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Department of Animal and Aquacultural Sciences
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Main Supervisor: Birger Svihus

Elevated Phosphorus Retention after Facilitating Phytase Efficacy via Intermittent Feeding and Acidification

Yunru Bai

Master in Feed Manufacturing Technology
Faculty of Biosciences

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Master of Science Thesis (30 Credits)

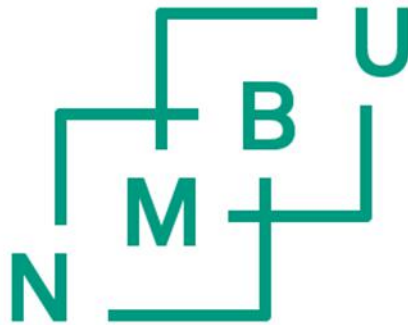
Yunru Bai

Department of Animal and Aquaculture Sciences

Faculty of Biosciences

Norwegian University of Life Sciences

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Yunru Bai

Abstract

The over-exploitation of phosphorus (P) reservoir is becoming one of the major concerns in poultry industry due to the limited stock of P. Secretion of endogenous phytase in monogastric animals is insufficient to break down the phytate in plant-based diet for broilers. Thus, the addition of exogenous enzyme, e.g. phytase, is becoming essential to release P from the complex structure of phytate. Present experiment was conducted to examine the effect of interaction among three main factors including phytase, formic acid and feeding regime on growth performance and bone mineralization. 800 of male Ross 308 broiler chickens one-d-old were allocated to 4 dietary treatments, including (1) negative control (NG) diet, (2) NG with 500 FTU kg⁻¹ of phytase, (3) NG with 1.1% of formic acid, (4) NG with same amount of phytase and formic acid, and all the treatments were fed intermittently and *ad libitum*. Each treatment had 10 pen replicates with 10 birds in each. Commercial starter feed was used in starter phase (1–10 d) while wheat-soy based experimental diets were provided during the grower phase (11–36 d) in pelleted form.

Overall, the results indicated that the combination of phytase, formic acid and feeding regime had a positive effect on growth performance, including feed intake, weight gain (WG) and feed conversion ratio (FCR), digestibility of P and the content of ash as well as deposition of P in tibiae. Phytase efficacy was enhanced through inclusion of formic acid, and the efficiency of such combination had more advantage under intermittent feeding. The average mortality rate was 2.38% during 11–36 d.

In addition, the interactions between and among three main factors were also observed. The interaction effect between acid and phytase on WG was found during 15–22 d. The interaction between feeding and phytase on FCR was observed between day 22–29 and this interaction also had effect on ileal P digestibility. The interaction between feeding and acid had effect on jejunal P digestibility. Also, the interaction among three main factors had no effect on ash content and deposition of P in tibiae, but, had effect on weight and width of tibiae. There were no significant differences among all treatments in terms of bone density of tibiae. The pH of the experimental diet with acid (T₃) increased significantly ($P < 0.001$) from 4.39 to 5.02 within the first 18 hours as it exposed to the same temperature (27–29 °C) with feeding experiment.

Keywords: Phytic acid, phytase, phosphorus, pH, formic acid, *ad libitum* feeding, intermittent feeding

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Abbreviation

ANOVA	analysis of variance
BW	body weight
FCR	feed conversion ration
FI	feed intake
FTU kg ⁻¹	unit of phytase in per kg of feed
IU kg ⁻¹	International Unit of vitamin in per kg of feed
WAD	weight after drying
WBD	weight before drying
WG	weight gain

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1. Introduction

Phosphorus (P) is a biologically important macro-mineral in the tissue of both plants and animals. For instance, phosphate is the critical composition of DNA (deoxyribonucleic acid), RNA (ribonucleic acid), energy storing molecule called ATP (adenosine triphosphate). In addition, phosphate also play important role in bone mineralization. Therefore, P is closely related to the metabolism of body tissues, including protein synthesis, formation of bone tissues, and energy transfer. In poultry industry, feed costs approximately 60–70% of the total expenditure, in which P is the third expensive ingredient in the diet formulation.

However, global storage of P is declining all the time mostly due its unsustainable exploration. However, the global demand for P is increasing all the time. It has been estimated that in a case of keeping present consumption of P, the current stock may be depleted in 50-100 years (Cordell et al., 2009). Therefore, the sustainable utilization of P is becoming crucial.

Facing the finite global resources of P, there are many innovations that are trying to recycle this non-renewable resource. Plants store P in a complex structure of phytic acid or in its salts phytate and phytin (Humer et al., 2015, Selle and Ravindran, 2007). However, monogastric animals cannot utilize phytate-P without breaking down the complex structure of phytic acid in plant tissues, and unfortunately, monogastric animals are lacking in ability to produce enzyme to release P from plant materials (Iqbal et al., 1994). The excessive phytate-P excreted with manure causes environmental pollution such as eutrophication.

In animal feed production, however, enzymatic approach is widely applied to exploit P from plant resources, in which phytase is the only known phytate-degrading enzyme to hydrolyze phytate to release inorganic P. From the perspective of poultry industry, the application of phytase in the diet of monogastric animals is critical step to minimize the addition of inorganic P, and at the same time, to maximize the bioavailability of phytate-P in plant materials.

The diversity of phytase is abundant based on their origin, and each type of phytase has the specific requirements for the ambient condition that phytate can be efficiently hydrolyzed, for example pH, temperature and concentration of substrate. Furthermore, structurally and functionally, the characteristics of digestive tract vary among different animals. Unlike other monogastric animals, proventriculus and gizzard are considered the stomach of chickens and they play an important role

in the activity of phytase (Svihus, 2014). The pH of foregut (crop, proventriculus and gizzard) can be reduced through prolonged retention time and intermittent feeding (Svihus, 2014). Broiler chickens have an ability to adapt discontinuous feeding rapidly due to the maximum storage capacity of crop. The ingested feed can be moisturized and fermented by microflora in crop, resulting in lower pH in foregut, which is fundamental for the activity of phytase (Svihus, 2014, Svihus et al., 2010).

In practice, some organic acids are added in animal feed to promote animal health by declining pH of foregut. However, there are not many studies regarding how feeding regime and organic acid facilitate phytase efficacy in poultry feed. The objective of this research was to test whether or not the combination of formic acid, phytase and feeding regime elevates phytase efficacy, availability of P and growth performance of broilers.

