase (Quantum Blue 5L, AB Vista Feed Ingredients, Marlborough, UK) was added at the rate of 300 g/kg diet to achieve phytase activity of 1500 FTU/kg. This experiment was conducted as a 2 x 2 factorial arrangement of treatments with 2 levels of dietary fat (low fat at 10 g/kg diet and high fat at 50 g/kg diet) and 2 levels of phytase (0 or 1500 FTU/kg diet). There were 2 additional diets having similar compositions as 5% fat diet with and without phytase except for filler (cellulose), designated as high density (HD) diets, were tested along with the 2 x 2 design. These diets were tested in order to investigate the effect of cellulose addition on the performance of broilers fed phytase supplemented diet. Internal acid insoluble ash (AIA) was used as marker to determine nutrient digestibility.

5.3.2. Bird Management

A total of 384 one-day-old male Ross 308 broiler chicks were obtained from a commercial hatchery and were vaccinated with Infectious Bronchitis (IB) and Mareks' disease vaccines. The birds were individually weighed and randomly allocated into 24 pens having 16 birds per pens with minimum variation of the means of body weight among pens. All birds were raised in floor pens that contained about 10 cm depth of fresh wood-shavings and had *ad libitum* access to feed and water.

Continuous lighting from d 1 to d 3 and a lighting program of 20 h light: 4 h dark (20L:4D) from d 4 onward and continuous ventilation were applied in this study. The room temperature was maintained at 32 ± 2°C for the first 7 d and then gradually reduced to 21°C at d 28. Individual body weights and pen feed intake were recorded weekly while health conditions and mortality were recorded daily.

5.3.3. Sample Collection and Measurements

At 20 d of age, following 1 h of feed withdrawal and 1 h of refeeding, 36 birds per treatment (6 birds per pen) were randomly selected and weighed. The birds were killed by cervical dislocation and dissected. In order to avoid post mortem digesta movement, crop, proventriculus-gizzard, duodenum-jejunum and ileum were clamped. The contents of each part of the gastrointestinal tract from each bird were collected, pooled within a pen and then pH values of the digesta were measured. The digesta samples were freeze-dried, ground and sieved to pass through 1.0 mm screen prior to determination of phytate, minerals and acid insoluble ash (AIA). The left legs were removed from the body and each of bone samples were individually packed in polyethylene bags and stored at -20^{o}C prior to bone preparation. After thawing, tibia bones were obtained by cutting the tibiometatarsal joint, the joints between femur and tibia/fibula and the joint between tibia and fibula. Tibiae were cleaned of the adhering tissues and dried in the oven at 100^{o}C for 24 h. The bone parameters including dry weight, length, volume and density were determined according to Zhang and Choon (1997). Dried tibiae were ashed in a muffle furnace (Carbolite 1100) at 600^{o}C for 24 h to determine the contents of tibia ash and tibia minerals. About 200g of each of the experimental diets were taken from representative feed bags weekly and stored at -20^{o}C prior to further analysis.

Table 5.1 Compositions and calculated nutrient contents of the experimental diets (%) for young broiler chickens [1]

Ingredients (%)		1% fat	5% fat	5% fat HD[2]
Wheat		38.25	38.25	40.47
Maize		19.20	19.20	20.32
Soybean meal		17.70	17.70	18.73
Corn gluten meal		1.40	1.40	1.48
Potato protein concentrate		6.44	6.44	6.81
Fishmeal		2.10	2.10	2.22
Soybean oil		1.00	5.00	5.29
Vitacell Cellulose[3]		-	5.49	-
Corn starch[3]		9.49	-	-
Dicalcium phosphate		0.39	0.39	0.41
Limestone		1.51	1.51	1.60
Salt		0.20	0.20	0.21
Sodium bicarbonate		0.16	0.16	0.17
L-Lysine HCl		0.32	0.32	0.34
DL Methionine		0.13	0.13	0.14
Threonine		0.06	0.06	0.06
Vitamin & mineral premix[4]		0.40	0.40	0.21
Binder (Ligno Bond DD)		1.25	1.25	1.32
Total		100.00	100.00	100.00
Calculated nutrients	*Recommended level*[5]			
AME Chick MJ/kg	12.65	12.65	12.65	13.39
Crude Protein	22-25	22.00	22.00	23.28
Energy: protein	0.575	0.575	0.575	0.575
Lysine	1.43	1.43	1.43	1.52
Methionine	0.51	0.51	0.51	0.54
Threonine	0.94	0.94	0.94	0.99
Calcium (Ca)	1.05	0.90	0.90	0.96
Phosphorus (P)		0.48	0.48	0.51
Available P	0.50	0.25	0.25	0.26
ca:aP	2.10	3.63	3.63	3.63
Phytate-P		0.21	0.21	0.23
Sodium	0.16	0.17	0.17	0.18
Chloride	0.16-0.23	0.23	0.23	0.24
Fibre		1.89	7.38	2.00
Fat		2.97	6.94	7.32

[1] Phytase diets have the same compositions as the above fat diets but supplemented with phytase Quantum Blue 5L at 300g/t to achieve 1500 FTU/kg diet.

[2] HD- High density diet has similar ingredients and nutrition compositions as 5% fat diet except without cellulose

[3] Corn starch and cellulose were used to make up the difference at ME of added soybean oil

[4] Premixes provided the following (per kg of diet): vitamin A, 13,200 IU; vitamin D3, 4,000 IU; vitamin E, 66 IU; vitamin B12, 39.6 µg; riboflavin, 13.2 mg; niacin, 110 mg; Dpantothenate, 22 mg; menadione (K3), 4 mg; folic acid, 2.2 mg; thiamine, 4 mg; pyridoxine, 8 mg; D-biotin, 252 µg; selenium (as Na2SeO3), 0.30 mg; manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; cobalt, 1.0 mg; choline chloride, 1,200 mg.

[5] The requirement was according to The Specification for As-Hatched Broilers Grown to <1.9kg (4.2lb) live weight for Ross 308 Broiler (Aviagen, 2007).

5.3.4. Chemical Analysis

Dry matter was determined by drying samples at 100°C for 24 h according to Method 930.15 (AOAC, 2000). Ashed samples of diets, digesta and tibiae were digested in 5M hydrochloric acid (HCl) for 1 h, filtered through ashless filter Whatman No. 541, washed several times with deionized water and the ash-asid filtrate were diluted to 100ml. Washed ashless fitters containing insoluble matter were ashed in a muffle furnace at 600°C for 24 h for AIA determination (Van Keulen and Young, 1977). Diets and digesta samples were analysed colorimetrically for total P using molybdovanadate method at 405 nm (Method 965.17, AOAC 2000) and Ca using Arsenazo III method at 610 nm according to Attin *et al.* (2004). Tibia minerals were analysed by ICP-OES Method 985.01 (AOAC 2000). Phytase activity in feed samples was determined by Enzyme Services and Consultancy Ltd, Wales according to the internal, validated methods of the producer (Quantum method)**.** One unit of phytase (FTU) is defined as the amount of enzyme which releases 1 μmol of inorganic P per minute from phytic acid at pH 4.5 and 60°C. Phytate contents in the diets and digesta were determined based on the difference between total P and free P. Free P was extracted from 1 g of sample in 20 ml 0.66M HCl by vigorously stirring for at least 3 h. One ml of the extraction mixture was centrifuged at 13,000 rpm for 10 min and the supernatant was subjected to P analysis using same method used for total P analysis. Phytate content was calculated as the followings with the assumption that the amount of P measured is exclusively released from phytate which is 28.2% of phytate is P.

$$\text{Phytate (g/100g)} = (\text{Total P} - \text{Free P}) / 0.282$$

Apparent nutrient and DM digestibility were calculated by the following equation using AIA as a marker.

$$\text{Digestibility (\%)} = [\,(\text{Nutrients in diet}/\text{AIA in diet}) - (\text{Nutrients in digesta}/\text{AIA in digesta})\,] / (\text{Nutrients in diet}/\text{AIA in diet}) \times 100$$

5.3.5. Statistical Analysis

Data were analysed using general linear model (GLM) to assess the inclusion of fat and phytase and their interactions with Minitab 17 Statistical Software (Minitab Inc, 2014). For tibia bone parameters, GLM was performed by adding body weight gain as a covariate. Significant differences among the treatments were identified at $P \leq 0.05$ by Multiple Comparison of Means using Tukey's method.

5.4. Results

5.4.1. Diet analysis

The analysed phytase activity and nutrient compositions of experimental diets are presented in Table 5.2. The recovered values of phytase activity in experimental diets were as expected. Analysed values for total P were generally in agreement with formulated values but total Ca values were higher than the calculated values especially for the diet with 5% fat level and HD diets giving average values of 2.15 for Ca: total P ratio, which is higher that the calculated value (1.88). In contrast, the detected amount of fibre in both 5% fat and HD diets was low in comparison to calculated values. The discrepancy between the analysed and the calculated values of nutrients in the diets particularly Ca and fibre is perhaps due to the differences in nutrient contents in feed ingredients used compared to the reference values used during formulation.

5.4.2. Broiler performance

Mortality during the trial was low (< 2%) and was not significantly different between the diet groups (data not shown). The deaths were not related to dietary treatment. The effect of fat and phytase supplementation on growth performance of male broiler chickens fed on wheat based diets from 0 d to 20 d is presented in Table 5.3. There were significant ($P < 0.05$) main effects due to fat inclusion level on growth performance of broilers aged between 0 to 7 d and 8 to 14 d. BWG of the chickens fed on diets containing 5% fat were higher than those fed on 1% fat diet from 0 to 20 d but the increase in BWG was significant ($P < 0.001$) only for the chickens aged

between 0 to 7 d. FI was also increased as fat inclusion level increased throughout the 3 week trial but higher FI was only significant ($P < 0.05$) for the chickens aged between 15 to 20 d. FCR was also affected significantly by different level fat for post-hatched chickens up to 7 d. An increase in fat inclusion level from 1 to 5% resulted in a significant ($P = 0.001$) improvement of FCR of chickens aged 0 to 7 d and insignificant improvement of FCR of the chickens aged 8 to 14 d. In contrast, higher fat inclusion level insignificantly increased FCR of the chickens aged 15 to 20 d, which in turn insignificantly increased the overall FCR.

Table 5.2 Amount of phytate-P, total P, total Ca, fat and fibre in experimental diets

Diet	1% fat	5% fat	HD[2]	1% fat + phytase[3]	5% fat + phytase	HD + phytase
Phytase activity (FTU/kg)						
Added	0.00	0.00	0.00	1500	1500	1500
Analysed[1]	129	< 50	< 50	1530	1630	1650
Phytate (%)						
Analysed	0.15	0.16	0.18	0.15	0.15	0.17
Total P (%)						
Expected	0.48	0.48	0.51	0.48	0.48	0.51
Analysed	0.43	0.45	0.46	0.43	0.45	0.43
Total Ca (%)						
Expected	0.90	0.90	0.96	0.90	0.90	0.96
Analysed	0.83	1.05	1.04	0.83	1.03	0.92
Fat (%)						
Expected	2.97	6.94	7.32	2.97	6.94	7.32
Analysed	3.17	6.92	7.20	3.61	6.94	7.03
Fibre (%)						
Expected	1.89	7.38	2.00	1.89	7.38	2.00
Analysed	1.80	4.20	2.30	1.90	3.60	2.35

[1]The values of phytase activity represent the means of triplicate samples per assay. One unit of phytase activity (FTU) is defined as the quantity of enzyme that liberates 1 µmol of inorganic P per minute from sodium phytate at pH 4.5 at 60°C.

[2] High density diet (HD) with same diet composition as 5% fat diet but without filler (cellulose).

[3] Supplementation of phytase at 1500 FTU/kg

Table 5.3 Effect of dietary fat and phytase supplementation on performance of male broilers at 20-d of age[1]

Item	Phytase	1 to 7 d			8 to 14 d			15 to 20 d			1 to 20 d		
	FTU/kg	FI (g)	BWG (g)	FCR	FI (g)	BWG (g)	FCR	FI (g)	BWG (g)	FCR	FI (g)	BWG (g)	FCR
Fat level, %													
1	0	133.9^{b}	128.2^{b}	1.05^{ab}	287.9^{b}	222.8^{b}	1.29	383.0^{c}	279.5^{b}	1.38	804.8^{c}	630.6^{c}	1.28
		(2.3)	(2.3)	(0.01)	(7.0)	(7.0)	(0.02)	(9.1)	(14.2)	(0.05)	(16.0)	(20.1)	(0.02)
1	1500	149.7^{a}	138.2^{b}	1.08^{a}	369.3^{a}	282.9^{a}	1.31	549.6^{b}	396.9^{a}	1.39	1,068.5^{b}	818.0^{b}	1.31
		(5.4)	(4.6)	(0.01)	(11.2)	(7.5)	(0.04)	(12.6)	(10.5)	(0.01)	(17.7)	(19.1)	(0.02)
5	0	128.2^{b}	126.5^{b}	1.01^{b}	272.3^{b}	224.0^{b}	1.22	376.6^{c}	270.0^{b}	1.40	777.1	620.5^{c}	1.25
		(1.8)	(1.6)	(0.01)	(2.7)	(3.3)	(0.01)	(10.1)	(7.7)	(0.03)	(12.1)	(7.9)	(0.01)
5	1500	152.3^{a}	153.9^{a}	0.99^{b}	377.0^{a}	310.1^{a}	1.22	644.1^{a}	442.5^{a}	1.45	1,173.4^{a}	906.4^{a}	1.29
		(2.5)	(1.9)	(0.01)	(8.5)	(5.8)	(0.02)	(29.1)	(8.6)	(0.05)	(35.2)	(12.0)	(0.03)
SEM		3.0	2.6	0.01	7.4	5.9	0.03	15.2	10.3	0.04	20.3	14.8	0.025
Main effects													
Fat level, %													
1		141.8	133.2^{b}	1.06^{a}	328.6	252.9	1.30	466.3^{b}	338.2	1.38	936.7	724.3	1.29
		(3.7)	(2.9)	(0.1)	(13.8)	(10.3)	(0.02)	(26.2)	(19.6)	(0.03)	(41.4)	(31.2)	(0.01)
5		144.3	140.2^{a}	1.00^{b}	324.6	273.0	1.22	510.3^{a}	356.2	1.43	975.3	763.4	1.27
		(2.9)	(3.1)	(0.1)	(11.8)	(9.6)	(0.01)	(31.7)	(18.6)	(0.03)	(45.2)	(30.6)	(0.02)
SEM		3.31	2.98	0.01	12.8)	9.93	0.02	28.95	19.2	0.03	43.3	30.9	0.01
Phytase, FTU/kg													
0		130.1^{b}	127.1^{b}	1.02	277.5^{b}	223.6^{b}	1.24	378.7^{b}	273.2^{b}	1.39	786.3^{b}	623.8^{b}	1.26
		(1.6)	(1.3)	(0.01)	(3.3)	(3.1)	(0.01)	(7.30	(6.8)	(0.02)	(9.9)	(8.3)	(0.01)
1500		151.5^{a}	148.7^{a}	1.02	374.4^{a}	301.0^{a}	1.24	612.6^{a}	427.3^{a}	1.43	1,138.5^{a}	876.9^{a}	1.33
		(2.4)	(2.6)	(0.01)	(6.7)	(5.5)	(0.02)	(22.3)	(8.4)	(0.04)	(26.6)	(14.1)	(0.02)
SEM		2.0	1.9	0.01	5.0	4.3	0.02	14.8	7.6	0.04	18.3	11.2	0.02
P-value													
Fat level		0.421	0.011	0.001	0.808	0.202	0.113	0.033	0.213	0.070	0.054	0.083	0.374
Phytase		<0.001	<0.001	0.241	<0.001	<0.001	0.639	<0.001	<0.001	0.396	<0.001	<0.001	0.071
Fat level x Phytase		0.257	0.024	0.030	0.052	0.059	0.959	0.039	0.049	0.430	0.021	0.017	0.466

[a-c] Means(±SE) without a different letter within a column were significantly different, $p < 0.05$; [1]Means represent 6 pens of 16 chicks each. FI = feed intake; BWG = body weight gain; FCR = feed conversion ratio;

Table 5.4 Effect of nutrient density and phytase supplementation on performance of male broilers at 20-d of age[1]

Diet[2]	Phytase (FTU/kg)	1 to 7 d			8 to 14 d			15 to 20 d			1 to 20 d		
		FI (g)	BWG (g)	FCR	FI (g)	BWG (g)	FCR	FI (g)	BWG (g)	FCR	FI (g)	BWG (g)	FCR
LD	0	132.8^{b} (1.6)	129.2 ^{b} (2.0)	1.03^{a} (0.01)	271.6^{b} (4.5)	217.3^{b} (4.3)	1.25 (0.01)	385.3^{b} (14.3)	269.0^{b} (13.0)	1.44 (0.04)	789.6^{b} (18.6)	615.6^{b} (14.8)	1.28^{ab} (0.01)
LD	1500	156.3^{a} (2.7)	153.8^{a} (2.3)	1.02^{a} (0.01)	390.1^{a} (11.4)	309.8^{a} (11.7)	1.26 (0.02)	675.5^{a} (51.9)	440.6^{a} (13.6)	1.52 (0.07)	1221.9^{a} (60.2)	904.2^{a} (23.2)	1.35^{a} (0.04)
HD	0	123.6^{b} (1.9)	123.8^{b} (1.9)	1.00^{ab} (0.00)	273.1^{b} (3.4)	230.6^{b} (3.6)	1.18 (0.01)	367.9^{b} (14.8)	270.9^{b} (9.7)	1.36 (0.02)	764.6^{b} (15.2)	625.4^{b} (6.8)	1.22^{b} (0.01)
HD	1500	148.5^{a} (3.7)	153.9^{a} (3.1)	0.96^{b} (0.02)	363.9^{a} (11.0)	310.3^{a} (3.4)	1.17 (0.04)	612.7^{a} (25.5)	444.3^{a} (11.7)	1.39 (0.07)	1125.0^{a} (29.8)	908.6^{a} (9.5)	1.24^{b} (0.03)
SEM		5.2	4.8	0.02	16.8	13.5	0.05	61.4	24.2	0.12	71.4	29.9	0.05
Main effects													
Diet													
LD		144.6^{a} (4.2)	141.5 (4.0)	1.02^{a} (0.01)	330.8 (18.8)	263.6 (15.2)	1.26^{a} (0.01)	530.4 (50.7)	354.8 (27.4)	1.48 (0.04)	1005.8 (71.8)	759.9 (45.5)	1.32^{a} (0.02)
HD		136.0^{b} (3.8)	138.9 (4.9)	0.98^{b} (0.01)	318.5 (14.7)	270.5 (12.3)	1.18^{b} (0.01)	490.3 (39.5)	357.6 (27.1)	1.37 (0.04)	944.8 (56.6)	767.0 (43.1)	1.23^{b} (0.02)
SEM		5.7	6.3	0.01	23.9	19.5	0.02	64.3	38.5	0.06	91.4	62.6	0.03
Phytase, FTU/kg													
0		128.2^{b} (1.8)	126.5^{b} (1.6)	1.01 (0.01)	272.3^{b} (2.7)	224.0^{b} (3.3)	1.22 (0.01)	376.6^{b} (10.1)	270.0^{b} (7.7)	1.40 (0.03)	777.1^{b} (12.1)	620.5^{b} (7.9)	1.25 (0.01)
1500		152.4^{a} (2.5)	153.9^{a} (1.9)	0.99 (0.01)	377.0^{a} (8.5)	310.1^{a} (5.8)	1.22 (0.02)	644.1^{a} (29.1)	442.5^{a} (8.6)	1.45 (0.05)	1173.4^{a} (35.2)	906.4^{a} (12.0)	1.29 (0.03)
SEM		3.1	2.4	0.01	8.9	6.7	0.03	30.8	11.5	0.06	37.2	14.3	0.03
P-value													
Diet		0.004	0.282	0.002	0.157	0.316	0.005	0.206	0.817	0.072	0.103	0.640	0.004
Phytase		<0.001	<0.001	0.070	<0.001	<0.001	0.993	<0.001	<0.001	0.341	<0.001	<0.001	0.138
Diet x Phytase		0.789	0.266	0.380	0.115	0.353	0.647	0.467	0.939	0.623	0.326	0.857	0.364

[a-c] Mean (±SE) without a different letter within a column were significantly different, $p < 0.05$; [1]Means represent 6 pens of 16 chicks each. FI = feed intake; BWG = body weight gain; FCR = feed conversion ratio; [2] HD (High density) diets contained 13.39 MJ/kg (AME) and 23.28 % crude protein (CP) and LD (low density) diets contained 12.65 MJ/kg AME and 22 % CP. Both diets have the same energy:protein ratio i.e. 0.575

There were significant ($P < 0.001$) main effects due to phytase supplementation on BWG and FI of the chickens throughout this trial. The chickens fed on diets supplemented with 1500 FTU/kg phytase had significantly ($P < 0.001$) higher BWG and FI compared to those fed on non-supplemented diets. Phytase supplementation also resulted in a non-significant increase in FCR. Significant ($P < 0.05$) interactions between fat inclusion level and phytase supplementation were observed on BWG and FCR of chickens aged between 0 to 7 d. Interaction effects between fat level and phytase supplementation on FI and BWG were also significant ($P < 0.05$) for the chickens aged between 15 d to 20 d and 0 to 20 d.