2. Literature review

2.1. Phytic acid

Phytic acid (myo-inositol-1, 2, 3, 4, 5, 6-hexakisphosphate, IP6 $C_6H_{18}P_6O_{24}$) is primary storage form of phosphorus (P) and inositol in plants containing six phosphate groups in a myo-inositol ring structure. The conformational state of phytic acid proposed by Anderson (Johnson and Tate, 1969) (Fig. 1) has been contributed to the comprehensive understanding and further study of its biochemical characteristics.

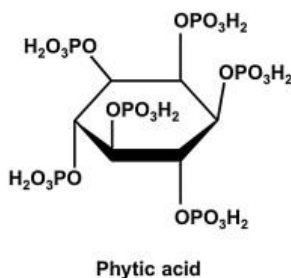


Fig. 1. Molecular structure of phytic acid

Phytic acid is unstable when present in a free acid form due to its high density of negative charge (Cowieson et al., 2016). The most representative compounds of phytic acid are phytate and phytin. Phytate is a compound of phytic acid and some elements, including Ca^{2+} , Mg^{2+} , K^+ , Fe^{2+} , Zn^{2+} ,

Mn²⁺ (Humer et al., 2015). Similar to phytate, phytin is formed between phytic acid and a few cations, including Ca²⁺, Mg²⁺ and K⁺ (Selle and Ravindran, 2007). It has been examined that phytate is predominantly distributed in the aleurone layer, i.e. the innermost layer of bran, of kernel of grains (Fig. 2), cereals and seeds, compared with roots, tubers and turions which are lower in phytate content (Schlemmer et al., 2009).

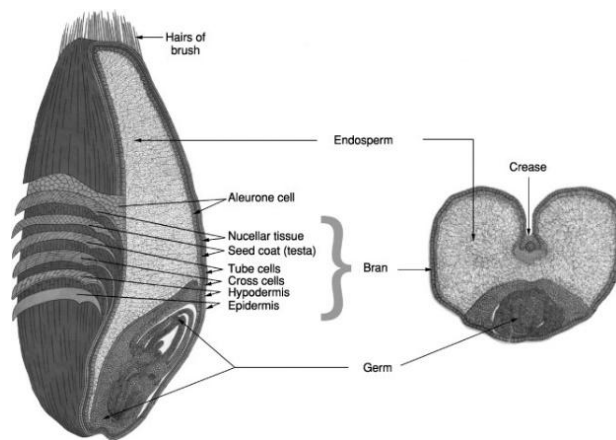


Fig. 2. Structure of wheat kernel

The diet for monogastric animals usually consists of a considerable amount of plant ingredients. For example, cereals, grains (e.g. corn, wheat) and legumes (e.g. soybeans) are widely used in the feed for broiler chicken. Phytate is a ubiquitous component and invariably present in plant-sourced feed ingredients. In cereals and grains, bran is a rich source of minerals, fiber and phytic acid. According to the data from previous studies (Table 1), total concentrations of phytate and phytate-P based on dry matter is much higher in by products, such as wheat bran and rice bran, than cereals. However, the proportion of total P ranges from 60% in general feed ingredients to 80% in brans (Selle et al., 2003, Selle and Ravindran, 2008). Although plant ingredients are rich in P, between 60–90% of total P are tightly locked up in the complex structure of phytic acid or its salts, i.e. phytate and phytin, existing in the form of phytate-P in plant feed ingredients (Johnson and Tate, 1969, Wu et al., 2009).

Table 1. Phytate and P content in major feed ingredients for broiler chicken

Feedstuffs	Phytate g 100g DM ⁻¹	Total P g kg ⁻¹	Phytate-P g kg ⁻¹	Phytate-P %
Cereals				
Barley	0.38–1.16	2.73–3.70	1.86–2.20	59–68
Maize	0.72–2.22	2.30–2.90	1.70–2.20	66–85
Wheat	0.39–1.35	2.90–4.09	1.80–2.89	55–79
Sorghum	0.57–3.35	2.60–3.09	1.70–2.46	65–83
By product				
Wheat bran	2.1–7.3	8.02–13.71	7.90–24.20	50–87
Rice bran	2.56–8.7	13.40–27.19	7.00–9.60	42–90
Oilseeds				
Soybeans	1.0–2.22	3.54–4.53	3.54–4.53	53–68
Rapeseed	2.50	8.791–1.50	4.00–7.78	36–76

Modified from previous studies (Nelson et al., 1968, Kirby and Nelson, 1988, Eeckhout and De Paepe, 1994, Viveros et al., 2000, Selle et al., 2003, Schlemmer et al., 2009)

Since the small intestinal mucosa of monogastric animals lacks secretion of phytase to dephosphorylate phytate (Iqbal et al., 1994), the utilization of phytate-P is challenging for monogastric animals. As a consequence, undigested phytate-P will be excreted with fecal materials aggravating agricultural run-off and eutrophication. Therefore, the research for improving phytate-P utilization efficacy is becoming a major concern.

Phytic acid, considered as a type of anti-nutrient, influences the digestion and absorption of macronutrients and minerals negatively, the interaction between phytate and protein for example. Either in acidic or alkaline medium, the interaction between phytate and amino acid residuals forms binary protein-phytate complex with a fairly insoluble nature (Selle et al., 2012). Phytic acid exacerbates the endogenous nitrogen loss due to its binding property with both digestive enzymes and dietary protein (Woyengo and M. Nyachoti, 2013). Both from scientific and practical standpoint, moreover, the negative effect of phytate on the availability of trace minerals is gaining more attention. Various insoluble chelates are formed from the interactions between phytic acid and multivalent cations such as Ca²⁺, Mg²⁺, Zn²⁺, Fe²⁺ and Cu²⁺ when the pH is close to neutral in small intestine (Svihus, 2010, Selle and Ravindran, 2008). However, these critical issues can be ameliorated by phytase which is the only known phytate-degrading enzyme that is capable of dephosphorylating phytate to facilitate the release of phytate-bound P from plant ingredients (Konietzny and Greiner, 2004).

2.2. Phytase

Phytase is classified into exogenous and endogenous form depending on its origin. Exogenous phytase can be derived from yeast, bacteria, fungi and plant. In contrast, endogenous phytase is generated by small intestinal mucosa of monogastric animals. The hydrolysing capacity of endogenous phytase is constrained by its low secretion amount. In order to deal with the issues mentioned above, dietary inclusion of exogenous phytase is essential for the monogastric animals to improve the nutrient utilizations, growth performance and P availability. Since 1990s, fungal phytase (*Aspergillus niger*) has been considered as the first generation of commercialized phytase in poultry feed (Selle and Ravindran, 2007, Dersjant-Li et al., 2015). More effective phytase derived from bacteria (*E. coli* acid phosphatases) was discovered in 1999 (Dersjant-Li et al., 2015). Phytase releases phytate-P from phytate, and through a complicated step-wise dephosphorylation procedure (Oh et al., 2004, Selle and Ravindran, 2008). Theoretically, the dephosphorylation procedure produces a series of lower myo-inositol phosphate esters ($IP_6 \rightarrow IP_5 \rightarrow IP_4 \rightarrow IP_3 \rightarrow IP_2 \rightarrow IP_1$), producing inositol and inorganic P (Selle and Ravindran, 2008).

The improvement of phytate-P utilization from plant-based ingredients is critical for the development of environmentally and economically sustainable agriculture. In addition, there are various factors influencing the efficacy of phytase, for example temperature. The optimum temperature range of their enzymatic activity is between 44–60 °C and, similar to other thermos-unstable feed additives, phytases are thermal intolerant above their optimum temperature (Oh et al., 2004). During the hydro-thermal treatment of feed manufacturing process, temperature goes up to 80–95 °C leading to inactivation of heat sensitive nutrients and enzymes. Unlike other enzymes, however, phytase can be added both pre-pelleting and post-pelleting due to the differences between various types of phytases in terms of their heat tolerance. Therefore, further research is necessary for coping with the potential factors influencing the functional properties of phytase in poultry feed to improve the utilization efficiency of P from the plant-based ingredients.

2.3. Factors influencing the efficacy of phytase in monogastric animals

2.3.1. Digestive tract of chicken

Generally, food is ground and mixed with saliva in the mouth of most monogastric animals. After the first step of reduction in size, digesta enters to stomach where further digestion occurs through

the combination of muscle contraction and gastric juice. Enzymatic digestion and absorption take place in small intestine in which most nutrients are absorbed in duodenum and jejunum. Retention time of digesta differs along the gastrointestinal tract whereas pH gradient increases during the passage from stomach to small intestine.

In poultry, however, function of stomach is replaced by proventriculus and gizzard, having a less storage capacity (Svihus, 2014). Ingested food accumulates in crop without experiencing mastication in mouth since teeth are absent in avian species. The main role of crop is to moisturize, soften and store ingested food temporarily before they enter proventriculus (Kierończyk et al., 2016). Proventriculus is a narrow glandular stomach located between crop and gizzard while gizzard is thick-walled muscular stomach with grinding function (Fig. 3). The proximal segment of digestive tract in a monogastric animal, usually from mouth to the entrance of bile duct in duodenum, is considered as an important place where exogenous phytase can be activated.

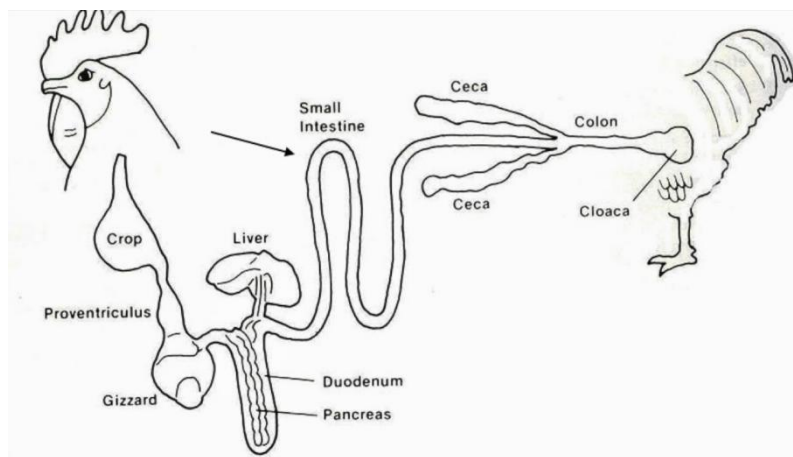


Fig. 3. Digestive system of chicken

2.3.2. Retention time and feeding regime

The time for ingested food to pass through digestive tract varies among different livestock. Retention time is defined as the length of time that ingested food is retained in a particular segment of digestive tract. The rate of food ingestion, nutrient assimilation, the efficacy of digestion and absorption of nutrients, and the mass of digesta carried are all influenced by retention time (Sibly, 1981, Weiner, 1992, Barton and Houston, 1994, Karasov and Cork, 1996).

Broilers respond differently to different feeding regimes. The growth and feed conversion ratio (FCR) of modern broiler flocks have improved, but the fast-growing live weight tends to cause sudden death syndrome (due to heart failure), ascites and skeletal deformities. In order to alleviate the incidence of those metabolic diseases and prolong the retention time in foregut, feed restriction, as an important strategy, is applied. Feed restriction can be categorized into two types based on the adjustment of quantity or quality. Quantitative feed restriction is a method that birds have restricted access to feed in order to improve feed efficiency (Lee and Leeson, 2001, Sunder et al., 2007) while birds have *ad libitum* access to lower caloric diet by qualitative restriction (Urdaneta-Rincon and Leeson, 2002, Sandilands et al., 2005). Quantitative feed restriction is usually employed in earlier stage through the adjustment of photoperiod (intermittent lightning) and feeding regime (e.g. intermittent feeding), and this quantitative feed restriction may lead to compensatory growth and efficient utilization of feed. Feed restriction is usually followed by *ad libitum* feeding in later stage to compensate the growth during the earlier stage. Buyse et al. (1996) showed that growth compensation and increase in N retention efficacy appeared in the later stage of life cycle (day 39–42) in broilers under the circulation of intermittent lightning with 1 h light (L) and 3 h darkness (D). However, the recent study by Rodrigues and Choct (2018) demonstrated that using similar intermittent lightning schedule (1h L: 3 h D, 1h L: 3 h D, 1h L: 3 h D, 1h L: 3 h D, 2 h L: 6 h D) broiler chickens ingested around 2.5 times the amount of feed that the birds consumed under continuous light, in the first one hour of darkness.

Feed restriction through intermittent lightning probably lead to an overestimation on FI because the ingestion amount in darkness cannot be neglected. Thus, intermittent feeding is more precise than intermittent lightning and it is merely achieved by feeding discontinuously for a few hours per day. Broiler chickens can adapt rapidly to consume few meals per day and to long period of starvation, which promotes the development of the holding capacity and function of crop and gizzard (Barash et al., 1992, Buyse et al., 1993). As a consequence, it not only prolong the retention time of nutrients in anterior part of digestive tract but also be a critical factor for the activity of exogenous enzyme (Svihus, 2014). Additionally, it has been suggested that an appropriate feed restriction in broiler not only led to a reduction in maintenance costs but also improve carcass quality by decreasing fat deposition in the carcass (Urdaneta-Rincon and Leeson, 2002).