Significant improvement of FCR ($P < 0.005$) was observed on 7 d, 14 d and overall trial period (0 d – 20 d) when chickens were fed with HD diets in comparison to LD diets. Whereas, phytase supplementation increased ($P < 0.001$) weekly and overall trial period (0 d – 20 d) FI and BWG but not affected FCR (Table 5.4).

5.4.3. pH of digesta

Digesta from each segment of the gastrointestinal tract (GIT) namely crop, proventriculus, gizzard and small intestine were collected. The content of stomach (proventriculus and gizzard) and upper small intestine (duodenum and jejunum) were pooled. The effects of fat inclusion level and phytase supplementation on pH of each segment of GIT are presented in Table 5.5. The level of fat inclusion significantly ($P < 0.05$) increased the pH of digesta in the small intestine but had no significant effect on the pH of stomach contents. Phytase supplementation resulted in a significant ($P < 0.05$) increase in pH of crop digesta and significant ($P < 0.005$) reduction of pH of duodenum-jejunal and ilea digesta. There was a significant ($P = 0.047$) interaction effect due to the increasing fat level and phytase inclusion on the pH of crop digesta. At high fat level inclusion, addition of phytase reduced crop pH from 5.76 to 5.19.

5.4.4. Digestibility of phosphorus and calcium

Duodenum-jejunal and ileal DM digestibility were significantly affected ($P < 0.005$) by fat inclusion level and phytase supplementation (Table 5.6). An increase of fat inclusion level from 1 to 5% resulted in reduction of DM digestibility in both

duodenum-jejunum and ileum. Phytase supplementaion at 1500 FTU/kg significantly increased duodenum-jejunal DM digestibility but decreased ileal DM digestibility. Significant interaction effects ($P < 0.001$) were also observed for both duodenum-jejunal and ileal DM digestibility. The duodenum-jejunum and ileal digesibility of P were significantly ($P < 0.05$) increased by both fat inclusion level and phytase supplementaiton but no interaction effect was observed. In contrast, the duodenum-jejunum and ileal digesibility of Ca were only affected by phytase supplementation. The digestibility of Ca in both duodenum-jejunum and ileum were significantly reduced ($P < 0.05$) when phytase at 1500 FTU/kg was suplemented

Table 5.5 Effect of dietary fat and phytase supplementation on pH of gastro intestinal tracts in male broiler chickens at 20-d of age[1]

Item	Phytase FTU/kg	Crop	Proventriculus+ Gizzard	Duodenum + Jejunum	Ileum
Fat level, %					
1	0	5.55^{ab} (0.08)	2.63 (0.09)	6.10^{a} (0.03)	7.65^{b} (0.03)
1	1500	5.53^{ab} (0.07)	2.35 (0.06)	6.27^{ab} (0.03)	7.79^{ab} (0.03)
5	0	5.76^{a} (0.07)	2.48 (0.08)	6.21^{b} (0.02)	7.73$_{ab}$ (0.03)
5	1500	5.19^{b} (0.09)	2.68 (0.10)	6.43^{a} (0.03)	7.92$_{a}$ (0.04)
SEM		0.14	0.13	0.03	0.04
Main effects					
Fat level, %					
1		5.54 (0.08)	2.49 (0.09)	6.18^{b} (0.04)	7.72^{b} (0.04)
5		5.48 (0.11)	2.58 (0.10)	6.32^{a} (0.04)	7.82^{a} (0.04)
SEM		0.10	0.09	0.04	0.04
Phytase, FTU/kg					
0		5.65^{a} (0.09)	2.56 (0.09)	6.15^{b} (0.03)	7.69^{b} (0.04)
1500		5.36^{b} (0.10)	2.51 (0.10)	6.35^{a} (0.04)	7.85^{a} (0.04)
SEM		0.10	0.09	0.03	0.04
P-value					
Fat level		0.655	0.502	0.007	0.047
Phytase		0.036	0.742	<0.001	0.003
Fat level x Phytase		0.047	0.076	0.645	0.687

[a-c] Means(± SE) without a different letter within a column were significantly different, $p < 0.05$

[1]Means represent 6 replicate cages of 16 chicks each.

Table 5.6 Effect of dietary fat and phytase supplementation on digestibility of dry matter, total phosphorus, total calcium in the duodenum-jejunum and ileum of male broilers at 20-d old age[1]

Item	Phytase FTU/kg	DM digestibility (%)		Phytate degradation (%)		P digestibility (%)		Ca digestibility (%)	
		Duodenum & jejunum	Ileum	Duodenum & jejunum	Ileum	Duodenum & jejunum	Ileum	Duodenum & jejunum	Ileum
Fat level, %									
1	0	43.28^{b}	61.13^{b}	11.04^{b}	14.09^{b}	37.33^{b}	48.41^{b}	57.48	59.46
		(0.27)	(0.10)	(3.7)	(0.98)	(3.40)	(3.41)	(3.21)	(3.73)
1	1500	47.20^{a}	61.32^{b}	22.24^{b}	43.17^{a}	43.56^{b}	59.84^{ab}	52.56	53.28
		(0.55)	(0.24)	(2.46)	(4.95)	(1.25)	(2.42)	(0.70)	(3.76)
5	0	37.55^{c}	65.52^{a}	11.95^{b}	40.43^{a}	45.52^{b}	58.42^{ab}	61.18	64.43
		(0.68)	(0.39)	(3.34)	(6.09)	(1.83)	(3.41)	(2.71)	(3.10)
5	1500	36.98^{c}	51.80^{c}	43.13^{a}	58.26^{a}	57.19^{a}	65.31^{a}	53.16	46.51
		(0.33)	(0.75)	(3.17)	(6.46)	(1.48)	(2.42)	(1.91)	(3.05)
SEM		0.46	0.37	3.34	4.62	1.81	2.03	2.13	3.41
Main effects									
Fat level, %									
1		45.24^{a}	61.23^{a}	16.64^{b}	28.63^{b}	40.44^{b}	54.12^{b}	55.02	56.37
		(0.66)	(0.13)	(2.71)	(5.55)	(1.97)	(2.63)	(1.73)	(2.69)
5		37.27^{b}	58.66^{b}	27.54^{a}	49.34^{a}	51.35^{a}	61.86^{a}	57.17	55.47
		(0.37)	(2.11)	(5.28)	(5.32)	(2.09)	(2.76)	(2.52)	(3.50)
SEM		0.52	1.12	4.00	5.44	2.03	2.70	2.13	3.10
Phytase, FTU/kg									
0		40.41^{b}	63.33^{a}	11.49^{b}	27.26^{b}	41.42^{b}	53.41^{b}	59.33^{a}	61.94^{a}
		(0.93)	(0.69)	(2.64)	(5.74)	(2.22)	(2.77)	(2.08)	(2.43)
1500		42.09^{a}	56.56^{b}	32.68^{a}	52.28^{a}	50.37^{a}	62.57^{a}	52.86^{b}	49.90^{b}
		(1.57)	(1.48)	(3.70)	(4.45)	(2.25)	(2.41)	(2.22)	(2.59)
SEM		1.25	1.09	3.17	5.10	2.24	2.59	2.15	2.51
P-value									
Fat level		<0.001	<0.001	0.005	0.001	<0.001	0.034	0.377	0.800
Phytase		0.003	<0.001	<0.001	0.001	0.001	0.014	0.014	0.003
Fat level x Phytase		<0.001	<0.001	0.004	0.303	0.222	0.512	0.522	0.108

[a-c] Means (± SE) without a different letter within a column were significantly different, $p < 0.05$; [1]Means represent 6 replicate cages of 16 chicks each[a-]

5.4.5. Tibia bone

In the present study, fat inclusion level and phytase supplementation had no influence on tibia weigh (g), tibia volume (cm^3), tibia density (g/cm^3) or tibia ash concentration (g/cm^3), although phytase supplementation showed a tendency to increase tibia ash concentration (Table 5.7). Tibia length (mm) was significantly reduced ($P < 0.05$) by fat level but not affected by phytase supplementation. The effects of fat level and phytase supplementation on tibia ash and tibia minerals of 20 d old broilers are presented in Table 5.8. Phytase supplementation significantly increased ($P < 0.001$) total ash, total Ca, total P, total Mg, total Na ($P = 0.031$) and total Fe ($P = 0.001$). Inclusion of dietary fat from 1 to 5% also increased ($P < 0.05$) total ash, total Ca, total P, total Mg but had no effect on total Na and Fe. There was no interaction effect observed between fat and phytase supplementation on bone mineralization.

Table 5.7 Effect of dietary fat and phytase supplementation on various tibia bone measurements of male broilers at 20-d old [1]

Item	Phytase FTU/kg	Tibia weight (g)	Tibia length (mm)	Tibia volume (cm^3)	Tibia density (g/cm^3)	Tibia ash concentration (g/cm^3)
Fat level, %						
1	0	1.98 (0.04)	66.52(1.06)	2.0 (0.08)	0.98(0.04)	0.36 (0.02)
1	1500	1.91 (0.11)	67.95(0.16)	1.95(0.12)	0.99 (0.01)	0.46 (0.01)
5	0	2.12 (0.04)	61.86(0.70)	2.04(0.04)	1.04(0.00)	0.37(0.01)
5	1500	2.04(0.13)	67.42(0.38)	2.08(0.15)	0.98(0.02)	0.45(0.02)
SEM		0.08	0.83	0.10	0.02	0.01
Main effects						
Fat level, %						
1		1.95(0.10)	67.23(0.95)	1.98(0.10)	0.98(0.01)	0.41(0.02)
5		2.08(0.15)	64.64(1.45)	2.06(0.15)	1.01(0.02)	0.41(0.02)
SEM		0.13	1.20	0.12	0.02	0.02
Phytase, FTU/kg						
0		2.05(0.04)	67.68(1.06)	2.02(0.08)	1.01(0.04)	0.36(0.02)
1500		1.98(0.11)	64.19(1.16)	2.02(0.12)	0.99(0.01)	0.46(0.01)
SEM		0.07	0.76	0.08	0.02	0.01
P-value						
Fat level		0.117	0.023	0.509	0.339	0.948
Phytase		0.757	0.249	0.982	0.776	0.059
Fat level x Phytase		0.922	0.068	0.689	0.335	0.476

[a-c] Means (± SE) without a different letter within a column were significantly different, $P < 0.05$

[1]Means represent 6 replicate cages of 16 chicks each.

Table 5.8 Effect of dietary fat and phytase supplementation on ash, calcium, phosphorus, iron, magnesium and sodium contents in tibia[1, 2]

Item	Phytase FTU/kg	Total Ash (mg)	Total Ca (mg)	Total P (mg)	Total Mg (mg)	Total Na (mg)	Total Fe (mg)
Fat level, %							
1	0	0.60[b] (0.02)	202.33[b] (11.2)	107.73[b] (5.30)	3.84[b] (0.35)	12.60 (0.95)	0.25[b] (0.02)
1	1500	1.05[a] (0.06)	403.30[a] (29.8)	209.30[a] (14.0)	7.66[a] (0.56)	14.38 (0.93)	0.36[ab] (0.05)
5	0	0.61[b] (0.03)	243.75[b] (15.0)	125.00[b] (06.57)	4.45[b] (0.25)	11.80 (0.44)	0.25[b] (0.01)
5	1500	1.21[a] (0.05)	453.30[a] (15.5)	237.50[a] (9.63)	9.12[a] (0.35)	13.85 (0.71)	0.49[a] (0.04)
SEM		0.04	20.39	10.09	0.38	0.76	0.03
Main effects							
Fat level, %							
1		0.82[b] (0.09)	302.79[b] (43.8)	158.49[b] (21.9)	5.75 [b] (0.84)	13.61 (0.71)	0.31 (0.04)
5		0.91[a] (0.12)	348.50[a] (40.8)	181.25[a] (21.9)	6.78 [a] (0.91)	12.83 (0.55)	0.37 (0.05)
SEM		0.03	14.43	7.15	0.87	0.63	0.04
Phytase, FTU/kg							
0		0.60[b] (0.02)	226.00[b] (12.3)	117.60[b] (5.34)	4.19[b] (0.23)	12.14[b] (0.46)	0.25[b] (0.01)
1500		1.13[a] (0.05)	428.25[a] (18.2)	223.38[a] (9.51)	8.39[a] (0.41)	14.11[a] (0.55)	0.42[a] (0.04)
SEM		0.03	14.43	7.15	0.32	0.50	0.02
P-value							
Fat level		0.048	0.047	0.046	0.028	0.408	0.092
Phytase		<0.001	<0.001	<0.001	<0.001	0.031	0.001
Fat level x Phytase		0.199	0.837	0.598	0.319	0.862	0.120

[a-c] Means (± SE) without a different letter within a column were significantly different, $p<0.05$

[1]Means represent 24 birds from 6 replicate cages per treatment.

[2]Ash and mineral contents are expressed as total ash/mineral per tibia (mg/tibia)

5.5. Discussion

The current study has clearly demonstrated significant effects due to increasing fat inclusion level and phytase supplementation on growth performance of young chickens fed on wheat based diet. The main effects of phytase supplementation were more evident on FI and BWG gain compared to the main effects exerted by fat level. In general, increasing the level of fat increased FI throughout the 20 d trial but a significant increase in FI was only observed for chickens aged 15 - 20 d. High fat

level also increased of BWG with improved FCR for chickens aged between 1 to 7 d and 0 to 14 d. Fat inclusion level has no significant effect on BWG and FCR of chickens aged 15 to 20 d and overall performance of the chickens from 0 to 20 d. Similar results were demonstrated by Atteh *et al*. (1989), who found non-significant effects of increasing fat levels from 5 % to 10 in corn based diets on FI and BWG for chickens aged 0 to 21 d, despite numerical increased in both parameters. Tabeidian *et al*.,(2005) and Monfaredi *et al*., (2011) also reported that FI and BWG of chickens aged 7 to 21 d and 11 to 28 d were not affected when fed on corn based diets containing increasing level of soybean oil from 0 to 5 % and 2 to 4 %, respectively.

Higher FI and BWG at high fat inclusion level may be attributed to better palatability of high fat supplemented diet and better digestibility of soybean oil in comparison to other sources of energy available in the diets such as corn starch, which used to compensate energy deficient in lower fat diet (Atteh *et al*., 1983; Baião and Lara, 2005). Although the trends of FI and BWG in the present study are consistent with the previous reports, addition of fat from 1 to 5 % insignificantly increased the FCR values. This is in contrast to those results of Atteh *et al.* (1983) and Atteh *et al.* (1989), who reported lower values of FCR, indicating better nutrient utilization with higher fat inclusion level (0 to 8 % and 5 to 10%, respectively).

Lower feed efficiency obtained in the present study could be due to the characteristics of wheat itself (Amerah, 2015). The presence of high amount of water soluble non-starch polysaccharides (NSPs), particularly arabinoxylans has significantly negatively affected the efficiency of nutrient utilization in wheat based diet in comparison to corn based diet though the level of fat added was higher in wheat based diets (Jia *et al*., 2009). Despite limited information on the effects of fat level on feed efficiency and nutrient utilization in chickens fed on wheat based diets, it is presumed that due to anti-nutritional effect of viscous arabinoxylans, the increasing fat level could contribute in further reducing feed efficiency and nutrient utilization in young chickens. This assumption is based on the studies by Laughout *et al*. (1997) and Meng *et a.l* (2004). Meng *et al*. (2004) showed that the FCR value of 1.4 and 87.1% faecal fat digestibility in 21 d old chickens fed on wheat based diets

containing 5% canola oil. As fat level increased to 6.5%, Laughout *et al*. (1997) found higher value of FCR (1.6) and lower faecal fat digestibility (80.2%) in young chickens fed on soybean oil-containing wheat-rye based diet, indicating possible negative effect of high fat level inclusion on feed efficiency and nutrient digestibility. In the present study, phytase supplementation significantly increased weekly and overall (0 to 21 d) FI and BWG. These results are consistent with the findings of Wu *et al*. (2004a) and Kühn *et al*. (2011), who reported significant increases in FI and BWG of young chickens fed on wheat based diets supplemented with 3% fat and between 1500 – 2000 FTU/kg phytase. However, the FCR of the present study was insignificantly higher than that of non-phytase supplemented diets, suggesting less efficient nutrient utilization with phytase supplementation and this is in contrast to the FCR found in the previous studies (Wu *et al*., 2004a; Kühn *et al*., 2011), which were significantly improved with phytase supplementation.