The feed for monogastric animals usually has a pH around 6 which is similar to the pH in the crop at the beginning of food storage (Ao et al., 2008). When birds consume large amount of feed at

once, the maximum storage of a crop appears and then the ingested food get more time to be soaked and slowly fermented by lactic acid bacteria (Kierończyk et al., 2016). With the accumulation of lactic acid, crop pH gradually drop to around 4.8 after 2 h feeding (Svihus, 2014). It was mentioned in previous studies that chickens consumed majority of feed during the first 20 min of a 1 h feeding bout under intermittent feeding and very little during the last third of hour (Svihus et al., 2010). Instead of filling their crop at once, however, birds tend to eat small meal approximately each 30 min under *ad libitum* feeding (Nielsen, 2004). As a consequence, digesta pass through proventriculus without sufficient acidification and hydration process (Svihus et al., 2013). It has been demonstrated that a considerable amount of phytic acid was broken down in crop due to the prolonged retention time by intermittent feeding (Svihus et al., 2010). The variation of pH in crop is associated with the retention time (Svihus, 2014), and the crop pH decreases with increasing retention time (Bolton, 1965) in broilers. Hence, it has been concluded that the extension of retention time in crop is achievable through interfering or manipulating feeding regime and feeding behaviour (Svihus, 2014).

Proventriculus and gizzard are considered as true stomach of poultry which is responsible for both chemical and mechanical digestion. A well-functioned gizzard is able to prolong the retention time of digesta in gizzard. It has been suggested that the inclusion of cereals with particle size larger than 1mm or structural component such as coarse oat and, wheat bran in broiler diet contributes to the stimulation of gizzard function by enhancing both size and volume of gizzard muscle (Svihus, 2011). Although gizzard does not have a storage capacity as crop does, its function is similar with a filter which selectively retains larger and hard materials and allows tiny and soluble particles to pass rapidly through. Remaining coarse digesta is continuously ground by the contraction of strong myelinated gizzard muscle and pushed back to the proventriculus, exposing the ground digesta to the gastric juice with pH around 2. The small muscle then assists the movement of digesta towards the grinding zone of gizzard again. The digesta is refluxed several times with such a manner until the size is small enough to pass through gizzard.

Svihus (2014) indicated that the retention time of food in proventriculus/gizzard varies from half an hour to one hour. Moreover, the foregut pH ranges between 1.9 and 4.5 depending on the content and size of the fibrous materials in broiler diet. Hence, the degree of reduction in pH is a closely intertwined with the retention time of digesta in gizzard. Hydrolytic characteristic of phytase is facilitated by gastric juice because the gastric pH is closer to the optimum pH of phytase activity

(Campbell and Bedford, 1992). In addition, phytate is more soluble in gastric acid (Campbell and Bedford, 1992). An earlier study demonstrated, on the other hand, that intermittent feeding and phytase improved growth performance of broiler (Svihus et al., 2013). But, it is still unclear whether intermittent feeding promotes phytase efficacy or not.

2.3.3. Foregut pH and usage of organic acids

Apart from retention time, gastrointestinal pH plays an important role in the optimization of phytase efficacy. The optimum pH and temperature differ slightly based on where the phytase is derived from. For instance, most bacterial phytases differ from fungal phytases in terms of their optimum pH range. Bacterial phytases show enzymatic activity at pH and temperature between 4.5–8.5 and 25–75 °C, respectively (Jain et al., 2016). In previous study, fungal (*A. niger*) phytase liberated 1 µmol inorganic orthophosphate min⁻¹ from 0.0051 mol L⁻¹ sodium phytate at pH 5.5 under 37 °C (Engelen et al., 1994). Heat stable phytases can withstand hydro-thermal treatment during pelleting process (above 80 °C) (Wyss et al., 1998, Garrett et al., 2004). Therefore, compared to temperature, phytase seems to demand a strict pH value to be active in the digestive tract.

Potential effect of various feed additives on phytase efficacy in monogastric animals has received more attention (Selle and Ravindran, 2007). Most prevalent feed additives related to phytate-P utilization include Vitamin D₃ (cholecalciferol) and organic acids (Selle and Ravindran, 2007).

Addition of organic acid or their salts in feed is an important approach to improve health and performance of animals, replacing the use of antibiotics in feed for monogastric animals. The antibiotics are banned to add in animal feed in European countries, since the new Feed Additives Regulation was released in 2006.

There are several types of organic acids used frequently in broiler industries such as formic, citric, lactic, fumaric and sorbic acid. These organic acids are usually characterized as weak acid and do not dissociate in water completely (E. Talebi, 2010).

Organic acid in feed acts as a chelating agent to lift the susceptibility of phytate to phytase hydrolysis (Selle and Ravindran, 2007). Otherwise, acidification promoted by organic acid inhibits the formation potency of mineral-phytate in foregut. Consequently, phytate tends to exist as non-conjugated form, which has a high affinity to phytase (Vieira et al., 2017). In this case, phytase has potential to release more P from phytate. Synergistic effect between phytase and organic acid is

probably strengthened through increasing dose of phytase instead of acid (Jongbloed et al., 2000). Previous study concluded that requirement of inorganic P decreased by 1.0 g kg⁻¹ after adding 40–60 g of citric acid per kg of corn and soy-based broiler diet (Boling-Frankenbach et al., 2001). Likewise, organic acid intensifies de-phosphorylation of phytate in *in vitro* experiment (Zyla et al., 1995) and increases the bone mineral deposition in *in vivo* experiment (Brenes et al., 2003). The result of a study on weaning pig (S Radcliffe et al., 1998) showed that application of phytase with citric acid increased linearly rib shear force, shear energy, dry bone weight, ash weight, ash percentage and digestibility of Ca and P. Therefore, the appropriate combination of organic acid and phytase has a great potential to improve bio-availability of phytate-P in poultry feed.