In the present study, it is also shown that phytase supplementation at 0 and 1500 FTU/kg resulted in relatively greater differences in overall FI and BWG (350 g FI and 240 g BWG) when compared to the differences in both performance parameters between 1% and 5% fat containing diets (70 g FI and 36 g BWG) (Table 5.3). However, chickens fed on phytase-containing diets achieved similar overall FCR as those fed on high fat diets, suggesting superior effects of phytase supplementation in comparison to the effect of fat inclusion level. Evidently, the significant interactions between fat level and phytase supplementation were observed on weight gain and feed intake of chickens aged 15 to 20 d and 0 to 20 d. The chickens fed on diets that supplemented with 5% soybean oil and 1500 FTU/kg have the highest FI and the heaviest BWG. The significant interactions between fat level and phytase supplementation were also observed on weight gain and feed efficiency of 0 to 7 d old chickens. Since the diets in this study were isocaloric and isonitrogenous (exclude HD diet), the increase of BWG and subsequent improvement of FCR in post hatched to 7 d old chickens due to increasing fat level indicate better utilization of fat as an energy source compared to other source of energy in the diets, e.g. wheat, corn and corn starch (Atteh *et al*., 1983). In addition, BWG and FCR were further improved with phytase supplementation (Table 5.3). Besides reducing phytate content in wheat based diets and improving phosphorus digestibility, supplementation of phytase was

shown to improve apparent metabolisable energy, protein, amino acid, calcium and other minerals (Afshamanesh *et al*., 2008; Juanpere *et al*., 2005, Rutherfurd *et al*., 2002; Ravindran *et al*., 2000; Shafey,1998).

Despite higher weekly FI, chickens fed with LD (5% fat with dietary fibre) diets have lower BWG and significantly poorer FCR compared to those fed with HD (5% fat without dietary fibre) diets (Table 5.4). This is in agreement with Brickett *et al.* (2007), who reported an increase in FCR with decreasing nutrient density. In the present study, all the diets given were in pelleted form and chickens fed on LD diets demonstrated their ability to regulate FI to meet their energetic requirement by eating more (Brickett *et al*., 2007) in order to yield similar body weight as those fed on HD diet. In the present study, although the energy to protein ratio was the same in both LD and HD diets, adding cellulose as a filler has diluted the nutrient concentration in LD diet that eventually led to poorer FCR (Hetland *et al*., 2004). However, with phytase supplementation, growth performance of chickens fed either LD or HD diets have significantly improved, which reflected by higher FI and BWG and these results were as expected (Selle and Ravindran, 2007). These results indicate that phytase supplementation could level out the effect of nutrient dilution in LD diet and enable the chickens to achieve better growth performance as good as those chickens fed on HD diet.

Crop, duodenum-jejunal and ileal pH were affected by fat level and phytase supplementation. The reduction of crop pH with phytase addition was in agreement with the results shown by Svihus *et al.* (2013) and Ptak *et al.* (2015), who reported that the reduction of crop pH from 5.30 to 5.00 and from 6.48 to 6.41 after supplementing wheat based diets with 500 and 5000 FTU/kg, respectively. The age of chickens when pH measurement was done is probably the reason for the notable variation in crop pH for non-phytase supplemented diets between the present study (pH 5.65 at 21 d) and the previous reports (Svihus *et al.* (2013) at 31 d; Ptak *et al.* (2014) at 42 d) as demonstrated by Morgan *et al.* (2014c), who observed increasing gut pH with chickens's age in chickens fed with wheat based diets. The mechanism of pH reduction with phytase supplementation is unclear. However, it could be due to digesta effluxing from the gizzard which contains high H ions and also could be due

to increased phytate ingested with increased feed intake. In the presence of phytate, pepsin activity in the stomach is reduced which leads to high HCl secretion and reduction of stomach and jejunal pH (Woyengo *et al*., 2010). Although no main effect of fat level was apparent, by increasing fat level in phytase supplemented diets crop pH was further reduced (Table 5.5). This reduction in pH is possibly because increased fat level prolonged ingesta retention in the crop (Mateos *et al*., 1982, Golian and Polin, 1984; Kim *et al*., 2013), therefore more H ions effluxing from gizzard and more phytate intake which leads to further increase in the acidogenic activity of phytate (Walk *et al*,. 2012d). While previous authors have reported incremental decrease in gizzard pH with phytase supplementation (Svihus *et al*., 2013; Walk *et al*,.2012), phytase had no significant effect on gizzard pH in the present study, despite a numerical decrease in pH value. In the present study, inclusion of higher fat level and phytase at 1500 FTU/kg significantly increased the pH of duodenum-jejunum and ileum. These results agreed with Shafey (1999) who found higher pH value of digesta collected from intestinal tract between the loop of duodenum and distal ileum from chickens fed on wheat-sorgum based diets containing 8 % sunflower oil than those fed on no-sunflower-containing diets. Furthermore, pH of duodenum, jejunum and ileum were increased when phytase was supplemented at 5000 FTU/kg in wheat based diets with 1.8% soybean oil (Ptak *et al*., 2015) and at 500 and 5000 FTU/kg in corn based diets with 0.77 % animal fat (Walk *et al*., 2012d). Walk *et al.* (2012d) also suggested that phytate degradation by high dietary phytase is responsible for the increase of pH in the gizzard and small intestine.

Currently, reports on the effect of fat addition in phytase supplemented diet on intestinal phytate degradation are limited. However, Zeller *et al*. (2015a; 2015b), Camden *et al.* (2001) and Tamim *et al*. (2004) could provide general indication related to fat addition effects on duodenum-jejunal and ileal phytate degradation. Zeller *et al.* (2015a) found inositol-6-phosphate ($InsP_6$) hydrolysis in duodenum-jejunum and ileum was 59 and 74%, respectively, when chickens were fed on non-phytase supplemented corn based diets that contained 2% soybean oil. With addition of *Escherichia coli* 6-phytase at 500 FTU/kg, duodenum-jejunal and ileal phytate degradation were increased to 68 and 82% with 9 and 10% improvement,

respectively. While Zeller *et al.* (2015b) reported lower $InsP_6$ degradation in duodenum-jejunum (48%) and ileum (51%) of chickens fed non-phytase supplemented with corn based diets containing 3% soybean oil which had similar Ca to total P ratio as Zeller *et al.* (2015a). Lower extent of duodenum-jejunal (2%) and ileal (7%) $InsP_6$ degradation was also observed by Zeller *et al.* (2015b) although the same type and level of phytase as Zeller et al. (2015a) was added. Camden *et al.* (2001), on the other hand, demonstrated that when chickens fed on a corn based diet containing 1.5% fat level, 0.59% total P and 0.80% Ca, ileal phytate degradation was 22% without supplemental phytase and was increased by 26 and 32% with 3-phytase supplementation at 500 and 1000 FTU/kg, respectively. At lower total P (0.40%) and Ca (0.65%), about 33% increase of ileal degradation from 25 to 59% was observed in diet containing 5.2 % crude soy oil and 500 FTU/kg *Aspergillus ficuum* 3-phytase (Tamim *et al*., 2004). These studies indicate that phytate degradation in small intestine improves with phytase supplementation regardless of different level of fat added, type and level of supplemental phytase. However, the extent of intestinal phytate degradation is affected by these factors. The percentages of intestinal phytate degradation in non-phytase supplemented diets may also be affected by dietary fat level and Ca: total P, as shown in previous studies, ranging from 22 to 74%.

Phytate degradation in duodenum-jejunum and ileum reported in the present study was significantly affected by fat level and phytase supplementation. Increased level of fat alone from 1 to 5% improved phytate degradation by 10% in duodenum-jejunum and 20% in the ileum, while 6-phytase supplementation alone, phytate degradation increased by 20 and 25% in duodenum-jejunum and ileum, respectively. Together both parameters brought about an additive effect on duodenum-jejunal and ileal phytate degradation (Table 5.6). Generally, the effect of fat and phytase in the present study seem in contrast to the previous reports by Zeller *et al.* (2015a; 2015b) although the same type of phytase (*E. coli* 6-phytase) was used. The extent of duodenum-jejunal and ileal phytate degradation reported by Zeller *et al.* (2015a; 2015b) were lower than those observed in the present study and the extent of intestinal phytate degradation were even lower with higher fat level. On the other hand, effects of fat and phytase in the present study seem to agree with Camden *et al.* (2001) and Tamim *et al*. (2004) who showed larger extent of ileal phytate

degradation with supplementation of 3-phytase and ileal phytate degradation was even higher with higher fat level. The inconsistency observed between the present study and previous reports may be due to the type of cereal used in the diets as well as level of fat, type and level of supplemented phytase and the ratio of Ca to total P as previously discussed.

Although there are no previous reports on the effect of fat level on P and Ca digestibility in duodenum-jejunum of chickens fed wheat base diet, Zeller *et al.* (2015a: 2015b) found 34 and 26% of duodenum-jejunal P digestibility in chickens fed on corn based diets containing 2 and 3% soybean oil, respectively and the digestibility of duodenum-jejunal P was improved by 5% with 500 FTU/kg phytase supplementation. In the present study, as fat level in the diets was increased from 1 to 5%, the digestibility of duodenum-jejunal P was improved by 11% and phytase supplementation at 1500 FTU/kg further improved the duodenum-jejunal P digestibility (Table 5.6). Although fat level numerically increased duodenum-jejunal Ca digestibility, phytase supplementation significantly reduced duodenum-jejunal Ca digestibility. Despite variable phytate degradation, similar values of ileal P digestibility were observed in previous studies regardless the content of fat, which ranged between 53 to 57% in chickens fed with corn based diets (Camden *et al*., 2001; Rutherfurd *et al*., 2004; Zeller *et al*., 2015a) and between 56 to 57% in chickens fed on wheat based diets (Leytem *et al*, 2008; Afshamanesh *et al*., 2008) and the ileal P digestibility were improved with phytase supplementation (Onyango *et al*., 2005; Tamim *et al*., 2004; Amerah *et al.*, 2014). Similar to the results of the present study, ileal P digestibility was 53% in non-phytase supplemented diets and increased by 9% with phytase supplementation. In addition, as fat level increased in the diets, the ileal P digestibility was further improved and a non-significant interaction between fat level and phytase supplementation was observed.

The effect of fat level and phytase supplementation on ileal Ca digestibility obtained in the present study was also in accordance to previous reports. Piyamas Thachoroerat (2012) found that ileal Ca digestibility reduced from 52 to 48% as the fat level in corn based diets increased from 0 to 4% and further reduced when fat level increased to 8% and the reduction of ileal Ca digestibility was evidently due to

the increase of digesta soap. Atteh *et al*., (1989) also reported significant reduction in Ca retention when young chickens fed corn based diets containing increased fat level from 5 to 10% and it was thought to be due to the formation of Ca-soap with increasing fat level. On the other hand, previous studies have demonstrated that chickens fed corn based diets supplemented with oleic acid or linoleic/oleic acid mixtures had less intestinal and faecal soap and these unsaturated fatty acids had no significant effect on Ca retention (Atteh *et al*., 1983; Atteh and Leeson, 1984). Whereas Atteh *et al.* (1989) found that the level of fat had no significant effect on the concentration of faecal soap and the soap formation due to vegetable oil, particularly soybean oil, was not considered as a limiting factor in nutrient utilization by chickens. It is because soybean oil generally contains more than 50% linoleic acid and around 25% of oleic acid (Jokic *et al*., 2013) and soap of unsaturated fatty acids were better utilized than those from saturated fatty acids (Atteh and Leeson, 1984). This could explain the insignificant difference of ileal Ca digestibility observed in the present study when soybean oil was increased from 1 to 5%, which is unlikely to lead to the Ca soap formation.

Phytase supplementation at 1500 FTU/kg reduced ileal Ca digestibility broilers by 12% compared to that in those fed an non-phytase supplemented diet (Table 5.6) and this finding is partially in agreement with Amerah *et al*., (2014) and Leytem *et al.* (2008), who reported numerical reduction of ileal Ca digestibility in chickens fed wheat and corn based diets supplemented with phytase at 1000 FTU/kg in comparison to non-phytase supplemented diets. Insignificant changes of ileal Ca digestibility when phytase was supplemented in broiler diets were also reported in previous studies (Onyango *et al*., 2005; Walk *et al*., 2012a, 2012b) even with superdosing phytase levels (Cowieson *et al*., 2006b). In contrary, Tamin *et al.* (2004) and Ravindran *et al.* (2006) demonstrated significant improvement of ileal Ca digestibility with phytase supplementation. According to Walk *et al*., (2012c), phytase contributes in balancing the concentrations of soluble Ca and P in the small intestine, which later reduces the precipitation of insoluble Ca-phytate complexes as phytate is broken down to smaller InsP esters (Zeller *et al*., 2015b) hence, improves Ca and P digestibility. As a result of phytate hydrolysis by high dietary phytate, concentrations of soluble Ca and P increase and may cause an increase of pH in

intestinal tract (Walk *et al*., 2012d). On the other hand, in high pH conditions, Ca is thought to mediate the formation of tertiary protein-mineral-phytate complexes (Singh, 2008). In addition, an increasing Ca concentration in the gut was reported to increase faecal soaps significantly when chickens fed on diets containing palmitic acid compared to those fed on diets containing linoleic acid/oleic acid mixture (Atteh and Leeson, 1983) and caused further reduction of Ca retention associated with addition of saturated fatty acid (SFA) (Atteh and Leeson, 1984). In addition, Atteh *et al*. (1989) observed higher amount of SFAs in faecal soap fat from chickens fed diets containing soybean oil in comparison to those fed on diets containing animal-vegetable (AV) blend fat although the amount of SFAs in the diets with soybean oil was lower than in the diets with AV-blend fat. Therefore, it is plausible to suggest that in the present study, the high pH environment in the small intestine (Table 5.5) would have caused the soluble Ca, released via phytate-phytase reaction; to bind to the remaining unhydrolysed phytate and protein to form insoluble complexes. With higher fat level in the diets, more free fatty acids would be released into intestinal tract environment during fat digestion and the free SFAs, particularly palmitic acid would bind with the soluble Ca to form insoluble soap. Insoluble tertiary protein-mineral-phytate complexes and insoluble Ca-soap together would have aggravated the effect of fat level and phytase supplementation on ileal Ca digestibility.

The influence of increasing dietary fat level on tibia ash and tibia minerals has been reported previously. By increasing fat level from 0 to 9%, Atteh et al. (1983) found no significant effect on tibia ash and tibia minerals, namely, P, Mg, Zn and Mn but tibia Ca was significantly reduced as fat level increased. Whereas, Atteh et al. (1989) reported that by increasing soybean oil from 5 to 10%, tibia ash was significantly reduced but tibia Ca, P and Mg were not affected. In the present study, addition of higher fat level increased total tibia ash and total tibia minerals, except for total Na and total Fe. In addition, phytase supplementation also significantly increased total tibia ash and total tibia minerals including P, Ca, Mg, Na and Fe. These findings are in agreement with Walk et al. (2014, 2012c), Kühn et al. (2011) and Amerah et al. (2014). Ptak et al. (2013) also demonstrated significant increase in tibia Mg and tibia Na with phytase supplementation. Tibia Fe and tibia Mg were also increased with phytase addition at 1000 FTU/kg (Pintar et al., 2005). However,

simultaneous addition of fat and phytase in broiler diet has no beneficial effect on bone mineralization.

5.6. Conclusion

In conclusion, phytase supplementation improved FI and BWG. With addition of higher dietary fat level in phytase supplemented diets, the growth performance of broilers were further improved. Phytate degradation and P digestibility were also improved with fat and phytase supplementation. On the other hand, phytase supplementation reduced duodenum-jejunal and ileal calcium digestibility and it may be related to the formation of insoluble tertiary protein-mineral-phytate complexes. Conversely, addition of dietary fat and phytase separately increased total ash and mineral retention in tibia. However, simultaneous addition of fat and phytase in broiler diet has no beneficial effect on bone mineralization. Adding cellulose as filler has diluted the nutrient concentration in 5% fat diet that eventually led to poorer FCR as compared to those fed 5% fat diet without cellulose addition (high density diet). However, phytase supplementation eliminated the negative effect of nutrient dilution due to cellulose addition by improving the growth performance of chickens as good as those fed on high density diet.

Chapter 6
Digesta transit time, gut development and phytate phosphorus degradation in broilers fed on phytase supplemented diet influenced by dietary fat and fibre level.

6.1. Abstract

The aim of this study was to investigate the effect of dietary fat and fibre on the digesta mean retention time (MRT) of broiler chickens fed on phytase supplemented diet compared to those fed non-phytase supplemented diet. It was hypothesised that digesta MRT would be increased with higher fat supplementation but not affected by addition of dietary fibre. Increased MRT should improve phytate phosphorus (P) degradation in each section of the gastrointestinal tract (GIT). This experiment was conducted as a 2 x 2 x 2 factorial arrangement of treatments with 2 levels of dietary fat (low fat at 10 g/kg diet, high fat at 50 g/kg diet), 2 levels of phytase (0 or 1500 FTU/kg diet) and 2 levels of dietary fibre at 0 or 40 g/kg diet and diets were in mash form. Body weight gain (BWG) and feed efficiency of chickens fed on the low P and calcium (Ca) diet were improved by simultaneous inclusion of high fat either with phytase or with high fibre. Higher dietary fat supplementation did not significantly increase the overall digesta MRT of chickens fed on low P and Ca diet, which is contradicted with the hypothesis of the present study. However, additive effect of high fat and phytate supplementation on phytate P degradation provides evidence for the role of fat in improving phytase efficacy in broilers. Similarly, addition of dietary fibre in phytase supplemented diet improved crop phytate degradation. The level of dietary fat also contributed to the extent of phytate P degradation in diet containing high dietary fibre. However, the effect of phytase supplementation on phytate P degradation was more pronounced than those of dietary fat and fibre. It is suggested that simultaneous addition of dietary fat as high as 5% and 1500 FTU/kg phytase in P and Ca deficient wheat mash diet is able to improve *in vivo* phytase efficacy and growth performance of broilers over and above those fed adequate P and Ca diet. Addition of high dietary fibre should be avoided.

6.2. Introduction

Gastrointestinal retention involves the movement of food from mouth into crop, proventriculus and gizzard, discharge of chyme through the pylorus into the duodenum, and the movement of digesta in the small intestine and finally the discharge of faeces from the colon. The gastrointestinal MRT of digesta is the time taken for feed to pass through the GIT. When the digesta MRT is prolonged, more time is available for the digesta to react with dietary enzymes in the crop and subsequently with endogenous enzymes further down the GIT, there will also be longer contact time of digesta with the absorptive surfaces in the small intestine, thus, improving the nutrient utilization efficiency in animal (Mateos and Sell, 1980a).