Importantly, organic acids have bacteriostatic and bactericidal properties. Organic acid, as an acidifier, restrain the growth of harmful bacteria and stabilize intestinal microflora to improve the health condition of animals (Khan and Iqbal, 2016). Additionally, it has been reported that mortality rate was reduced significantly by adding propionic acid in turkey feed (Roy et al., 2002). Organic acids have a positive effect on nutrient utilization, growth (Luckstadt and Mellor, 2011) and feed conversion efficiency (Aherne and Falkowski, 1984). For example, supplementation of citric acid in P deficient diet increased linearly with weight gain and tibia ash content (E. Talebi, 2010, Boling et al., 2000, Snow et al., 2004, Liem et al., 2008). In an experiment with pig, combination of phytase (750 FTU kg⁻¹) and citric acid (15.0 g kg⁻¹) or vitamin D₃ (2000 IU kg⁻¹) in a negative control diet (P deficient diet) increased nitrogen (N) digestibility and lowered fecal P and N excretion by 27–39% and 8.3%, respectively, compared with positive control diet (Li et al., 1998, Madrid et al., 2013), and in the same study, FI, daily weight gain (WG), and feed efficiency of swine elevated by adding phytase in corn-soy based diet. During the post-weaning period of swine, the stress associated with insufficient production of hydrochloric acid, pancreatic enzymes and rapid change in feed consistency and feed intake can be alleviated by adding weak acid in the feed, improving digestion and absorption of nutrients (Suiryanrayna and Ramana, 2015). Conversely, S Radcliffe et al. (1998) showed that phytase did not affect growth performance and no synergistic effect was found between phytase and citric acid in weanling pig. Each specific organic acid contributes to growth performance differently and the interaction between organic acid and phytase may varies in different species, age and gender.

2.3.4. Other factors

A proper Ca and P ratio is also an important factor that contributes to the efficient utilization of phytase in the diet of monogastric animals (Angel et al., 2002). It was suggested that narrower ratio between Ca and P can have positive effect on phytase efficacy in swine, at approximately 1.1:1 Ca: p ratio (Lei et al., 1994). The growth performance of young pigs increased considerably when dietary Ca reduced from 8.8 to 4.8 g kg⁻¹ in phytase supplementation diets at 750 and 1200 FTU kg⁻¹ (Lei et al., 1994).

The recommended ratio for poultry were 2.22 to 2.67 Ca to 1 non-phytate P depending on growth stage (NRC, 1994). But, total Ca to digestible P ratio has been standardized at 2:1 over three decades and these ratios are not for optimal performance and bone mineralization. Thus, more precise methodologies has been developed based on digestible Ca and P (Angel, 2013). It has been suggested that the inclusion of high level of Ca exacerbates P-deficiency (Waldroup et al., 1962, Nelson et al., 1965, Gardiner, 1971). Driver et al. (2005) showed that the ash content of tibia reached maximum level in the treatment with total Ca to non-phytate P ratio of 1.07 to 1.35. Excessive Ca interact with phytate to form both soluble and insoluble Ca-phytate compound between pH 2 to 12 (Marini et al., 1985) due to the high acid binding capacity of Ca (Lawlor et al., 2005). The pH in crop rose from 4.89 to 5.32 after supplementation of Ca in broiler feed (Shafey et al., 1991, Driver et al., 2005), which may lead to a significant reduction in phytase activity as mentioned by Selle and Ravindran (2007). Selle et al. (2009) demonstrated that the formation of Ca-phytate compound is mainly in small intestine, and the efficacy of phytase is not directly inhibited by insoluble compounds. However, both Ca and P are firmly captured in Ca-phytate compounds and these compounds cannot be absorbed by intestinal wall according to Selle et al. (2009).

Overall, the comprehensive knowledge about digestion and absorption along the gastrointestinal tract is fundamental to optimize phytase efficacy in broiler chicken. Further research is required to determine the effect of combination of multiple factors on phytase efficacy.

3. Material and methods

The experiment was designed using 2 × 2 × 2 factorial arrangement (Table 2), which integrated formic acid (diet with and without formic acid), phytase (diet with or without phytase) and feeding regime (intermittent and *ad libitum*) for each treatment.

Table 2. Experimental design and treatments

Item	Intermittent	<i>Ad libitum</i>
Negative control* (NC)	T ₁	T ₁
NC + Phytase	T ₂	T ₂
NC + Acid	T ₃	T ₃
NC + Phytase + Acid	T ₄	T ₄

* Less available phosphorus, no phytase

This study was a part of collaborative project between Norwegian University of Life Sciences (NMBU) and Olszowa PIAST Broiler Chicken Experimental Station in Poland. Feeding experiment was carried out in Poland and the analysis of tibiae was conducted in a chemistry lab at the Department of Animal and Aquacultural Sciences (IHA) of NMBU. Part of the data regarding growth performance, digestibility of P, content of ash and deposition of P in tibiae were presented in this paper.

3.1. Experimental diets

Experimental diets were based on wheat and soy, manufactured at the Centre for Feed Technology (FôrTek) of Norwegian University of life Sciences in Ås, Norway. All experimental diets (Table 3) were formulated to meet or exceed the nutritional requirement of broiler chicken recommended by NRC (1994). All the ingredients were mixed with some water in a twin-shaft paddle mixer (PEGASUS, 400 Liters, Model 1992 OB-1078), and the feed mash was conditioned at 70–85 °C, and the malleable dough from the conditioner was pressed through a pellet press.

One size of nozzle (size 6505) was used for adding phytase, acid and oil. In total, 5.8% of soya oil was added in each diet in which 3% of oil was sprayed to feed mash during the mixing process and remaining 2.8% of oil was added after pelleting in all diets. For T₂, phytase was diluted with 0.2% of water and then sprayed into the diets before adding oil. For T₃, formic acid was directly sprayed before adding rest of oil (2.8%). For T₄, phytase was diluted with 0.2% of water and sprayed and formic acid and oil were added thereafter.