The digesta MRT is affected by numerous dietary and husbandry factors including dietary fat, particle sizes and types of fibre and carbohydrate. Mateos *et al.* (1982) showed that the digesta MRT increased as inclusion level of yellow grease increased from 193 min with 0% fat to 270 min with 30% fat in chickens fed a corn-based diet. For rye-based diets, the MRT was higher with the addition of 100 g/kg soybean oil (499 min) compared to beef tallow (414 min) at the same level of inclusion (Danicke *et al.*, 1999). There is only one report in the literature evaluating the effect phytase on MRT (Watson *et al.*, 2006) which found faster digesta MRT in 1 day old chicks fed on corn based diet with phytase supplementation. The effect of fat addition, type and inclusion level of fat in the presence of phytate on MRT has not yet been reported at the time of thesis writing. On the other hand, increasing the level of oat hulls as the source of insoluble fibre was reported to have no significant effect on the digesta MRT in broilers (Hetland and Svihus, 2001; Svihus *et al.*, 2002). Conversely, Cao *et al*. (2003) demonstrated that increasing the level of dietary cellulose to 10% significantly reduced digesta MRT.

Based on our previous study, addition of phytase to a wheat/corn based diet significantly increased BWG and feed intake (FI) of three week old broiler chickens. This effect was further enhanced in terms of BWG and FI by the addition of fat (Chapter 5). However, in the previous study, it was not established whether or not the improved performance by addition of dietary fat was due to prolonged MRT.

The effect of adding dietary cellulose into the diet on digesta TT was also not determined.

Therefore, the aim of this study was to investigate the effect of dietary fat and fibre on the digesta TT of broiler chicken fed a phytase supplemented diet. Their effects on gut development, digesta pH and phytate P degradation were also studied. It was hypothesised that TT would be prolonged with higher fat supplementation but not affected by addition of dietary fibre, thereby improving phytate P degradation in each section of the GIT

6.3. Materials and methods

All experimental procedures were conducted in accordance with The Animals (Scientific Procedures) ACT 1986, under Animal Ethical Review Committee of University of Leeds..

6.3.1. Animals and housing

A total of 225 day-old male Ross chicks were vaccinated with Infectious Bronchitis (IB) and Marek's disease vaccines at the hatchery. Upon arrival at the research farm, the chicks were individually weighed and randomly allocated into 45 pens (floor space of 82 cm x 50 cm) with 5 birds per pen. The floor of each pen was covered with AstroTurf with a layer of wood shavings on top. The pens complied with DEFRA recommendations and meet the Welfare of Farmed Animals (England) Regulations 2007 and Code of Practice for the housing and care of animals used in scientific procedures (1989). The chicks were raised with *ad libitum* access to the experimental diet and water from d 0 till d 23 in a controlled environment. Room temperature was initially set at 32^{o}C ± 2^{o}C and gradually reduced to 22°C by d 23. Relative humidity (RH) was monitored. Continuous lighting from 1 to 3 d of age, a lighting program of 20 h light: 4 h dark (20L: 4D) from 4 to 23 d of age and continuous ventilation were applied. Individual body weights and pen FI were recorded weekly while health and mortality were recorded daily.

6.3.2. Experimental design and diet

This trial was conducted as a 2 x 2 x 2 factorial arrangement of treatments with 2 levels of dietary fat (low fat at 10 g/kg diet, high fat at 50 g/kg diet), 2 levels of phytase (0 or 1500 FTU/kg diet) of an enhanced *Escherichia coli*-derived phytase (Quantum Blue 5G, AB Vista Feed Ingredients, Marlborough, UK) and 2 levels of fibre (Vitacel®) at 0 or 40 g/kg diet. Wheat/corn based diets were formulated to meet or exceed the commercial broiler requirements for starter feed except for the level of available P (aP), Ca and Na which were reduced to 0.34, 0.84 and 0.13%, respectively(Table 6.1 and 6.2). All diets contained titanium dioxide (5g TiO_2/kg) as an indigestible marker for measurement of digesta MRT and as reference for nutrient digestibility. The diets differed in the level of dietary fat and fibre level. Diet A and Diet B were supplemented with 1 and 5% dietary fat level, respectively. Diet C and Diet D were also supplemented with 1 and 5% fat, respectively but both diets were supplemented with 4% dietary fibre. While, Diet E, F, G and H had the same compositions as Diet A, B, C and D, respectively but supplemented with phytase at 1500 FTU/kg. All diets were formulated to have similar energy to protein ratio (Table 6.2) and the calculated differences in the ME of soybean oil were overcome by corn starch and corn gluten meal. A positive control (PC) diet with adequate P, Ca and Na, 1% fat level and without the addition of dietary fibre was also prepared. All diets were in mash form.

Table 6.1 Dietary treatments and measured activity of phytase in the diet

Diet	Dietary Fat %	Dietary Fibre %	Dietary enzyme	Added activity (FTU/kg)	Measured activity[1] (FTU/kg)
A	1	-	- phytase	-	<50
B	5	-	- phytase	-	<50
C	1	4	- phytase	-	<50
D	5	4	- phytase	-	<50
E	1	-	+ phytase	1500	1580
F	5	-	+ phytase	1500	1285
G	1	4	+ phytase	1500	1485
H	5	4	+ phytase	1500	1400
PC_2	1	-	- phytase	-	<50

[1] Means of duplicate assays; [2] Adaquate P, Ca and Na

Table 6.2 Compositions of the experimental diets (%)

Ingredients (%)	PC[1]	Diet A	Diet B	Diet C	Diet D	Diet E	Diet F	Diet G	Diet H
Wheat	33.00	33.00	33.00	33.00	33.00	33.00	33.00	33.00	33.00
Corn	18.97	18.97	18.97	18.97	18.97	18.97	18.97	18.97	18.97
Soybean oil	1.00	1.00	5.00	1.00	5.00	1.00	5.00	1.00	5.00
Vitacel® Cellulose	-	-	-	4.00	4.00	-	-	4.00	4.00
Soya extract (48%)	21.38	23.81	29.77	20.00	27.70	23.81	29.77	20.00	27.70
Potato protein (79%)	8.00	6.47	2.00	7.25	3.33	6.47	2.00	7.25	3.33
Corn starch	13.00	13.00	5.00	11.08	3.34	13.00	5.00	11.08	3.34
Corn gluten meal (62%)	-	-	2.45	0.85	0.80	-	2.45	0.85	0.80
Lysine HCl	0.22	0.24	0.33	0.31	0.33	0.24	0.33	0.31	0.33
Methionine	0.15	0.16	0.16	0.16	0.18	0.16	0.16	0.16	0.18
Threonine	0.03	0.05	0.08	0.07	0.09	0.05	0.08	0.07	0.09
Dicalcium phosphate, anhyd, 18% P	2.47	1.40	1.37	1.40	1.38	1.40	1.37	1.40	1.38
Limestone	0.49	0.69	0.67	0.70	0.67	0.69	0.67	0.70	0.67
Salt	0.21	0.21	0.20	0.20	0.20	0.21	0.20	0.20	0.20
Sodium carbonate	0.18	0.10	0.10	0.11	0.11	0.10	0.10	0.11	0.11
Vitamin & mineral premix[2]	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Titanium dioxide (TiO_2)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Diet E, F, G and H have the same compositions as Diet A, B, C and D, respectively but supplemented with phytase at 1500 FTU/kg.

[1]Positive control (PC)l has similar compositions as Diet A but have adequate P, Ca and Na.

[2]Premixes provided the following (per kg of diet): vitamin A, 13,200 IU; vitamin D3, 4,000 IU; vitamin E, 66 IU; vitamin B12, 39.6 µg; riboflavin, 13.2 mg; niacin, 110 mg; Dpantothenate, 22 mg; menadione (K3), 4 mg; folic acid, 2.2 mg; thiamine, 4 mg; pyridoxine, 8 mg; D-biotin, 252 µg; selenium (as Na2SeO3), 0.30 mg; manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; cobalt, 1.0 mg; choline chloride, 1,200 mg;.

Table 6.3 Calculated and analysed nutrient contents of the experimental diet (%)

Calculated nutrients	Recommended level[1]	Diet PC[2]	Diet A	Diet B	Diet C	Diet D	Diet E	Diet F	Diet G	Diet H
DM	As it is	88.20	88.04	88.30	88.34	88.61	88.04	88.30	88.34	88.61
AME Chick	12.65	12.65	12.66	13.16	12.24	12.66	12.66	13.16	12.24	12.66
CP	22-25	22.05	22.04	23.00	21.44	22.05	22.04	23.00	21.44	22.05
ME: CP	0.575	0.574	0.574	0.572	0.571	0.574	0.574	0.572	0.571	0.574
Lys	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43	1.43
Met	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51
Thr	0.94	0.95	0.94	0.94	0.94	0.94	0.94	0.94	0.94	0.94
Trp	0.24	0.28	0.28	0.28	0.27	0.28	0.28	0.28	0.27	0.28
Ca	1.05	1.05	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84
Total P		0.78	0.59	0.61	0.58	0.60	0.59	0.61	0.58	0.60
Available P (aP)	0.50	0.50	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
Ca:aP	2.10	2.13	2.45	2.47	2.48	2.46	2.45	2.47	2.48	2.46
Phytate		0.13	0.12	0.13	0.12	0.12	0.12	0.13	0.12	0.12
Na	0.16	0.16	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Cl	0.16-0.23	0.21	0.21	0.20	0.20	0.20	0.21	0.20	0.20	0.20
Fibre		1.89	1.97	2.19	5.90	6.10	1.97	2.19	5.90	6.10
Fat		2.80	2.79	6.76	2.78	6.74	2.79	6.76	2.78	6.74
Analysed nutrients[3]										
Gross energy		15.22	16.58	17.71	16.61	17.26	16.35	17.54	16.60	17.40
Crude protein		23.63	24.47	24.00	23.00	23.20	23.37	23.97	22.67	22.80
Ether extract		2.97	3.62	8.87	2.91	7.04	3.02	7.87	2.83	7.13
Crude fibre		2.13	2.37	2.67	3.43	3.30	2.13	2.30	3.23	3.47
Ash		5.67	6.33	6.23	5.17	5.43	5.17	5.73	4.73	5.43
Ca		1.03	1.06	1.03	0.85	0.87	0.81	0.84	0.77	0.80
Total P		0.78	0.63	0.69	0.55	0.62	0.63	0.62	0.57	0.57
Phytate P		0.20	0.19	0.21	0.18	0.22	0.21	0.22	0.19	0.20

The requirement was according to The Specification for As-Hatched Broilers Grown to <1.9kg (4.2lb) live weight for Ross 308 Broiler (Aviagen, 2007). The levels of aP and Ca are reduced to 0.34 and 0.84, i.e. about 31% and 20% lower than the values recommended for Ross Broiler; [2]Positive control(PC) diet with adequate P, Ca andNCa. [3]Means of triplicate assay

6.3.3. Measurement of mean retention time

The estimation of digesta MRT was conducted according to Method 1 by Van Der Klis *et al*. (1990). On day 21, after the weekly weighing of birds and feed residues, the birds were fed with fresh pre-weighed diet and the time of feeding was recorded as a start time. At the time of slaughter, the end time for feeding period was recorded and feed residues were weighed. The birds were individually weighed and killed by intravenous injections of sodium pentobarbitone followed by cervical dislocation. The abdomen was opened and the GIT was exposed. The skin over the crop was incised and both the upper and lower junctions of the crop were carefully clamped. Three other sections of GIT were individually clamped; 1) proventriculus-gizzard which is between upper junction of proventriculus and lower junction of gizzard; 2) duodenum-jejunum, which is between lower junction of gizzard till just before the Meckel's diverticulum and 3) ileum that is from Meckel's diverticulum and ileocaecal junction. The full weight of crop, gizzard, duodenum-jejunum and ileum were recorded, then digesta were removed and the empty weight of each section was recorded for determining the total weight of digesta. The digesta from all birds in the same pen were pooled and mixed before pH measurement. All collected digesta samples were stored at -20^{o}C until determination of dry matter (DM) and titanium (Ti) content. The digesta TT in each part of the digestive tract was calculated according to the following equations where 1440 is minutes per day (24 h);

Feed intake (FI) in g = weight of pre-weighed diet - weight of feed residue

Total weight of digesta (g) = full weight of a segment - empty weight of a segment

Ti in segment (mg) = Total DM in a segment (g) x Ti concentration (mg/g digesta)

$$\text{Ti Intake (mg/min)} = \frac{\text{FI (g) x Ti concentration (mg/g diet)}}{\text{(End time - start time of feeding x 1440)}}$$

$$\text{Digesta MRT (min)} = \frac{\text{Ti in segment (mg)}}{\text{Ti intake (mg/min)}}$$

6.3.4. Chemical Analysis

Prior to analysis, digesta samples were freeze dried and ground to pass through a 1.0 mm sieve. DM was determined according to Method 930.15 (AOAC, 2000). Ashed samples of diet and digesta were digested in 7.4 M sulphuric acid for about 1 h, filtered through ashless filter Whatman No. 541 and diluted to 100ml with ultrapure water. Diet and digesta samples were analysed for Ti and other minerals by ICP-OES. Phytase activity in feed samples was determined by Enzyme Services and Consultancy Ltd, Wales according to the internal, validated methods of the producer (Quantum method). One unit of phytase (FTU) is defined as the amount of enzyme which releases 1 μmol of inorganic P per minute from phytic acid at pH 4.5 and 60°C. Phytate contents in the diet and digesta were determined based on the difference between total P and free P. Free P was extracted from 1 g of sample in 20 ml 0.66M HCl by vigorously stirring for at least 3 h. One ml of the extraction mixture was centrifuged at about 19,000 x g for 10 min and the supernatant was analysed for P as above. Phytate content was calculated with the assumption that the amount of P measured is exclusively released from phytate which is 28.2% P.

$$\text{Phytate (g/100g)} = (\text{Total P} - \text{Free P}) / 0.282$$

Phytate-P degradation, DM digestibility and apparent nutrient digestibility were calculated by the following equation using Ti as a marker.

$$\text{Nutrient digestibility (\%)} = [(\text{Nutrients/Ti})_{diet} - (\text{Nutrients /Ti})_{digesta}] / (\text{Nutrients/Ti})_{diet} \times 100$$

6.3.5. Statistical Analysis

The performance data was derived from pen means. Digesta MRT and digesta content were first subjected to log transformation to achieve near normal distribution on normality test in Minitab 17 Statistical Software (Minitab Inc, 2014). In order to assess the inclusion of fat, fibre and phytase and their interactions, data from the PC group was excluded from the data sets. All data sets were analysed using general linear model (GLM) in Minitab 17 with batch (the experiments were conducted in 2 batches) as the covariate except for gut weight which was analysed with batch and BWG at 21 d as the covariates. Significant differences between means were identified at $P \leq 0.05$ by Multiple Comparison of Means Tukey's Method. Comparisons between PC (adequate P and Ca at 1% dietary fat level) and negative control (NC; low P and Ca at 1% dietary fat level), PC and fat (average of all diet containing 5% fat level), PC and fibre (average of all diet containing 4% fibre level), PC and phytase (average of all diet containing 1500 FTU/kg phytase) and PC and average of all treatments were made using orthogonal.

6.4. Results

6.4.1. Diet analysis

The analysed nutrient contents and measured phytase activity in the experimental diet are presented in Table 6.1 and Table 6.3. The analysed dietary Ca and P generally agreed with formulated values and were within acceptable ranges. The measured values of phytase activity in the experimental diets were as expected and in agreement with the experimental design.

6.4.2. Broiler performance

Mortality during the trial was low (2%) and was not significantly different between the diet groups (data not shown). Deaths were not related to dietary treatment. FI, BWG and feed conversion ratio (FCR) of broiler chicken from 0 to 21 d are presented in Table 6.4. There were significant ($P < 0.05$) main effects due to fat inclusion level

on growth performance of broilers aged between 0 to 7 d, 0 to 14 d and 0 to 21 d. Fat inclusion at 5% significantly increased FI ($P < 0.01$) and BWG ($P < 0.001$) and improved FCR ($P < 0.001$). There were also significant ($P < 0.05$) main effects due to dietary fibre inclusion on growth performance of broilers. Addition of dietary fibre at 4% significantly reduced both FI and BWG of chickens aged 0 to 7 d ($P < 0.05$) and 0 to 14 d ($P < 0.01$) and BWG of chickens aged 0 to 21d ($P = 0.05$). However, dietary fibre inclusion had no significant effect on FCR. While phytase supplementation only had a significant effect on FI ($P < 0.05$) and FCR ($P < 0.01$) of chickens aged 0 to 21 d. With phytase supplementation at 1500 FTU/kg, FI was reduced by 6% from 1.111 kg to 1.043 kg per bird and FCR improved by 7% from 1.707 to 1.595.

There were also significant interactions ($P < 0.05$) between fat and fibre inclusion and between fat and phytase inclusion. From 0 to 14 d, chickens fed on diet containing 5% dietary fat either with or without addition of 4% dietary fibre had significantly higher BWG ($P = 0.04$) and lower FCR ($P = 0.02$) compared to those fed diets containing 1% dietary fat either with or without addition of 4% dietary fibre. However, addition of 4% dietary fibre to the 5% fat diet significantly ($P = 0.04$) lowered BWG. Supplementing phytase in the 1% fat diet significantly ($P = 0.002$) improved FCR from 2.016 to 1.774 and when the level of fat in the diet increased from 1% to 5%, FCR was further improved from 1.774 to 1.416.

From 0 to 7 d, FI, BWG and FCR of chickens fed the PC diet, were similar to those fed on the NC diet, the high fat diet, the high fibre diet and the phytase supplemented diet. From 0 to 14 d, chickens fed the PC diet had significantly higher BWG ($P = 0.032$) and better FCR ($P = 0.012$) compared to those fed the NC diet, whereas chickens fed the high fat diet had significantly higher BWG ($P = 0.043$) and better FCR ($P = 0.004$) than those fed on the PC diet. Similarly, from 0 to 21 d,

Table 6.4 Effects of dietary fat, dietary fibre and phytase supplementation on performance of broilers fed on wheat/corn starter diet (0 to 20 d posthatch)[1]

Fat,	Fibre,	Phytase,	d 0 to 7			d 0 to 14			d 0 to 21		
%	%	FTU/kg	FI (%)	BWG (%)	FCR	FI (%)	BWG (%)	FCR	FI (%)	BWG (%)	FCR
1			124(3)^{b}	98(4)^{b}	1.32(0.04)^{a}	466(17)^{b}	266(10)^{b}	1.77(0.05)^{a}	1031(34)^{b}	550(23)^{b}	1.90(0.04)^{a}
5			135(2)^{a}	119(3)^{a}	1.16(0.03)^{b}	509(10)^{a}	372(8)^{a}	1.38(0.03)^{b}	1123(31)^{a}	806(18)^{a}	1.41(0.02)^{b}
SEM			3	4	0.04	14	9	0.04	31	20	0.03
	0		133(3)^{a}	112(4)^{a}	1.23(0.04)	505(13)^{a}	335(14)^{a}	1.58(0.06)	1105(34)	703(32)^{a}	1.65(0.06)
	4		126(3)^{b}	105(4)^{b}	1.25(0.04)	470(14)^{b}	304(11)^{b}	1.58(0.04)	1049(31)	653(26)^{b}	1.65(0.05)
SEM			3	4	0.04	14	12	0.05	32	29	0.05
		0	127(3)	107(4)	1.24(0.04)	486(15)	319(13)	1.59(0.05)	1111(36)^{a}	681(30)	1.71(0.06)^{a}
		1500	133(3)	110(4)	1.24(0.03)	488(13)	319(12)	1.57(0.04)	1043(26)^{b}	675(28)	1.60(0.04)^{b}
SEM			3	4	0.04	14	12	0.05	31	29	0.05
1	0		130(4)^{ab}	101(6)^{b}	1.34(0.05)^{a}	488(23)^{ab}	271(15)^{c}	1.83(0.06)^{a}	1068(66)^{ab}	551(34)^{c}	1.96(0.06)^{a}
1	4		118(3)^{b}	96(6)^{b}	1.30(0.07)^{a}	444(25)^{b}	262(15)^{c}	1.71(0.07)^{a}	993(58)^{b}	549(32)^{c}	1.83(0.06)^{a}
5	0		136(4)^{a}	124(4)^{a}	1.12(0.04)^{b}	521(13)^{a}	398(10)^{a}	1.32(0.04)^{b}	1142(25)^{a}	855(23)^{a}	1.47(0.03)^{b}
5	4		134(3)^{a}	113(4)^{a}	1.20(0.03)^{ab}	496(15)^{a}	346(10)^{b}	1.44(0.04)^{b}	1104(26)^{ab}	758(23)^{b}	1.34(0.03)^{b}
SEM			4	5	0.05	19	12	0.05	44	28	0.04
1		0	122(4)^{b}	96(6)^{b}	1.33(0.06)^{a}	466(25)^{b}	263(16)^{b}	1.82(0.07)^{a}	1090(68)^{ab}	546(35)^{b}	2.02(0.05)^{a}
1		1500	127(4)^{ab}	101(6)^{b}	1.30(0.06)^{ab}	465(23)^{ab}	270(12)^{b}	1.73(0.06)^{a}	971(41)^{b}	554(27)^{b}	1.77(0.06)^{b}
5		0	132(4)^{ab}	118(4)^{a}	1.14(0.04)^{bc}	506(15)^{ab}	376(11)^{a}	1.36(0.04)^{b}	1131(26)^{a}	816(25)^{a}	1.40(0.03)^{c}
5		1500	138(3)^{a}	119(5)^{a}	1.18(0.03)^{c}	511(14)^{a}	368(12)^{a}	1.40(0.04)^{b}	1115(25)^{a}	796(26)^{a}	1.42(0.04)^{c}
SEM			4	5	0.05	19	13	0.05	40	28	0.04
	0	0	130(4)^{ab}	111(5)	1.22(0.06)	510(19)^{a}	338(19)	1.58(0.08)	1151(55)	719(46)	1.69(0.09)
	4	1500	136(3)^{a}	114(6)	1.24(0.05)	499(18)^{ab}	332(20)	1.56(0.07)	1059(36)	686(45)	1.62(0.08)
	0	0	123(3)^{b}	103(6)	1.25(0.06)	462(21)^{b}	301(17)	1.59(0.07)	1070(47)	642(38)	1.72(0.07)
	4	1500	129(4)^{ab}	107(5)	1.24(0.04)	478(20)^{ab}	307(12)	1.57(0.05)	1028(37)	664(33)	1.57(0.05)
SEM			4	6	0.05	20	17	0.07	44	41	0.07

Continue..

Table 6.4 Effects of dietary fat, dietary fibre and phytase supplementation on performance of broilers fed on wheat/corn starter diet (0 to 20 d posthatch)[1]

Fat, %	Fibre, %	Phytase, FTU/kg	d 0 to 7			d 0 to 14			d 0 to 21		
			FI (%)	BWG (%)	FCR	FI (%)	BWG (%)	FCR	FI (%)	BWG (%)	FCR
….continue											
1	0	0	129(6)^{ab}	101(9)^{cd}	1.34(0.08)	500(36)^{ab}	273(24)^{b}	1.87(0.10)^{a}	1150(107)	564(54)^{b}	2.06(0.06)^{a}
1	0	1500	132(5)^{ab}	101(7)^{bcd}	1.34(0.06)	476(28)^{ab}	268(17)^{b}	1.80(0.09)^{ab}	985(47)	537(26)^{b}	1.86(0.09)^{ab}
1	4	0	115(4)^{b}	91(8)^{d}	1.33(0.10)	433(32)^{b}	252(22)^{b}	1.76(0.11)^{ab}	1030(86)	527(46)^{b}	1.97(0.08)^{a}
1	4	1500	122(6)^{ab}	101(11)^{bcd}	1.27(0.11)	454(40)^{ab}	272(17)^{b}	1.66(0.08)^{abc}	957(73)	570(40)^{b}	1.68(0.06)^{bc}
5	0	0	132(7)^{ab}	121(4)^{ab}	1.10(0.07)	521(16)^{a}	402(9)^{a}	1.30(0.03)^{d}	1152(34)	875(28)^{a}	1.32(0.02)^{d}
5	0	1500	141(3)^{a}	126(8)^{a}	1.14(0.05)	521(22)^{a}	395(18)^{a}	1.33(0.06)^{cd}	1132(39)	835(37)^{a}	1.37(0.05)^{d}
5	4	0	131(4)^{ab}	115(7)^{abc}	1.18(0.06)	491(24)^{ab}	349(17)^{a}	1.42^{c}(0.06)^{d}	1110(41)	757(33)^{a}	1.48^{c}(0.04)^{d}
5	4	1500	136(5)^{a}	112(4)^{abc}	1.22(0.03)	501(19)^{ab}	342(11)^{a}	1.47(0.05)^{bcd}	1098(33)	758(33)^{a}	1.46(0.05)^{cd}
SEM			5	7	0.070	27	17	0.072	57	38	0.057
PC diet			131(4)	114(6)	1.16(0.04)	548(32)	338(14)	1.616(0.06)	1210(70)	712(28)	1.70(0.08)
P-value											
Fat			0.002	<0.001	<0.001	0.001	<0.001	<0.001	0.007	<0.001	<0.001
Fibre			0.031	0.027	0.620	0.006	0.004	0.946	0.096	0.050	0.903
Phytase			0.086	0.401	0.865	0.875	0.993	0.679	0.047	0.819	0.008
Fat x fibre			0.168	0.390	0.141	0.435	0.040	0.020	0.582	0.061	0.003
Fat x phytase			0.813	0.594	0.381	0.790	0.490	0.211	0.126	0.582	0.002
Fibre x phytase			0.991	0.845	0.663	0.266	0.552	0.964	0.455	0.266	0.349
Fat x fibre x phytase			0.570	0.182	0.660	0.503	0.552	0.813	0.526	0.774	0.862
Contrast PC[2] vs NC[3]			0.768	0.252	0.080	0.342	0.032	0.012	0.659	0.007	0.002
Contrast PC vs fat			0.442	0.525	0.970	0.279	0.043	0.004	0.271	0.025	0.004
Contrast PC vs fibre			0.392	0.231	0.133	0.054	0.054	0.668	0.066	0.190	0.560
Contrast PC vs phytase			0.775	0.522	0.103	0.108	0.233	0.533	0.045	0.382	0.227
Contrast PC vs all treatments			0.805	0.389	0.089	0.099	0.220	0.608	0.100	0.399	0.553

[a,b,c] Means (± SE) within the same column with no common superscript differ significantly (P<0.05). [1]Means represent the average response of 5 chicks per replicate pen and 10 replicates (50 chicks) per treatment.[2]Positive control (PC) diet contains adequate available P (0.50%) and Ca (1.05%) and 1% supplemental fat. [3]Negative control (NC) diet contains 0.34% aP, 0.84% Ca and 1% supplemental fat.

significantly higher BWG (P = 0.007) and (P = 0.002) better FCR were observed in chickens fed with the PC diet compared to those fed with the NC diet, while FCR (P = 0.004) and BWG (P = 0.025) of chickens fed on the high fat diet was better in comparison to the PC diet. The FI of chickens fed the phytase supplemented diet was significantly lower (P = 0.045) than those fed the PC diet.

6.4.3. Digesta mean retention time

The effects of inclusion of dietary fat, dietary fibre and phytase supplementation on digesta MRT of each section of GIT are presented in Table 6.5. There was no significant main effect due to inclusion of dietary fat, dietary fibre and phytase on MRT of digesta in crop, proventriculus-gizzard, ileum and overall digestive tract. The MRT of digesta in duodenum-jejunum was significantly (P = 0.049) increased by dietary fat, tended to be increased by dietary fibre (P = 0.087) but was not affected by phytase supplementation. Whereas, MRT of ileal digesta in chickens fed the phytase supplemented diet showed a tendency (P = 0.058) towards shorter duration (58 vs 67 min) in comparison to those fed the non-phytase supplemented diet. Significant interaction (P < 0.001) between dietary fat and phytase supplementation was only observed in the crop. At 1% fat level, phytase supplementation reduced crop MRT but at 5% fat level, higher crop MRT was observed with phytase supplementation. However, crop MRT in the diet with 5% fat with supplemental phytase was numerically higher (25 min) than that observed in the diet with 1% fat without phytase supplementation.

Digesta MRT in each section of GIT in chickens fed the PC diet was similar to that of those fed the NC diet and all other treatment diets, except in proventriculus-gizzard and ileum of chickens fed the NC and the phytase supplemented diets, respectively. Chickens fed the PC diet had significantly longer digesta MRT in proventriculus-gizzard (P = 0.040) and in ileum (P = 0.048) than those chickens fed on the NC and the phytase supplemented diets in their respective GIT sections.

Table 6.5 The effects of dietary fat, dietary fibre and phytase supplementation on mean retention time in intestinal tract of 20 d broilers fed on wheat/corn diet[1]

Fat level,%	Fibre level,%	Phytase,FTU/kg	Digesta transit time (min) Crop	Proventriculus + Gizzard	Duodenum +jejunum	Ileum	Overall TT
1			14(1.2)	46(1.1)	44(1.1)[b]	63(1.1)	173(1.1)
5			17(1.2)	44(1.0)	51(1.1)[a]	62(1.0)	186(1.0)
SEM			1.2	1.1	1.1	1.1	1.1
	0		14(1.1)	43(1.1)	45(1.1)	64(1.1)	172(1.1)
	4		17(1.2)	47(1.1)	51(1.1)	62(1.1)	188(1.1)
SEM			1.2	1.1	1.1	1.1	1.1
		0	16(1.2)	43(1.1)	49(1.1)	67(1.1)	182(1.1)
		1500	15(1.2)	47(1.1)	47(1.1)	58(1.1)	177(1.1)
SEM			1.2	1.1	1.1	1.1	1.1
1	0		15(1.2)	40(1.1)	40(1.2)[b]	61(1.1)	163(1.1)
1	4		13(1.3)	52(1.1)	49(1.2)[ab]	65(1.1)	184(1.1)
5	0		14(1.2)	45(1.1)	50(1.1)[ab]	66(1.1)	180(1.1)
5	4		22(1.3)	44(1.1)	52(1.1)[a]	58(1.1)	192(1.1)
SEM			1.2	1.1	1.2	1.1	1.1
1		0	21(1.2)[a]	43(1.1)	45(1.2)	69(1.1)	179(1.1)
1		1500	9(1.2)[b]	49(1.1)	44(1.2)	57(1.1)	167(1.1)
5		0	12(1.3)[ab]	42(1.1)	53(1.2)	65(1.1)	185(1.1)
5		1500	25(1.2)[a]	46(1.0)	50(1.1)	59(1.1)	188(1.1)
SEM			1.2	1.1	1.2	1.1	1.1
	0	0	16(1.2)	40(1.1)	45(1.2)	67(1.1)	175(1.1)
	0	1500	13(1.2)	45(1.1)	44(1.2)	60(1.1)	168(1.1)
	4	0	15(1.3)	45(1.1)	52(1.2)	67(1.1)	189(1.1)
	4	1500	18(1.3)	50(1.1)	49(1.2)	56(1.1)	187(1.1)
SEM			1.2	1.1	1.2	1.1	1.1
1	0	0	24(1.3)[ab]	37(1.2)	39(1.3)	66(1.1)	168(1.2)
1	0	1500	9(1.3)[b]	44(1.2)	42(1.3)	56(1.1)	159(1.2)
1	4	0	18(1.4)[ab]	51(1.2)	52(1.3)	73(1.2)	191(1.2)
1	4	1500	9(1.3)[b]	53(1.1)	46(1.3)	58(1.1)	177(1.1)
5	0	0	11(1.2)[ab]	44(1.1)	53(1.2)	67(1.1)	182(1.1)
5	0	1500	18(1.3)[ab]	45(1.1)	48(1.2)	65(1.1)	179(1.1)
5	4	0	13(1.5)[ab]	40(1.1)	52(1.3)	63(1.1)	188(1.1)
5	4	1500	35(1.2)[a]	47(1.1)	52(1.1)	54(1.1)	197(1.1)
SEM			1.3	1.1	1.2	1.1	1.1
PC			17(1.2)	54(1.1)	49(1.4)	72(1.1)	208(1.1)
P-value							
Fat			0.281	0.625	0.049	0.871	0.274
Fibre			0.504	0.202	0.087	0.660	0.179
Phytase			0.844	0.232	0.564	0.058	0.691
Fat x fibre			0.142	0.101	0.260	0.198	0.694
Fat x phytase			<0.001	0.867	0.855	0.469	0.533
Fibre x phytase			0.356	0.939	0.747	0.539	0.864
Fat x fibre x phytase			0.801	0.382	0.284	0.865	0.761
Contrast PC[2] vs NC[3]			0.298	0.040	0.571	0.565	0.322
Contrast PC vs fat			0.998	0.176	0.898	0.136	0.407
Contrast PC vs fibre			0.875	0.395	0.922	0.137	0.429
Contrast PC vs phytase			0.602	0.382	0.889	0.048	0.248
Contrast PC vs all treatments			0.645	0.194	0.935	0.145	0.247

[a,b] Means(± SE) within the same column with no common superscript differ significantly (P<0.05). [1]Means represent the average response of 5 chicks per replicate pen and 10 replicates (50 chicks) per treatment.[2]Positive control (PC) diet contains adequate available P (0.50%) and Ca (1.05%) and 1% supplemental fat. [3]Negative control (NC) diet contains 0.34% aP, 0.84% Ca and 1% supplemental fat

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6.4.4. Digesta content and weight of GIT sections and pancreas

The weight of freeze-dried digesta, empty GIT sections and pancreas are presented in Tables 6.6 and 6.7, respectively. Digesta contents in all GIT sections were significantly heavier ($P < 0.005$) in chickens fed diets containing 5% fat compared to those fed the lower fat level. Ileal contents in broilers fed phytase supplemented diets were also lesser ($P = 0.004$) in comparison to those fed diets without supplemental phytase. A significant interaction ($P = 0.009$) between fat and phytase supplementation on crop digesta content was also observed. At 1% fat level, crop content was lesser with phytase supplementation but at 5% fat level, crop content was higher with phytase supplementation. However, the crop content at 1% fat level without supplemental phytase was similar as in the diet at 5% fat with supplemental phytase.

There were significant main effects ($P < 0.05$) of dietary fat, dietary fibre and phytase supplementation on the empty weights of proventriculus-gizzard, duodenum-jejunum, ileum and pancreas. Inclusion of 5% dietary fat significantly increased ($P < 0.001$) the empty weight of the ileum, whereas inclusion of 4% dietary fibre significantly increased ($P = 0.043$) the empty weight of the proventriculus-gizzard. Conversely, supplemental phytase at 1500 FTU/kg significantly reduced the weight of the empty duodenum-jejunum ($P < 0.001$), empty ileum ($P < 0.001$) and pancreas ($P = 0.019$). There was no significant interaction observed on the empty weight of all GIT sections and pancreas.

The weights of empty GIT sections in PC diet fed chickens were similar to those of chickens fed on the NC and the high fibre diets. The empty weight of crop in chickens fed with the high fat ($P = 0.005$) and the supplemental phytase ($P=0.010$) were lower compared to those fed on the PC diet. PC diet fed chickens also had significantly heavier ($P = 0.005$) empty duodenum-jejunum compared to birds fed phytase supplemented diets and had significantly heavier ($P < 0.05$) pancreas compared to those fed on NC and all other treatment diets.