Table 3. Composition of the experimental diets used in the feeding trial

Ingredients, g kg ⁻¹	T ₁	T ₂	T ₃	T ₄
Wheat	361.0	361.0	361.0	361.0
Wheat bran	301.0	301.0	301.0	301.0
Soybean meal extracted	121.0	121.0	121.0	121.0
Rapeseed meal	120.0	120.0	120.0	120.0
Soybean oil	58.0	58.0	58.0	58.0
Limestone	12.0	12.0	12.0	12.0
Titanium dioxide	5.0	5.0	5.0	5.0
<i>L</i> -lysine	3.0	3.0	3.0	3.0
Salt	2.0	2.0	2.0	2.0
<i>DL</i> -methionine	2.0	2.0	2.0	2.0
Micromineral	1.0	1.0	1.0	1.0
<i>L</i> -threonine	1.0	1.0	1.0	1.0
Sodium bicarbonate	0.9	0.9	0.9	0.9
Monteban G100 (narasin)	0.7	0.7	0.7	0.7
ADKB	0.6	0.6	0.6	0.6
Vitamin A	0.8	0.8	0.8	0.8
Vitamin D ₃	0.9	0.9	0.9	0.9
Vitamin E	0.3	0.3	0.3	0.3
Selenium	0.1	0.1	0.1	0.1
Rovabio excel AP	0.05	0.05	0.05	0.05
Soya oil, %	5.8	5.8	5.8	5.8
Formic acid, g kg ⁻¹	—	—	11	11
Phytases, FTU kg ⁻¹	—	500	—	500

3.2. Feeding experiment

Over the entire feeding period, two types of diets were used depending on the growing phases, i.e. commercial starter and experimental diets. The whole flocks were fed *ad libitum* with commercial starter feed from day 1 to 10 followed by experimental feed during the grower phase which was from day 11 to 36.

Feeding experiment was conducted using birds with same WG, FI and FCR as they aged 10 d (Table 4), and lasted from 11 to 36 d.

Table 4. Weight gain (WG), feed intake (FI) and feed conversion ratio (FCR) during the 10 days of starter phase

Group	Starter phase (from 1–10 d)		
	WG (g)	FI (g)	FCR
G ₁	294	319	1.09
G ₂	290	316	1.09
G ₃	286	315	1.10
G ₄	292	317	1.09
G ₅	292	317	1.09
G ₆	292	313	1.07
G ₇	290	313	1.08
G ₈	286	304	1.06
SEM	1.01	1.19	0.003
p-value	0.411	0.083	0.104

The feeding trial was carried out at a commercial broiler farm of PIAST PASZE Sp. Zo.o.Olszowa, Poland. A total of 800 one-d-old male Ross 308 broiler chicks were allocated randomly in 80 cages with a size of 1 m² and fresh straw bedding for each. There were 10 cages of birds as replicates for per treatment and 10 birds in each cage. To restrict access to feed, automatic feeders were applied in the intermittent feeding treatments. The feeding periods were distributed among 08:00–09:00, 12:00–13:00, 16:30–17:30, 21:00–22:00 and 02:00–04:00 for all intermittent treatments. Both *ad libitum* and intermittently fed chicks had free access to nipple drinkers. Lights were turned off from 22:00 to 02:00 and from 04:00 to 08:00 for all treatments. Temperature of chicken house was set to 32–33 °C at the beginning and gradually decreased by 2–3 °C. Since the birds aged 28 d, the temperature remained at 21 °C, until the termination of feeding trial.

3.3. Performance and data collection

Body weight (BW) and FI were determined at day 10, 15, 22, 29 and 36 in order to calculate FCR of both starter and grower phases. Mortality rate was also recorded during the entire feeding period. The birds in one pen were weighted together and the average weight was taken. FI was determined by subtracting the amount of remaining feed from the total feed given.

3.4. Sample collection

One bird was randomly selected from each cage. In total, therefore, 10 birds were sampled for each treatment. Dissection was performed on day 36 for *ad libitum* and day 37 for intermittent treatments. All birds were euthanized by cervical dislocation and then tie up the neck by plastic strips in order to prevent regurgitation of crop content. The left tibia of each bird was removed and frozen immediately at $-20\text{ }^{\circ}\text{C}$ until determination of ash content.

The pH of crop and gizzard was measured. The contents of crop, jejunum and ileum were collected to determine further the digestibility of P along with the digestive tract and these samples were analyzed at Poznan University of Life Sciences, Poland. But, not all the relevant data were presented in this thesis.

The excreta was collected at day 28. A paper sheet was placed on the bottom of each pen at the start of feeding at 12:00 and removed at 16:00 just before next feeding. And excreta was manually plucked several times during these hours. The samples from same pen were pooled together for further analysis.

3.5. Tibia sample analysis

The attached meat or tissues were carefully removed using a scalpel and a small knife before the analysis of tibiae. The tibiae were then dried at $104\text{ }^{\circ}\text{C}$ for 16 h using forced conductive air in an oven (FP 53, BinderTM9010-0153, Germany). The fresh and dry weight of tibiae were measured before and after drying. The length and breadth of tibiae were also measured after drying.

The dried tibiae were crushed into smaller pieces using an iron plier and collected in a separate crucible for each tibia in order to ash them using a muffle furnace (LT 5/12, Nabertherm, Germany) at $550\text{ }^{\circ}\text{C}$ for 16 h.

After the combustion overnight, tibiae were completely ashed and then the ash were placed into an exicator until cooling down at room temperature. The ashed tibiae were ground into fine powder by a mortar and pestle after weighing.

The fine powders were then collected into the scintillation glasses with the same numbering system and stored at $-4\text{ }^{\circ}\text{C}$ until the analysis of P content.

3.6. Measurement of total phosphorus in the tibia ash

To measure P content of tibiae, 0.05 g of fine powder was taken from each sample and added 2 ml of HCl with 1 mol of concentration. When the fine powder completely dissolved into HCl, 5 ml of ion exchange water was added to each sample to dilute the solution. If there were no visible floating particles, 400 ul of solution was taken from the upper layer of each sample and transfer them to micro-centrifugal tubes using a pipette. Micro-centrifugal tubes with liquid samples were then placed into an Automatic Clinical Chemistry Analyser (RX daytona+, Randox Laboratories Ltd, UK) to quantify P content.

3.7. pH measurement of diet 3

Approximately 5 g of feed samples was taken from diet 3 (with 1.1% of formic acid). 0.5 ul of formic acid with 85% of concentration was added for each sample after weighing. A pH meter (VWR pH 100 INTERNATIONAL) was used in the test and calibrated using standard buffer with pH of 4 and 7.

The pH was measured at different time. The first treatment was tested immediately after adding acid and others were recorded after 8, 18, 24 and 48 h respectively. Each treatment had 10 replicates. The rest of 40 samples were placed in an oven (FP 53, BinderTM 9010-0153, Germany) at $27\text{--}29\text{ }^{\circ}\text{C}$ to expose them to forced conductive air. In order to effectively dissolve samples in distilled water, feed samples were ground and then mixed with distilled water at a ratio of 1: 5. The pH was measured three times for each sample in order to calculate the average.