Table 6.6 Effects of dietary fat, dietary fibre and phytase supplementation on freeze dried content (g) of each GIT section in broilers fed on wheat/corn diet[1]

Fat level, %	Fibre level, %	Phytase, FTU/kg	Crop (g)	Proventriculus + Gizzard (g)	Duodenum +jejunum (g)	Ileum (g)
1			4.71(1.18)	14.80(0.81)^{b}	9.89(1.12)^{b}	5.66(0.28)^{b}
5			6.29(1.19)	17.94(0.69)^{a}	14.54(1.10)^{a}	7.35(0.25)^{a}
SEM			1.18	0.75	1.11	0.26
	0		5.54(1.14)	16.38(0.82)	11.79(1.11)	6.21(0.28)
	4		5.34(1.22)	16.35(0.76)	12.20(1.12)	6.80(0.31)
SEM			1.18	0.79	1.12	0.29
		0	6.17(1.20)	16.43(0.79)	12.82(1.12)	7.04(0.30)^{a}
		1500	4.80(1.17)	16.30(0.80)	11.22(1.11)	5.97(0.26)^{b}
SEM			1.18	0.79	1.12	0.28
1	0		5.71(1.20)	14.74(1.14)	9.74(1.16)^{b}	5.19(0.32)^{c}
1	4		3.88(1.30)	14.85(1.18)	10.04(1.20)^{b}	6.14(0.45)^{bc}
5	0		5.38(1.21)	18.03(1.09)	14.26(1.15)^{a}	7.23(0.31)^{ab}
5	4		7.35(1.33)	17.84(0.88)	14.83(1.14)^{a}	7.47(0.39)^{a}
SEM			1.26	1.07	1.16	0.37
1		0	7.28(1.25)^{a}	15.74(1.26)^{ab}	10.70(1.18)^{bc}	6.32(0.47)^{b}
1		1500	3.05(1.22)^{b}	13.85(1.00)^{b}	9.14(1.17)^{c}	5.01(0.24)^{c}
5		0	5.24(1.33)^{ab}	17.12(0.96)^{ab}	15.35(1.16)^{a}	7.75(0.32)^{a}
5		1500	7.54(1.20)^{a}	18.75(0.99)^{a}	13.78(1.13)^{ab}	6.94(0.37)^{ab}
SEM			1.25	1.05	1.16	0.35
	0	0	6.51(1.21)	16.59(1.24)	12.38(1.18)	6.47(0.37)^{ab}
	0	1500	4.72(1.18)	16.18(1.11)	11.23(1.15)	5.94(0.41)^{b}
	4	0	5.86(1.37)	16.27(1.01)	13.28(1.18)	7.60(0.46)^{a}
	4	1500	4.87(1.29)	16.42(1.17)	11.21(1.17)	6.01(0.35)^{b}
SEM			1.26	1.13	1.17	0.40
1	0	0	9.13(1.25)	15.45(1.93)	9.99(1.26)^{abc}	5.65(0.50)^{bc}
1	0	1500	3.58(1.20)	14.03(1.28)	9.50(1.20)^{bc}	4.72(0.36)^{c}
1	4	0	5.81(1.45)	16.03(1.73)	11.47(1.29)^{abc}	7.00(0.76)^{ab}
1	4	1500	2.60(1.42)	13.67(1.60)	8.79(1.30)^{c}	5.29(0.31)^{bc}
5	0	0	4.65(1.32)	17.73(1.57)	15.33(1.24)^{a}	7.30(0.41)^{ab}
5	0	1500	6.23(1.29)	18.33(1.60)	13.27(1.21)^{abc}	7.15(0.51)^{ab}
5	4	0	5.91(1.71)	16.51(1.15)	15.38(1.24)^{a}	8.20(0.47)^{a}
5	4	1500	9.14(1.30)	19.17(1.25)	14.30(1.18)^{ab}	6.73(0.54)^{abc}
SEM			1.36	1.51	1.24	0.48
PC			7.08(1.48)	16.70(1.29)	18.45(4.14)	6.62(0.38)
P-value						
Fat			0.215	0.004	<0.001	<0.001
Fibre			0.872	0.973	0.649	0.095
Phytase			0.276	0.902	0.081	0.004
Fat x fibre			0.134	0.887	0.953	0.313
Fat x phytase			0.009	0.099	0.744	0.476
Fibre x phytase			0.766	0.791	0.633	0.141
Fat x fibre x phytase			0.990	0.480	0.344	0.705
Contrast PC[2] vs NC[3]			0.124	0.560	0.230	0.157
Contrast PC vs fat			0.927	0.815	0.214	0.231
Contrast PC vs fibre			0.318	0.837	0.428	0.731
Contrast PC vs phytase			0.260	0.464	0.808	0.181
Contrast PC vs all treatments			0.533	0.837	0.349	0.822

[a,b] Means(± SE) within the same column with no common superscript differ significantly (P<0.05). [1]Means represent the average response of 5 chicks per replicate pen and 10 replicates (50 chicks) per treatment.[2]Positive control (PC) diet contains adequate available P (0.50%) and Ca (1.05%) and 1% supplemental fat. [3]Negative control (NC) diet contains 0.34% aP, 0.84% Ca and 1% supplemental fat.

Table 6.7 Effects of dietary fat, dietary fibre and phytase supplementation on empty weight of GIT sections and pancreas of broilers fed on wheat/corn diet[1]

Fat level,%	Fibre level, %	Phytase, FTU/kg	Crop (g)	Proventriculus + Gizzard (g)	Duodenum +jejunum (g)	Ileum (g)	Pancreas (g)
1			3.52(0.12)	24.56(0.56)	21.11(0.56)	9.11(0.23)^{b}	2.60(0.08)
5			3.49(0.10)	24.44(0.45)	21.44(0.54)	9.89(0.21)^{a}	2.52(0.06)
SEM			0.11	0.50	0.55	0.22	0.07
	0		3.43(0.14)	24.00(0.74)^{b}	21.26(0.86)	9.55(0.39)	2.52(0.10)
	4		3.58(0.12)	25.0(0.65)^{a}	21.29(0.73)	9.45(0.31)	2.60(0.09)
SEM			0.13	0.69	0.79	0.35	0.09
		0	3.61(0.13)	24.84(0.66)	22.12(0.80)^{a}	9.85(0.35)^{a}	2.64(0.09)^{a}
		1500	3.41(0.13)	24.16(0.72)	20.43(0.76)^{b}	9.15(0.34)^{b}	2.49(0.10)^{b}
SEM			0.13	0.69	0.78	0.34	0.09
1	0		3.49(0.19)	24.15(0.85)	21.08(0.81)	9.13(0.34)	2.55(0.11)
1	4		3.55(0.15)	24.98(0.74)	21.14(0.78)	9.09(0.31)	2.65(0.11)
5	0		3.37(0.14)	23.86(0.60)	21.44(0.75)	9.97(0.32)	2.49(0.08)
5	4		3.61(0.14)	25.02(0.67)	21.44(0.70)	9.81(0.21)	2.55(0.09)
SEM			0.15	0.72	0.76	0.30	0.09
1		0	3.64(0.18)	24.97(0.82)	21.70(0.77)^{ab}	9.40(0.31)^{b}	2.65(0.11)
1		1500	3.41(0.15)	24.15(0.72)	20.51(0.74)^{b}	8.81(0.31)^{b}	2.55(0.10)
5		0	3.57(0.12)	24.70(0.54)	22.53(0.74)^{a}	10.30(0.29)^{a}	2.62(0.07)
5		1500	3.4(0.15)1	24.17(0.72)	20.36(0.69)^{b}	9.49(0.26)^{b}	2.43(0.10)
SEM			0.15	0.70	0.73	0.29	0.10
	0	0	3.61(0.20)	24.33(1.00)	22.23(1.20)^{a}	9.95(054)^{a}	2.60(0.13)^{ab}
	0	1500	3.25(0.17)	23.68(1.08)	20.29(1.15)^{c}	9.15(0.53)^{b}	2.45(0.14)^{b}
	4	0	3.60(0.15)	25.35(0.87)	22.00(1.02)^{ab}	9.75(0.42)^{ab}	2.67(0.12)^{a}
	4	1500	3.56(0.19)	24.65(0.97)	20.58(1.02)^{bc}	9.14(0.44)^{b}	2.53(0.13)^{ab}
SEM			0.18	0.98	1.10	0.48	0.13
1	0	0	3.64(0.31)	24.83(1.34)	21.72(1.14)^{ab}	9.47(0.50)^{ab}	2.68(0.16)
1	0	1500	3.35(0.20)	23.47(0.90)	20.43(1.01)^{ab}	8.79(0.41)^{b}	2.66(0.12)
1	4	0	3.65(0.21)	25.12(1.02)	21.68(1.07)^{ab}	9.34(0.39^{ab}	2.63(0.16)
1	4	1500	3.46(0.22)	24.83(1.12)	20.59(1.13)^{ab}	8.84(0.48)^{b}	2.62(0.17)
5	0	0	3.59(0.14)	23.83(0.81)	22.75(0.78)^{a}	10.43(0.40)^{a}	2.57(0.07)
5	0	1500	3.16(0.19)	23.89(0.91)	20.14(1.04)^{b}	9.52(0.43)^{ab}	2.48(0.13)
5	4	0	3.56(0.18)	25.57(0.74)	22.31(1.03)^{ab}	10.17(0.31)^{a}	2.44(0.10)
5	4	1500	3.66(0.22)	24.46(1.14)	20.57(0.91)^{ab}	9.45(0.27)^{ab}	2.41(0.15)
SEM			0.21	1.00	1.01	0.40	0.13
PC			4.13(0.29)	26.08(0.74)	22.85(0.67)	9.87(0.30)	2.90(0.10)
SEM							
P-value							
Fat			0.859	0.867	0.586	0.004	0.403
Fibre			0.201	0.043	0.939	0.542	0.211
Phytase			0.081	0.154	<0.001	<0.001	0.019
Fat x fibre			0.444	0.732	0.942	0.724	0.764
Fat x phytase			0.760	0.751	0.198	0.505	0.485
Fibre x phytase			0.177	0.955	0.488	0.567	0.904
Fat x fibre x phytase			0.347	0.233	0.658	0.994	0.511
Contrast PC[2] vs NC[3]			0.396	0.291	0.180	0.293	0.031
Contrast PC vs fat			0.005	0.230	0.606	0.158	0.037
Contrast PC vs fibre			0.067	0.365	0.070	0.312	0.014
Contrast PC vs phytase			0.010	0.054	0.005	0.056	0.001
Contrast PC vs all treatments			0.018	0.114	0.084	0.485	0.006

[a,b] Means (± SE) within the same column with no common superscript differ significantly (P<0.05). [1]Means represent the average response of 5 chicks per replicate pen and 10 replicates (50 chicks) per treatment.[2]Positive control (PC) diet contains adequate available P (0.50%) and Ca (1.05%) and 1% supplemental fat. [3]Negative control (NC) diet contains 0.34% aP, 0.84% Ca and 1% supplemental fat.

6.4.5. pH of digesta

Table 6.8 shows the effect of dietary fat, dietary fibre and phytase supplementation on pH of digesta in each section of the GIT. pH in the proventriculus-gizzard was significantly increased by inclusion of fibre ($P = 0.010$) and supplementation of phytase ($P = 0.001$) but was not affected by dietary fat inclusion. Whereas, pH in the duodenum-jejunum was increased ($P = 0.040$) as dietary fat level increased but was reduced ($P = 0.002$) with phytase supplementation. However, dietary fibre inclusion had no influence on pH in the duodenum-jejunum. On the other hand, ileal pH was significantly increased by inclusion of dietary fat ($P = 0.003$), dietary fibre ($P = 0.031$) and phytase supplementation ($P < 0.001$). There was no significant effect of dietary fat, dietary fibre and phytase supplementation on pH in the crop.

The effect of fat x phytase interaction on the pH of small intestine was also observed in the present study. Phytase supplementation increased duodenum-jejunal pH at 1% fat level but had no effect at 5% fat level. Whereas, ileal pH was increased with phytase supplementation at 1% fat level but at higher fat level, the ileal pH was already high regardless with or without phytase supplementation. Ileal pH was also affected ($P = 0.040$) by fibre x phytase interaction. Ileal pH was higher with phytase supplementation at higher dietary fibre inclusion but no effect of phytase supplementation was observed at 0% fibre level.

pH of proventriculus-gizzard, duodenum-jejunum and ileum were significantly ($P < 0.05$) higher with chickens fed the phytase supplemental diet compared to those fed the PC diet. pH of proventriculus-gizzard in chickens fed on the fibre diet was also higher ($P = 0.036$) than those fed on the PC diet.

Table 6.8 Effects of dietary fat, dietary fibre and phytase supplementation on pH in gastrointestinal tract of 20 d broilers fed on wheat/corn diet[1]

Fat level,%	Fibre level,%	Phytase, FTU/kg	Crop	Proventriculus + Gizzard	Duodenum +jejunum	Ileum
1			5.43(0.10)	2.63(0.06)	6.14(0.03)^{a}	6.92(0.07)^{b}
5			5.61(0.08)	2.62(0.05)	6.08(0.02)^{b}	7.11(0.05)^{a}
SEM			0.09	0.02	0.05	0.06
	0		5.48(0.10)	2.54(0.04)^{b}	6.10(0.02)	6.95(0.05)^{b}
	4		5.56(0.09)	2.72(0.06)^{a}	6.11(0.02)	7.09(0.06)^{a}
SEM			0.09	0.02	0.05	0.06
		0	5.46(0.10)	2.52(0.05)^{b}	6.06(0.02)^{b}	6.83(0.06)^{b}
		1500	5.58(0.09)	2.74(0.05)^{a}	6.15(0.02)^{a}	7.20(0.04)^{a}
SEM			0.09	0.02	0.05	0.05
1	0		5.36(0.15)	2.51(0.07)	6.13(0.04)	6.86(0.09)^{b}
1	4		5.50(0.14)	2.76(0.09)	6.15(0.04)	6.98(0.10)^{ab}
5	0		5.60(0.12)	2.57(0.05)	6.08(0.02)	7.03(0.06)^{ab}
5	4		5.62(0.10)	2.68(0.08)	6.07(0.02)	7.19(0.07)^{a}
SEM			0.13	0.03	0.07	0.08
1		0	5.25(0.15)	2.49(0.07)^{b}	6.06(0.03)^{b}	6.65(0.08)^{b}
1		1500	5.61(0.13)	2.78(0.08)^{a}	6.22(0.04)^{a}	7.18(0.05)^{a}
5		0	5.67(0.11)	2.55(0.06)^{ab}	6.06(0.03)^{b}	7.01(0.07)^{a}
5		1500	5.55(0.12)	2.70(0.08)^{ab}	6.09(0.02)^{b}	7.21(0.06)^{a}
SEM			0.13	0.03	0.07	0.07
	0	0	5.40(0.15)	2.48(0.06)^{b}	6.06(0.03)	6.83(0.09)^{b}
	0	1500	5.56(0.13)	2.60(0.06)^{b}	6.15(0.03)	7.06(0.05)^{b}
	4	0	5.53(0.13)	2.55(0.07)^{b}	6.06(0.03)	6.84(0.08)^{b}
	4	1500	5.60(0.12)	2.89(0.08)^{a}	6.16(0.03)	7.34(0.05)^{a}
SEM			0.13	0.03	0.07	0.07
1	0	0	5.40(0.22)	2.40(0.09)^{b}	6.05(0.04)^{b}	6.63(0.13)^{d}
1	0	1500	5.56(0.17)	2.62(0.09)^{ab}	6.20(0.05)^{ab}	7.09(0.06)^{ab}
1	4	0	5.53(0.20)	2.58(0.11)^{ab}	6.06(0.05)^{ab}	6.68(0.10)^{cd}
1	4	1500	5.60(0.21)	2.94(0.10)^{a}	6.23(0.05)^{a}	7.28(0.09)^{ab}
5	0	0	5.40(0.16)	2.57(0.08)^{ab}	6.07(0.04)^{ab}	7.03(0.10)^{abc}
5	0	1500	5.56(0.20)	2.57(0.08)^{ab}	6.09(0.02)^{ab}	7.03(0.09)^{abc}
5	4	0	5.53(0.15)	2.53(0.10)^{ab}	6.05(0.04)^{ab}	6.99(0.10)^{bcd}
5	4	1500	5.60(0.15)	2.83(0.12)^{a}	6.09(0.03)^{ab}	7.39(0.03)^{a}
SEM			0.18	0.04	0.09	0.10
PC			5.27(0.17)	2.52(0.07)	5.93(0.19)	6.76(0.14)
SEM						
P-value						
Fat			0.179	0.883	0.040	0.003
Fibre			0.530	0.010	0.927	0.031
Phytase			0.373	0.001	0.002	<0.001
Fat x fibre			0.639	0.294	0.611	0.724
Fat x phytase			0.075	0.293	0.025	0.011
Fibre x phytase			0.711	0.102	0.786	0.040
Fat x fibre x phytase			0.447	0.558	0.934	0.286
Contrast PC[2] vs NC[3]			0.509	0.304	0.455	0.720
Contrast PC vs fat			0.184	0.137	0.442	0.061
Contrast PC vs fibre			0.202	0.036	0.194	0.125
Contrast PC vs phytase			0.171	0.017	0.049	0.017
Contrast PC vs all treatments			0.224	0.083	0.129	0.117

[a,b] Means(± SE) within the same column with no common superscript differ significantly (P<0.05). [1]Means represent the average response of 5 chicks per replicate pen and 10 replicates (50 chicks) per treatment.[2]Positive control (PC) diet contains adequate available P (0.50%) and Ca (1.05%) and 1% supplemental fat. [3]Negative control (NC) diet contains 0.34% aP, 0.84% Ca and 1% supplemental fat.

6.4.6. Phytate-P degradation

The effects of dietary fat, dietary fibre and phytate supplementation on phytate P degradation in each GIT section are presented in Table 6.9. Phytate P degradation was significantly increased in the crop ($P = 0.017$) and all other GIT sections ($P < 0.001$) with supplemental phytase at 1500 FTU/kg. The inclusion of dietary fat resulted in an increase ($P = 0.005$) in crop phytate P degradation from 23.84 to 34.657% but had no significant influence on phytate P degradation in other GIT sections. Dietary fibre inclusion also has no influence on phytate P degradation in all GIT sections.

Significant fat x fibre interactions ($P < 0.05$) on phytate P degradation in all GIT sections were observed except in proventriculus-gizzard. Phytate P degradation in crop, duodenum-jejunun and ileum were higher with fibre inclusion in 1% fat diet but lower with fibre inclusion in 5% fat diet. Whereas, phytate P degradation in the crop was higher ($P = 0.026$) with supplemental phytase in diet containing 4% fibre level but was not affected by supplemental phytase in diet without addition of dietary fibre.

Phytate P degradation in all GIT sections of chickens fed on the PC diet were significantly higher ($P < 0.05$) compared to phytate P degradation in those fed on the NC, the high fat (except crop) and the high fibre diet. Crop phytate P degradation in chickens given the PC diet was also higher ($P = 0.047$) compared to those with phytase supplemented diet. Whereas, the PC diet fed chickens have similar phytate P degradation in proventriculus-gizzard, duodenum-jejunum and ileum as observed in the chickens fed on diet with supplemental phytase.

Table 6.9 Effects of dietary fat, dietary fibre and phytase supplementation on phytate P degradation in intestinal sections of broilers fed on wheat/corn diet[1]

Fat level,%	Fibre level,%	Phytase, FTU/kg	Crop (%)	Proventriculus + Gizzard (%)	Duodenum +jejunum (%)	Ileum (%)
1			23.84(3.76)^{b}	30.28(4.73)	41.91(3.43)	52.09(2.99)
5			34.65(2.67)^{a}	27.17(3.08)	42.02(2.12)	51.66(2.99)
SEM			2.90	3.31	2.78	2.82
	0		26.08(3.54)	29.02(3.64)	42.07(2.86)	49.92(2.64)
	4		32.41(3.13)	28.43(4.15)	41.86(2.84)	53.83(2.97)
SEM			3.00	3.37	2.85	2.81
		0	24.74(3.35)^{b}	13.08(2.90)^{b}	28.64(2.06)^{b}	37.21(1.66)^{b}
		1500	33.75(3.34)^{a}	44.37(2.32)^{a}	55.29(1.71)^{a}	66.54(1.48)^{a}
SEM			2.95	2.28	1.89	1.57
1	0		14.94(4.29)^{b}	26.77(6.48)	37.44(5.30)^{b}	47.79)4.34)^{b}
1	4		32.74(5.08)^{a}	33.78(6.47)	46.38(4.26)^{a}	56.39(3.99)^{a}
5	0		37.23(3.84)^{a}	31.26(3.63)	46.70(1.81)^{a}	52.05)3.05)^{ab}
5	4		32.08(3.79)^{a}	23.08(4.80)	37.34(3.59)^{b}	51.27(4.42)^{ab}
SEM			3.85	4.62	3.74	3.95
1		0	19.07(4.96)^{b}	11.84(4.80)^{b}	24.53(3.14)^{b}	36.47(2.81)^{b}
1		1500	28.60(5.6)^{ab}	48.71(4.04)^{a}	59.30(2.60)^{a}	67.70(1.76)^{a}
5		0	30.41(4.29)^{ab}	14.32(3.39)^{b}	32.75(2.42)^{b}	37.95(1.83)^{b}
5		1500	38.89(2.88)^{a}	40.02(2.25)^{a}	51.29(1.88)^{a}	65.37(2.39)^{a}
SEM			3.97	3.07	2.78	2.32
	0	0	25.78(5.14)^{b}	15.53(4.11)^{b}	29.91(3.69)^{b}	37.02(2.45)^{b}
	0	1500	26.39(4.97)^{b}	42.50(2.94)^{a}	54.24(2.10)^{a}	62.82(2.27)^{a}
	4	0	23.71(4.41)^{b}	10.63(4.18)^{b}	27.37(1.92)^{b}	37.40(2.31)^{b}
	4	1500	41.11(3.52)^{a}	46.23(3.51)^{a}	56.35(2.72)^{a}	70.25(1.54)^{a}
SEM			4.02	3.20	2.61	2.14
1	0	0	17.24(7.12)^{bc}	12.71(5.35)^{d}	18.64(5.07)^{d}	31.61(3.84)^{b}
1	0	1500	12.63(4.40)^{c}	40.84(5.30)^{ab}	56.25(3.80)^{a}	63.97(2.59)^{a}
1	4	0	20.90(7.28)^{bc}	10.98(6.61)^{d}	30.41(2.85)^{cd}	41.34(3.67)^{b}
1	4	1500	44.57(5.13)^{a}	56.58(4.18)^{a}	62.34(3.45)^{a}	71.43(1.82)^{a}
5	0	0	34.31(6.68)^{abc}	18.36(3.42)^{cd}	41.18(1.91)^{bc}	42.43(2.02)^{b}
5	0	1500	40.14(3.48)^{ab}	44.17(2.63)^{ab}	52.23(1.83)^{ab}	61.66(3.84)^{a}
5	4	0	26.52(5.4)^{abc}	10.28(4.91)^{d}	24.33(2.32)^{d}	33.46(2.38)^{b}
5	4	1500	37.65(4.77)^{ab}	35.88(3.28)^{bc}	50.35(3.37)^{ab}	69.07(2.51)^{a}
SEM			4.94	4.04	3.08	2.83
PC			45.30(4.62)	51.70(2.94)	55.45(1.35)	61.57(1.56)
P-value						
Fat			0.005	0.313	0.962	0.834
Fibre			0.091	0.848	0.926	0.062
Phytase			0.017	<0.001	<0.001	<0.001
Fat x fibre			0.003	0.529	<0.001	0.026
Fat x phytase			0.887	0.072	0.001	0.358
Fibre x phytase			0.026	0.162	0.316	0.091
Fat x fibre x phytase			0.124	0.152	0.028	0.026
Contrast PC[2] vs NC[3]			<0.001	<0.001	<0.001	<0.001
Contrast PC vs fat			0.067	<0.001	<0.001	0.002
Contrast PC vs fibre			0.027	<0.001	<0.001	0.016
Contrast PC vs phytase			0.047	0.122	0.942	0.119
Contrast PC vs all treatments			0.004	<0.001	<0.001	0.002

[a,b] Means(± SE) within the same column with no common superscript differ significantly (P<0.05). [1]Means represent the average response of 5 chicks per replicate pen and 10 replicates (50 chicks) per treatment.[2]Positive control (PC) diet contains adequate available P (0.50%) and Ca (1.05%) and 1% supplemental fat. [3]Negative control (NC) diet contains 0.34% aP, 0.84% Ca and 1% supplemental fat.

6.5. Discussion

This study was conducted to investigate the effect of dietary fat and fibre on the digesta MRT, gut development, digesta pH and phytate P degradation of broiler chicken fed on phytase supplemented diet. It was hypothesised that MRT would be prolonged with higher fat level in the diet but would not be affected by addition of dietary fibre. Increased MRT would improve phytate P degradation in each segments of the gastrointestinal tract.

6.5.1. Effect of dietary fat inclusion

In the present study, BWG and feed efficiency of broilers given a wheat/corn diet were significantly reduced when the contents of available P and Ca were lowered from 0.50 to 0.34 % and from 1.05 to 0.84 %, respectively. The negative effects of the P-and-Ca deficient diet were restored with supplemental phytase at 1500 FTU/kg. This is likely due to the liberation of phytate P by phytase (Table 6.8) thus improving P availability in the diet. Similar improvement of growth parameters were reported when phytase was added to wheat/corn diet with deficient P and Ca (Wu *et al*., 2004a; Kühn *et al*., 2011). The results of this study also showed further improvement on weight gain and feed efficiency with 5% inclusion of dietary fat in phytase supplemented wheat diet. The results are in agreement with other studies that showed significant increase in weight gain and feed efficiency as fat level in the diet increased (Atteh *et al*., 1983, Polycarpo *et al*., 2014). The improvement of weight gain and feed efficiency in chickens fed on high fat-phytase supplemented diet is presumably not due to P utilization *per se* but also possibly because of better utilization of fat as energy source compared to other energy sources available in the diet and also due to higher nutrient intake as indicated by significantly higher feed intake with 5% fat inclusion (Table 6.4). It is evident in this study that the effect on broiler growth performance imposed by fat inclusion was greater than that of phytase supplementation as the magnitude of improvement especially in feed efficiency was greater with dietary fat inclusion (0.488 vs 0.112 point of improvement).

In the present study, MRT for ileal digesta and the overall GIT digesta were faster in chickens fed the phytase supplemented diet compared to those fed the PC

diet (Table 6.5). However, the 15% difference of overall digesta MRT between phytase supplemented diet and PC diet was not significant. These results were in partially in agreement with previously reported by Watson *et al*. (2006), who showed that with phytase supplementation, the overall digesta MRT was faster in chickens fed the P and Ca deficient diet in comparison to those fed the P and Ca adequate diet without supplemental phytase. However, Watson *et al*. (2006) demonstrated that the effect of phytase on overall digesta MRT was significant in chickens with the average age of 23 d but not in older chickens with the average age of 30 d. The overall MRT observed in the present study was about 50% longer than those reported by Watson *et al*. (2006). This is probably due to different cereal (wheat versus corn) based diets used in these studies, which may be related to the viscosity of intestinal digesta. According to previous studies (Chiang et al., 2005; Jia et al., 2009), chickens fed wheat based diet had higher intestinal viscosity compared to corn based diet and viscous digesta may increase digesta MRT (Sacranie et al 2012).

In improving phytase efficacy, delaying digesta MRT particularly in crop could provide a favourable environment for phytase-phytate reaction (Selle *et al*., 2010, Mateos and Sell, 1980a) and addition of fat into the diet could be expected to prolong crop digesta TT (Mateos *et al.*, 1982). In the present study, however, the inclusion of dietary fat did not influence the digesta TT in each GIT segment and overall GIT except in duodenum-jejunum, which digesta TT was significantly increased as the fat level increased from 1 to 5%. This observation was partly agreed with previous studies by Golian and Maurice (1992), who demonstrated insignificant increase in overall digesta TT when the level of poultry fat in corn/soybean meal-mash diet was increased from 0 to 20%. On the other hand, contradictory findings were reported by Kim *et al*. (2013), who observed significantly higher overall digesta TT in broilers fed corn/soybean diet containing 5% tallow compared to diet without tallow inclusion.

Previous studies demonstrated the presence of endogenous phytase in small intestine that may contribute in intestinal phytate degradation and its production is regulated by the concentration of dietary P (Maenz and Classen, 1998; Onyango *et al*., 2006). Maenz and Classen (1998) measured phytase activity in the small intestinal

brush border membrane of the chicken and found the highest specific activity was in duodenum, followed by jejunum and ileum. In the absence of high concentration of dietary P, the specific activity of endogenous phytase was significantly increased as detected in broilers fed on P-deficient corn/soybean meal diet (Onyango *et al*., 2006). Whereas, dietary phytate was shown to inhibit trypsin *in vitro* (Caldwell, 1992) and has significantly reduced pancreatic lipase activity in broilers (Liu *et al*., 2010). The adverse effect of phytate on pancreatic enzyme was restored with phytase supplementation (Liu *et al.*, 2010). In contrast, phytase supplementation has been shown to reduce proteolytic activities in both proventriculus mucosa and pancreatic tissue in broilers fed on P-deficient corn based diet (Kapika and Puzio, 2004). These authors suggested that the formation of phytate-protein complex which is resistance to proteolytic digestion has caused the increase in proteolytic and trypsin activity in pancreatic tissue. With phytase supplementation, phytate degradation may release the protein from the phytate-protein complexes and become susceptible for enzymatic digestion. Pancreatic amylolytic and trypsin activities were also significantly decreased by supplementation with phytase with numerically reduced weight of pancreas (g/kg body weight). Generally, phytate reduces protein digestion in upper GIT and causes the growth of pancreatic tissue and intestinal mucosa in order to synthesis more pancreatic and intestinal enzymes, consequently increasing the mass of pancreas. However, supplemental phytase reverses this effect (Selle *et al.*, 2003). Akyurek *et al*. (2009) also investigated the effect of phytase on the weight of GIT and pancreas in 21 d old broilers and they found that supplemental phytase in P-deficient diet reduced the weight of anterior and posterior segments of GIT. Liu *et al.* (2014) also found a significant decrease in pancreas weight when phytase was supplemented in P-adequate wheat based diet.

In the present study, significantly lower weight of small intestine and pancreas in phytase diet fed chicken was observed. The plausible explanation for this is that dephosphorylation of phytate by phytase reduces the binding capacity of phytate, releases phytate bound minerals, proteins and starch and make the nutrients more susceptible to enzymatic digestion. Therefore, lower levels of pancreatic and intestinal enzymes are synthesised and secreted. Consequently, this limits the growth of pancreatic tissue and intestinal mucosa, therefore less mass and smaller size of

pancreas and GIT. However, the findings of the present study were in contrast to previously reported by Selle *et al.* (2003) who did not observe any changes in pancreatic mass with fungal phytase supplementation at 600 FTU/kg in P and Ca deficient wheat based diet. However, a numerically increase in pancreas weight was observed in the previous study when adequate level of P and Ca was used (Selle *et al.*, 2003). Different phytase effect on pancreas weight observed in the present study and the previous study probably due to the level of phytase, P and Ca used in the diet. Higher dietary phytase level (1500 FTU/kg) used in the present study may results in greater extent in phytate degradation in comparison to lower dietary phytase level (600 FTU/kg) and with adequate P and Ca (Walk *et al*., 2014) which leads to a greater improvement in protein digestion consequently limits the increase of pancreatic mass and pancreatic enzymes synthesis.

On the other hand, an increased fat inclusion level increased the level of pancreatic lipase activity in jejunum (Krogdhal, 1985) and in pancreatic tissue (Polycarpo *et al*., 2014), but the occurrence of pancreatic hypertrophy was not observed. Similar result was found in the present study that there was no significant increase in pancreas weight with higher inclusion of dietary fat. The plausible explanation for this observation is that despite high requirement for pancreatic lipase, the level of pancreatic secretion probably is not extreme enough to cause significant enlargement of pancreas. Furthermore, bile secretion was also increased as level of dietary fat increased which enhanced lipase activity in the small intestine (Krogdahl, 1985). Another explanation may be related to the slow digesta passage in small intestine due to high dietary fat, especially in duodenum and jejunum, facilitates longer contact time between digesta and bile salts, lipase and other digestive enzymes (Leeson, 1993). This could result in an improved nutrient utilization without the need of excessive secretion of pancreatic and intestinal enzymes and it was considered as an adaptation undertaken to optimise nutrient digestion in chickens (Krogdahl, 1985).

Addition of phytase increased pH of digesta in all GIT segments, which is in agreement with results reported by Amerah *et al*. (2014) and Walk *et al*. (2012). These authors suggested that the presence of phytate in the stomach reduced activation of pepsin which later stimulated greater gastric secretion of HCl by gastrin and resulted in the reduction of pH and the emptying of gastric content. Lower pH

and slower digesta passage in the proventriculus-gizzard would increase phytate P degradation and reverse the effect of phytate on pepsin activity, consequently reduce HCl secretion and increase digesta pH. This explains the increase of pH in the gizzard (Table 6.8) and supported by stomach MRT (Table 6.5) and phytate P degradation (Table 6.9) observed in the present study. Conversely, significant effect of high fat inclusion on pH of crop and gizzard was not observed in the present study. On the other hand, high fat inclusion and phytase supplementation affected intestinal pH and the results were in agreement with previous studies that showed intestinal pH increased with increasing level of fat inclusion (Shafey, 1999) and phytase supplementation (Ptak *et al*., 2013). The reason for high ileal pH in the high fat diet in the present study is not clear, however, it may be indirectly related to the bacteriostatic effect of bile acids that may limit the growth of intestinal microflora consequently preventing the reduction of intestinal pH due to microbial activity (Dänicke *et al*., 1999). Whereas, with phytase supplementation, more phytate molecules are degraded and release more chelated ions including Ca, Na, P consequently increase the pH of small intestine (Walk et al., 2012d).

Most published work demonstrated the improvement of phytate degradation in small intestine especially in the ileum following supplementation with phytase (Zeller *et al*., 2015b; Amerah *et al*., 2014;S hastak *et al*., 2014). In the present study, the efficacy of supplemental phytase in wheat/corn diet was also observed in the upper part of GIT. Inclusion of higher fat level to low P and Ca diet resulted in significant increases in degradation of phytate P in crop, providing direct evidence for the role of fat in improving phytase efficacy. However, higher fat inclusion did not affect phytate P degradation in gizzard and small intestine. This finding contradicted the results found in previous study (Chapter 5), that demonstrated phytate P degradation was significantly improved by higher fat level inclusion. Although the mechanism of phytate P degradation in high fat diet is not clear, this inconsistency may be related to the higher content of aP (0.34%) and lower Ca:aP (2.46) in the present study compared to previous study (0.25% aP; 3.63 Ca:aP). Whereas, supplementation of phytase increased phytate P degradation in all segments of GIT, in agreement with previous reports (Camden *et al*., 2001; Rutherfurd *et al*., 2004; Leytem *et al*., 2008; Zeller *et al*., 2015). Conversely, significant fat x phytase interaction was not observed

in the present study. Nonetheless, there was a 20% increase in crop phytate P degradation when high fat and phytase were simultaneously added in the diet. The degradation magnitude was similar to the sum of phytate P degradation in high fat and phytase supplemented diet, indicating additive effect between fat level and phytase supplementation. The additive effects were also observed in previous study (Chapter 5).

6.5.2. Effect of dietary fibre inclusion

In the present study, BWG and FI were significantly reduced by addition of 4% dietary fibre but no change in FCR. These findings were in contrast to data reported by Jimenez-Moreno *et al.* (2009), who found that by adding either oat hull or sugar beet pulp as the source of dietary fibre at 3% inclusion level improved BWG and FCR but FI remained unaffected. Cao *et al*. (2003) reported that inclusion of dietary cellulose at 3.5% resulted in insignificant increase in BWG but further fibre inclusion (10%) did reduce BWG. Whereas, Hetland and Svihus (2001) demonstrated that increasing dietary fibre (oat hulls) as high as 10% did not affect BWG but increased FI, subsequently increasing FCR. Nonetheless, there was a significant interaction effect due to simultaneous addition of high dietary fibre and high fat observed in the present study. At high fibre and fat inclusion, FCR was improved from 1.962 to 1.345 with higher BWG but similar FI. However, there was no significant interaction effect between fibre inclusion and phytase supplementation on growth performance of 21 d old broilers fed on wheat/corn diet.

In the present study, inclusion of high fibre did not affect digesta MRT though there was a tendency for high fibre to reduce the digesta passage in duodenum-jejunum. High fibre inclusion also did not affect the weight of digesta content, empty segment of GIT and pancreas except for the weight of empty gizzard. These results were not consistent with Jimenez-Moreno *et al.* (2009), who demonstrated digesta TT increased with increasing level of fibre and suggested that diet containing more lignified dietary fibre was retained longer in GIT, particularly in gizzard because they were more resistant to grinding. Whereas, the insoluble fibre with high water holding capacity swelled, increased the bulkiness of the digesta and consequently, retained longer in the enlarged gizzard. Conversely, Cao *et al*. (2003)

reported shorter digesta MRT as level of dietary fibre increased and supposed to be related to physical structure of the diet (Hetland and Svihus, 2001). Furthermore, Jimenez-Moreno *et al.* (2010) demonstrated that due to the lack of physical structure, the added cellulose in the diet did not accumulate in the gizzard, which indicates fast transit time.. Rezaei et al. (2011) also reported that inclusion of Vitacel® (contains 74% cellulose) at 0.5% did not affect the weight of gizzard and small intestine. In the present study, the source of fibre used was Vitacel® Powdered Cellulose that contains 100% cellulose. Different effect of fibre inclusion on gizzard weight observed in the present study and the study by Rezaei et al. (2011) probably due to the level of fibre, which was higher in the present study. Higher gizzard weight shown in the present study indicates better functioning of the gizzard due to high dietary fibre inclusion (Mateos et al., 2012).