3.8. Calculation and statistical analysis

FCR was calculated for each treatment using the equation (Eq. 1) below:

$$FCR = \frac{\text{total amount of feed consumed (g)}}{\text{total amount of weight gain (g)}} \quad Eq. 1$$

Mortality rate was calculated using the equation (Eq. 2) below:

$$\text{Mortality rate (\%)} = \frac{\text{total number of dead birds}}{\text{Number of live birds}} \times 100\% \quad Eq. 2$$

Apparent digestibility coefficient (ADC) of phosphorus (P) was calculated using the equation (Eq. 3) below:

$$ADC_{\text{nutrient}} = \left[1 - \frac{(\text{Marker}_{\text{feed}} \times \text{Nutrient}_{\text{digesta/faeces}})}{(\text{Marker}_{\text{digesta/faeces}} \times \text{Nutrient}_{\text{feed}})} \right] \times 100 \quad Eq. 3$$

where the marker was titanium dioxide (TiO₂); the digesta was taken from both jejunum and ileum.

Density of tibia was calculated (Eq. 4) based on previous study (Seedor et al., 1991):

$$\text{Density of tibia (mg mm}^{-1}\text{)} = \frac{\text{Dry weight of tibia (mg)}}{\text{Length of tibia (mm)}} \quad Eq. 4$$

Total P content of the tibia ash was calculated by using the equation (Eq. 5) below:

$$\text{Total P (mg g}^{-1}\text{)} = \frac{\text{value (mmol l}^{-1}\text{)} \times 30.97 \times 0.007}{\text{ash weight (g)}} \quad Eq. 5$$

where the value (mmol L⁻¹) in the numerator was provided by the Automatic Clinical Chemistry Analyser; atomic mass of P was 30.97; dilution of the samples was 0.007 L. Ash weight was around 0.05 g for each sample. Total P (mg g⁻¹) was the total P content in each gram of tibia ash.

Statistical analyses were conducted using R (Version 3.4.3). The model of $2 \times 2 \times 2$ factorial design with three main factors (feeding regime, acid, phytase) with its interaction terms was applied to investigate the potential correlation between dependent (response) and independent variables. Three-way analysis of variance (ANOVA) test was carried out to test whether there was an overall difference among the treatments, followed by a Tukey HSD pair-wise comparison as a *post hoc* test. A liner model was applied to test the correlation between dependent (response) and independent variables (Eq 6). One-way ANOVA was applied to test the overall difference among the pH measured at different time points using T₃ diet, followed by Tukey HSD as a *post hoc* test. All the statistical analyses were carried out at a significance level of $P = 0.05$.

$$y_{ijk} = u_0 + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijk} \quad \text{Eq. 6}$$

where y was response variable; the feeding regime, formic acid and phytase, were illustrated by α , β and γ , respectively. The interaction effects were indicated by $\alpha\beta$ (feeding \times acid), $\alpha\gamma$ (feeding \times phytase), $\beta\gamma$ (acid \times phytase) and $\alpha\beta\gamma$ (feeding \times acid \times phytase). ε represents the error term. $i, j, k = 1, 2, 3 \dots 40$.

4. Result

4.1. Growth performance

The result showed that three main factors, i.e. feeding regime, phytase and formic acid, had significant effect on FI during the period of 15–36 d (Table 5). In total, the FI of *ad libitum* feeding was higher than that of intermittently fed one. Under both feeding regimes, the lowest FI was found in the birds raised on the diet with acid (T₃) whereas the highest FI was observed in the birds raised on the diet with phytase (T₂). The FI of birds exposed to feed with phytase and acid (T₄) was slightly higher than NC (T₁) treatment under different feeding regimes, but not statistically higher. No interaction effect was observed between and among the main factors.

WG was significantly influenced by feeding regime ($P < 0.001$) and phytase ($P < 0.001$), i.e. the growth of the birds was improved by addition of phytase and *ad libitum* feeding. The interaction between acid and phytase was found during the period of 15–22 d but not the period of 15–36 d.

Phytase also had significant effect on FCR, i.e. the FCR was better in treatments with phytase compared to those without phytase. The influence of feeding and interaction between phytase and feeding were observed during a period of 22–29 d, with $P < 0.001$ and $P = 0.048$, respectively.