Despite lack of fibre effect on phytate P degradation in all segment of GIT, there was significant interaction between dietary fibre and dietary fat in crop and small intestine. It is interesting to note that in the low fat diet, addition of dietary fibre increased phytate P degradation, whereas with the high fat diet, phytate P degradation reduced with dietary fibre inclusion in all GIT segments. These results suggest effect of dietary fibre on phytate P degradation is dependent on level of fat, which could be explained as the following; At low fat level, the population of GIT microbiota may utilize cellulose as the source of energy and produces short-chain fatty acids (SCFAs) (Cisek and Binek, 2014) that reduces the pH of the intestinal content. In a lower pH condition, phytate become more soluble (Grynspan and Cheryan, 1983) and more susceptible toward degradation by either dietary (Naves *et al.*, 2012) or endogenous phytase (Morgan *et al.*, 2015). At higher fat level, dietary fibre may stimulate bile acids secretion and assist in fat digestion (Mateos *et al.*, 2014) and due to its bacteriostatic effect, high bile acids concentration in small intestine may limit the growth of lactic acid bacteria and other intestinal microflora (Dänicke *et al.*, 1999), including those with the capability of degrading phytate (Raghavendra and Halami, 2009)

6.6. Conclusion

In conclusion, BWG and feed efficiency of chickens fed on low P and Ca diet were improved by simultaneous inclusion of high fat either with phytase or high fibre but were not affected by simultaneous inclusion of high fibre with phytase. Higher dietary fat supplementation did not significantly prolong the overall digesta MRT of chickens fed on low P and Ca diet, which is contradicted with the hypothesis of the present study. However, additive effect of high fat and phytate supplementation on phytate P degradation provides evidence for the role of fat in improving phytase efficacy in broilers. Similarly, addition of dietary fibre in phytase supplemented diet improved crop phytate-P degradation. The level of dietary fat also contributed to the extent of phytate-P degradation in diet containing high dietary fibre. Phytate P degradation was increased in low fat diet but reduced in high fat diet. However, the effect of phytase supplementation on phytate P degradation was more pronounced than those of dietary fat and fibre. For mash diet, it is suggested that simultaneous addition of dietary fat as high as 5% and 1500 FTU/kg phytase in P and Ca deficient wheat diet is able to improve in vivo phytase efficacy and the growth performance of broilers over and above those fed adequate P and Ca diet. Addition of high dietary fibre should be avoided.

Chapter 7
General Discussion and Conclusion

7.1. Feed withdrawal and feed refeeding

Digesta collection was considered as a useful tool for evaluating nutrient digestibility in different sections of the digestive tract in poultry (Rodehutscord *et al*., 2012) and adequate amount of samples are required from each part of the GIT in order to evaluate the degradation of phytate ($InsP_6$) and lower InsPs. In some of digestibility studies, feed withdrawal (FW) and feed refeeding (FR) were applied prior to the killing and sampling in order to stimulate feed intake in order to obtain adequate amounts of digesta samples.

In our experiment (Chapter 3, Study 1), FW and FR prior to the killing and sampling resulted in higher dry matter (DM) of crop digesta but has similar amount of DM content in proventriculus-gizzard, duodenum-jejunum and ileum when compared to continuous feeding. Longer duration of FW and FR also resulted in similar amounts of DM in each GIT segment to continuous feeding. The time of sampling was also shown to affect the amount of crop and ileal DM. The most amount of crop DM was obtained when sampling was done in the afternoon i.e. about 3 to 4 h prior to the start of dark period. These results were in agreement with observation reported by Scanes et al. (1987), who found large quantities of feed present in the crop at the start of dark period. The most amount of ileal DM was obtained during morning sampling i.e. about 8 to 9 h after the start of light period with continuous feeding.

In other study (Chapter 4), it was shown that sampling shortly after the start of light period results in higher phytate degradation, higher P digestibility and higher concentration of InsPs compared to those samples collected after 3 h (with 1 FW and 1 h FR) and 5 h (continuous feeding) especially for diets fed without phytase. This might overestimate the phytate degradation for the majority of the feed digested over

the daytime. The effect of different feeding methods prior to sampling was more prominent in the ileum compared to in the gizzard. It was also shown that FW and FR had similar effect on phytate degradation and P digestibility as continuous feeding.

Therefore, based on these studies, it is recommended to conduct digesta sampling at least 3 h after the start of light period, without FW and avoid sampling in less than 4 h prior to dark period in order to obtain more consistent amount of digesta from each part of gut section and more reliable measurement of phytate degradation. Increasing the number of chickens and sample pooling could be the alternatives to obtain higher amount of digesta sample as described by Morgan et al. (2013).

7.2. Effect of different feeding methods prior to sampling

This study (Chapter 4) was an extension from previous experiment reported in Chapter 3 (Study 1). In this study, the samplings of the digesta were done shortly after 6 h dark period ended with the aim to investigate whether or not the different feeding methods i.e. 1 h feeding, 1 h feeding with 1 FW and 1 h FR and 5 h continuous feeding prior to sampling would change the measured effectiveness of dietary phytase, which would be reflected in the hydrolysis of phytate, relative concentrations of InsPs and apparent digestibility of P. The data showed greater InsPs hydrolysis and P disappearance in the gizzard at the start of the photoperiod and became less rapid as chickens were exposed to longer periods of light and free excess to food. Similarly, ileal hydrolysis of InsPs and P digestibility were higher at 1 h feeding method compared to other feeding methods that applied before sampling. However, the hydrolysis of $InsP_5$ and $InsP_6$ in the ileum was lower than those in the gizzard at 1 h feeding method. As the feeding methods changed, the hydrolysis of $InsP_5$ and $InsP_6$ was even lower in the ileum compared to in the gizzard. These findings conformed to Zeller et al (2015a) who suggested that there is better InsPs hydrolysis in the proventriculus-gizzard.

The interaction between phytase supplementation and feeding method on $InsP_6$ hydrolysis in the gizzard, $InsP_6$ and $InsP_{5-6}$ hydrolysis and phytate P digestibility

in the ileum indicates the importance of feeding method prior to sampling on the analysed effect of supplemented phytase. Digesta sampling immediately after dark or scotoperiod ends may result in overestimated phytate degradation for the majority of feed digested during the daytime, particularly for diets fed without phytase supplementation.

Phytase supplementation was shown to increase digesta MRT in both gizzard and ileum (Chapter 4) and these results confirmed the contribution of slower digesta passage in improving phytate hydrolysis in the gizzard and P absorption in the ileum. Further increase in ileal digesta MRT due to the interaction between phytase supplementation and feeding method prior to sampling has further increased the hydrolysis efficiency of $InsP_6$ with greater reduction in $InsP_6$ concentration at 5 h feeding method than at 1 h feeding method.

Therefore, it is vital to standardise the feeding method prior to digesta collection before comparing phytase effects between studies. As recommended in 7.1, digesta sampling should be conducted at least 3 h after the start of light period and without FW in order to obtain more reliable measurement of phytate degradation.

7.3. A common sample preparation

With limited amount of digesta samples, data reported in Chapter 3 (Study 2) showed that analysis of titanium (Ti), P and Ca can be done on digesta samples containing titanium dioxide via a single acid digestion. Sulphuric acid (H_2SO_4) could be used for replacing hydrochloric acid (HCl) in sample digestion prior to P and Ca analysis. Colorimetric methods for determining Ti and P in H_2SO_4 solution and Ca in HCl were as effective as ICP-OES method though ICP-OES is more sensitive and able to simultaneously analyse multi-elements (Morgan *et al.*, 2014b). Finally, a sample preparation protocol for sequential analysis in determining of DM, total ash, Ti and other minerals involving H_2SO_4 digestion for diet and digesta was proposed.

7.4. Blood myo-inositol, P and Ca

Myo-inositol is becoming important in nutrition research particularly to understand its role in improving growth of broilers fed diets containing supplemental phytase (Cowieson *et al*., 2011). In order to minimize variation during blood analysis, several steps in sample preparation including type of anticoagulant and deproteinization were evaluated. In our study (Chapter 3, Study 3), it was shown that circulating myo-inositol, Ca and P in chickens fed diet containing supplemental phytase can be determined from plasma or serum with similar results. Heparinized or EDTA plasma can be used for analysis of myo-inositol and P but only heparinized plasma is suitable for Ca determination. Myo-inositol could be analysed using the colorimetric method as effectively as by the HPLC method but with special attention given to the method of deproteinization since incomplete sample deproteinization may interfere with the colorimetric assay. The concentration of plasma my-inositol, P and Ca in chickens fed low P and Ca deficient diets either with or without supplemental phytase were compared in Chapter 4. It was shown that phytase supplementation increased plasma myo-inositol but did not affect the concentration of plasma P and Ca. Although data presented in Chapter 4 showed that phytate P and InsPs hydrolysis were reduced with different feeding methods prior to killing and sampling, the effects were not observed in plasma myo-inositol, P or Ca.

7.5. Effect of phytase supplementation

Phytase supplementation at 1500 FTU/kg in wheat based diets was shown to effectively hydrolyse $InsP_6$ and lower InsPs resulting in high myo-inositol concentration in the gizzard. More complete hydrolysis of $InsP_{5\text{-}6}$ occurred in the ileum as indicated by high concentrations of $InsP_4$, $InsP_3$ and inositol. This finding confirmed the hypothesis by Walk et al. (2014), who proposed that phytase at 1500 FTU/kg could hydrolyse phytate almost completely and result in high concentration of inositol. Walk et al. (2014) also suggested that better performance in broilers fed high dose phytase diets was because of phytate degradation and myo-inositol rather than excess P and Ca.

7.6. Effect of dietary fat and phytase

In an effort to increase phytase efficacy in wheat based diets, the effect of dietary fat inclusion in a phytase supplemented diet was investigated (Chapter 5). This study showed that high dietary fat inclusion had no significant effect on growth performance in broilers fed a wheat based diet except for 7 d old chicks, while phytase supplementation improved feed intake (FI) and body weight gain (BWG). Whereas, inclusion of high dietary fat in a phytase supplemented diet increased phytase efficacy by increasing BWG and FI of broilers, particularly post hatch to 7 d old chicks with better feed efficiency.

On the other hand, data presented in Chapter 6 showed that, high dietary fat improved feed efficiency with birds having higher FI and BWG and these results were as expected when broilers fed with mash diet. Similarly, phytase supplementation also improved feed efficiency but with reduced FI and comparable BWG to those chickens fed on the non-supplemented phytase diet. In addition, the inclusion of both high dietary fat and phytase improved feed efficiency though no significant interaction effects on FI and BWG were observed. The inconsistent findings between this study and previous study (Chapter 5) may be due to different form of diet (mash versus crumble) and phytase (granular versus liquid). Better growth performance in the study of Chapter 6 indicates high fat diet in mash form could be better utilized than crumble diet (Chapter 5). According to Latsaw (2008), the energy intake (ME) per gram diet was not affected by the form of diet although FI was higher with pellet than mash diet. In addition, Jafarnejad *et al.*, (2011) showed that diet with higher energy level which is due to higher level of fat inclusion improved BWG and FCR of broilers fed mash diet compared to lower energy diet but energy level in the diet did not influence the performance of broilers fed crumble-pellet diet. Crumbled diet is prepared in such a way that the feed mixture is pelleted before being crushed into a consistent coarser diet than mash, therefore similar effects of feeding crumbled diet as those observed with pelleted diet in broilers are expected (Agah and Norollahi, 2008).

Also phytase in granular form may be more efficacious than in liquid form. In Chapter 6, granular phytase was added to mash diet. Chun *et al*. (2007) reported that

supplementation of granular phytase in low P diet resulted in higher daily gain, feed efficiency and total P digestibility in 42 d old broilers compared to those with liquid phytase at same supplementation level (500 FTU/kg). Improvement of BWG was also observed in Chapter 4 with supplementation of granular phytase in crumbled diet and is in agreement with Kirkpinar and Basmacioglu (2006).

For avoiding harsh condition during pelleting, liquid phytase was applied by spray-coating the enzyme onto the pelleted feed (Chapter 5). However, this technique has several limitations including incompatibility of liquid phytase with other liquid ingredients and higher percentage of activity loss during storage compared to other form of phytase consequently negatively affects the efficacy of phytase in animal, particularly broilers (Zwart, 2006; Bedford and Cowieson, 2009).

Significant improvement of phytate degradation and P digestibility in duodenum-jejunum and ileum with either high dietary fat or supplemental phytase was also shown in data presented in Chapter 5. With high fat inclusion in phytase supplemented diets, an additive effect was observed on phytate degradation and P digestibility in duodenum-jejunum and ileum. In contrast, Ca digestibility in both duodenum-jejunum and ileum was not affected by high dietary fat but reduced with phytase supplementation. In this study, it was also shown that phytase supplementation increased tibia ash, P and Ca, which indicate that the availability of P and Ca from the phytate-mineral complex is increased due to the action of phytase.

7.7. Effect of dietary fibre

In the study presented in Chapter 5, cellulose was added to the high fat diet to compensate for the differences in the metabolisable energy of soybean oil among the experimental diets. Removal of cellulose from the high fat diet has created a high density diet and this diet was evaluated against the high fat diet containing cellulose, designated as low density diet, either with or without supplemental phytase. The negative effects of the low density diet on feed efficiency were consistent with published reports (Brickett *et al*., 2007, Hetland et al., 2004). With phytase supplementation, BWG and FI of broilers fed either high or low density diets were significantly improved.

Similarly, data presented in Chapter 6 also showed that addition of dietary fibre in the diet reduced overall BWG and FI up to 14 d of age but did not affect feed efficiency of young broilers. This study also showed that dietary fibre addition imposed different interaction effects with either dietary fat or phytase on phytate P degradation although there was no main effect observed on phytate P degradation in the GIT. Addition of dietary fibre affected phytate P degradation in each segment of GIT by increasing it at the low level of dietary fat but reducing it at the high level of dietary fat. Dietary fibre also further increased phytate P degradation with supplemental phytase in the crop. However, the effect of dietary fibre on phytate P degradation was not associated with digesta MRT.

7.8. Digesta mean retention time

Previously, it was shown that inclusion of high dietary fat in a phytase supplemented diet has further enhanced growth performance, phytate degradation and P digestibility in broiler chickens above phytase supplementation alone (Chapter 5). It was also shown that prolonged digesta MRT in proventriculus-gizzard and ileum due to phytase supplementation may contribute in reducing the concentration of InsPs in the GIT of chickens fed wheat based diet (Chapter 4). Therefore, the final experiment in this thesis (Chapter 6) was conducted to establish whether or not the improvement in phytase efficacy by adding high dietary fat was due to the prolonged digesta MRT through the crop and the whole digestive tract.

In this study, higher dietary fat supplementation did not significantly increase the digesta MRT of all sections of the GIT and the overall digesta MRT except in duodenum-jejunum, which is in contrast to the hypothesis of the present study. However, phytate P degradation in the crop was significantly improved with high fat level. In addition, an additive effect of supplementing phytase and high dietary fat together can be observed in the crop which indicated by the highest digesta MRT and it corresponded to the highest phytase degradation. These results provide evidence for the role of fat in improving phytase efficacy in broilers. On the other hand, in this experiment phytase supplementation did not influence the digesta MRT overall or in any section of the GIT. These results contradicted the previous findings in Chapter 4,

which showed increased digesta MRT in proventriculus-gizzard and ileum with supplemental phytase.

The discrepancy in digesta MRT in the studies of both Chapter 4 and Chapter 6 may be related to the form of the feed. Generally, when feeding mash diet, the chickens would prefer to eat larger sized particles compared to finer particles and these large size particles tend to remain longer in the gizzard and stimulate its grinding activity. Whereas, moistened pelleted diet will rapidly dissolve after consumption and since there is no requirement for grinding, pelleted diet may pass through the gizzard at faster rate (Amerah, 2008). This could explain the shorter gizzard digesta MRT in chickens fed pelleted diet (Chapter 4) in comparison to those fed mash diet without phytase supplementation (Chapter 6). Finer microstructure of pelleted diet may enhance the efficacy of supplemental phytase by allowing better access of phytase to phytate (Abdollahi and Ravindran, 2012) that in mash diet. Increased viscosity of digesta due to the action of phytase (Juanpere *et al*., 2005) could result in an increase of digesta MRT in crop, proventriculus-gizzard and small intestine. Therefore, this explains the observed effect of phytase supplementation on delaying gizzard digesta MRT in pelleted diet.

7.9. Suggestion for future research

Data presented in Chapter 4 indicated that the ileal concentration of insoluble $InsP_6$ was significantly increased at 1+1+1 h and 5 h feeding method. Similar findings were reported by Jongbloed *et al*. (1992) who hypothesised that *de novo* synthesis of $InsP_6$ in the small intestine of pigs may contribute in low value of ileal phytate digestibility. The synthesis of $InsP_5$ in chick red cells and *in vivo* synthesis of $InsP_6$ by the action of human $InsP_5$ 2-kinase have been reported (Isaacks *et al*., 1982, Versky *et al*., 2005). Further investigation is warranted to confirm the existence of any such *de novo* synthesis in chicken's gut to support this hypothesis.

In the present study, there was inconsistent effect of fat and phytase on the digesta MRT along the GIT due to diet form (Chapter 4 and 6) and diet form was reported to affect the development of each section of GIT, particularly the length of the small intestine (Amerah, 2008). Although the overall digesta MRT was not

affected by different diet form, it is might be useful to investigate the possibility of different gross morphology of each section of GIT resulted from feeding mash and pelleted-crumble diet in influencing digesta MRT in broilers fed diet containing high fat and supplemental phytase. Also, it is worth to expend the scope of investigation to the effect of the morphological changes of each GIT section due to diet form, dietary fat level and supplemental phytase on the digestion and absorption of starch, protein, fat and minerals.

7.10. General Conclusion

The research work presented in this thesis demonstrated that different feeding methods prior to sampling and the time of sampling significantly affected the concentration of InsPs, degradation and digestibility of phytate P in different segments of the GIT which could lead to overestimation or underestimation of degradation and digestibility values. Therefore, it is vital to standardise the feeding method prior to digesta collection before comparing the degree of phytase effects between studies. It is recommended to conduct digesta sampling at least 3 h after the start of light period, without FW and avoid sampling in less than 4 h prior to dark period. This is to obtain more consistent amount of digesta from each part of gut section, more reliable measurement of phytate degradation and ease of handling before and during sampling. Inclusion of high dietary fat in phytase supplemented diets further improved the growth performance of broilers, additively increased phytate P degradation and P digestibility in upper and lower segments of the GIT. Adding cellulose as filler in pellet-crumbled diet has diluted the nutrient concentration in 5% fat diet that eventually led to poorer FCR as compared to those fed 5% fat diet without cellulose addition (high density diet). However, phytase supplementation eliminated the negative effect of nutrient dilution due to cellulose addition by improving the growth performance of chickens as good as those fed on high density diet. Additive effect of high fat and phytate supplementation on phytate P degradation also provides evidence for the role of fat in improving phytase efficacy in broilers by increased digesta MRT in the crop. Dietary fibre also contributed in enhancing crop phytate P degradation in phytase supplemented diet. However, the

effect of phytase supplementation on phytate P degradation was more pronounced than those of dietary fat and fibre. For mash diet, it is suggested that simultaneous addition of dietary fat as high as 5% and 1500 FTU/kg phytase in P and Ca deficient wheat diet is able to improve *in vivo* phytase efficacy and the growth performance of broilers over and above those fed adequate P and Ca diet. Addition of high dietary fibre should be avoided.

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