Table 5. Growth performance of broiler chickens from day 11 to 36

Treatment		Feed intake, g bird ⁻¹					Weight gain, g					FCR					Mortality	
Feeding	Acid	Phytase	10–15 d	15–22 d	22–29 d	29–36 d	15–36 d	10–15 d	15–22 d	22–29 d	29–36 d	15–36 d	10–15 d	15–22 d	22–29 d	29–36 d	15–36 d	11–36 d
Intermittent	No	No	325 ^b	861 ^b	1249 ^{ab}	1403 ^{bc}	3511 ^{bc}	199 ^d	535 ^{cd}	737 ^{abc}	777 ^c	2049 ^{cd}	1.63	1.61	1.69 ^{abc}	1.82	1.72 ^{ab}	2
Intermittent	No	Yes	335 ^b	866 ^b	1295 ^a	1449 ^{abc}	3609 ^{ab}	202 ^{cd}	537 ^{cd}	787 ^a	810 ^{abc}	2133 ^{abc}	1.66	1.61	1.65 ^c	1.79	1.69 ^{ab}	2
Intermittent	Yes	No	320 ^b	841 ^b	1207 ^b	1389 ^c	3434 ^c	196 ^d	509 ^d	726 ^{bc}	761 ^c	1995 ^d	1.65	1.65	1.67 ^{bc}	1.83	1.72 ^{ab}	4
Intermittent	Yes	Yes	330 ^b	860 ^b	1269 ^{ab}	1404 ^{bc}	3533 ^{bc}	204 ^{bcd}	542 ^c	773 ^{ab}	815 ^{abc}	2130 ^{bc}	1.65	1.59	1.65 ^c	1.73	1.66 ^b	1
<i>Ad libitum</i>	No	No	370 ^a	938 ^a	1250 ^{ab}	1473 ^{ab}	3661 ^{ab}	219 ^{abc}	578 ^{ab}	716 ^c	830 ^{abc}	2124 ^c	1.62	1.62	1.75 ^{ab}	1.78	1.72 ^{ab}	4
<i>Ad libitum</i>	No	Yes	371 ^a	942 ^a	1296 ^a	1506 ^a	3744 ^a	221 ^{ab}	592 ^{ab}	773 ^{ab}	871 ^a	2237 ^{ab}	1.69	1.59	1.68 ^{bc}	1.73	1.67 ^b	1
<i>Ad libitum</i>	Yes	No	372 ^a	922 ^a	1239 ^{ab}	1435 ^{abc}	3596 ^{abc}	223 ^a	562 ^{bc}	699 ^c	796 ^{bc}	2057 ^{cd}	1.67	1.64	1.77 ^a	1.81	1.75 ^a	1
<i>Ad libitum</i>	Yes	Yes	369 ^a	965 ^a	1300 ^a	1497 ^a	3748 ^a	227 ^a	594 ^a	783 ^a	863 ^{ab}	2240 ^a	1.63	1.60	1.66 ^{bc}	1.74	1.67 ^b	4
SEM			3.20	5.95	6.07	7.94	0.006	1.95	3.96	5.32	6.92	12.36	0.008	0.006	0.008	0.010	0.006	0.51
Feeding regime																		
Intermittent			328 ^b	857 ^b	1255	1411 ^b	3522 ^b	200 ^b	531 ^b	756	791 ^b	2077 ^b	1.65	1.61	1.66 ^b	1.79	1.70	2.25
<i>Ad libitum</i>			371 ^a	942 ^a	1271	1478 ^a	3687 ^a	222 ^a	582 ^a	743	840 ^a	2164 ^a	1.65	1.61	1.71 ^a	1.77	1.70	2.50
Acid																		
No			350	902	1272	1458 ^a	3631 ^a	210	560	753	822	2136	1.67	1.61	1.69	1.78	1.70	2.25
Yes			348	894	1254	1431 ^b	3578 ^b	212	552	745	809	2105	1.64	1.62	1.69	1.77	1.70	2.50
Phytase																		
No			347	891	1236 ^b	1425 ^b	3550 ^b	209	546 ^b	719 ^b	791 ^b	2056 ^b	1.66	1.63 ^a	1.72 ^a	1.81 ^a	1.73 ^a	2.75
Yes			351	905	1290 ^a	1464 ^a	3659 ^a	213	566 ^a	779 ^a	840 ^a	2185 ^a	1.65	1.60 ^b	1.66 ^b	1.75 ^b	1.68 ^b	2.00
P values																		
Feeding			< 0.001	< 0.001	0.127	< 0.001	< 0.001	< 0.001	< 0.001	0.131	< 0.001	< 0.001	0.065	0.995	< 0.001	0.162	0.431	0.808
Acid			0.574	0.314	0.082	0.049	0.048	0.478	0.076	0.327	0.261	0.086	0.076	0.276	0.842	0.792	1.000	0.808

Phytase	0.278	0.066	< 0.001	0.005	< 0.001	0.183	< 0.001	< 0.001	< 0.001	< 0.001	0.422	0.012	< 0.001	0.003	< 0.001	0.478
Feeding × acid	0.556	0.543	0.156	0.817	0.385	0.333	0.726	0.563	0.489	0.926	0.347	0.697	0.563	0.303	0.220	0.808
Feeding × phytase	0.209	0.762	0.995	0.529	0.724	0.751	0.6042	0.179	0.659	0.279	0.347	0.799	0.048	0.999	0.326	0.466
Acid × phytase	0.802	0.204	0.465	0.991	0.507	0.537	0.011	0.475	0.315	0.083	0.114	0.083	0.753	0.257	0.142	0.467
Feeding × acid × phytase	0.783	0.691	0.978	0.266	0.516	0.814	0.506	0.388	0.938	0.780	0.779	0.224	0.228	0.494	0.805	0.031

^{a-d} Means with different superscripts within the columns indicates those values are significantly different from each other ($P < 0.05$).

SEM: standard error of the mean

4.2. Digestibility of phosphorus

The apparent digestibility of P in jejunum, ileum and excreta showed that there were significant effects due to the three main factors and the interactions between them (Table 6). Feeding regime and phytase had positive effect on digestibility of P in jejunum, and the interaction between feeding and acid was also observed ($P = 0.016$) in this case. The apparent ileum digestibility of P was positively influenced by inclusion of acid and phytase, and there was also the interaction effect between feeding and phytase.

The P digestibility of birds reared intermittently on diet T₂ and T₄ was similar that of birds fed *ad libitum* on T₄. And no significant difference was found between NC diet (T₁) and diet with acid (T₃).

Both jejunal and ileal P digestibility were highest in birds reared on the diet with phytase (T₂) and diet with phytase and acid (T₄), and there were not statistically different between two diets under two feeding regimes. The faecal P digestibility was significantly affected by the three main factors. The highest faecal P digestibility was found in chickens fed intermittently on diet with phytase (T₂) and diet with phytase and acid (T₄), and they were significantly higher than those of *ad libitum* feeding.

Table 6. Apparent digestibility data of phosphorus (P) in jejunum, ileum and excreta

Treatment			Content of P, %		
Feeding	Acid	Phytase	Jejunum	Ileum	Excreta
Intermittent	No	No	42.36 ^{cd}	44.64 ^{bc}	33.89 ^{cd}
Intermittent	No	Yes	67.93 ^a	59.66 ^{ab}	42.02 ^{ab}
Intermittent	Yes	No	38.45 ^{cd}	45.35 ^{bc}	37.18 ^{bc}
Intermittent	Yes	Yes	69.21 ^a	65.60 ^a	46.03 ^a
<i>Ad libitum</i>	No	No	18.53 ^e	31.67 ^c	22.31 ^e
<i>Ad libitum</i>	No	Yes	49.03 ^{bc}	60.51 ^{ab}	32.24 ^{cd}
<i>Ad libitum</i>	Yes	No	26.13 ^{de}	39.18 ^c	28.12 ^{de}
<i>Ad libitum</i>	Yes	Yes	65.58 ^{ab}	72.02 ^a	30.79 ^{cd}
SEM			2.44	2.01	0.99
Feeding					
Intermittent			54.80 ^a	53.81	39.58 ^a
<i>Ad libitum</i>			39.82 ^b	50.85	28.36 ^b
Acid					
No			44.51	49.12 ^b	32.61
Yes			49.84	55.54 ^a	34.86
Phytase					
No			31.09 ^b	40.21 ^b	30.02 ^b
Yes			62.94 ^a	64.45 ^a	37.34 ^a
P values					
Feeding			< 0.001	0.302	< 0.001
Acid			0.052	0.027	0.018
Phytase			< 0.001	< 0.001	< 0.001
Feeding × acid			0.016	0.282	0.542
Feeding × phytase			0.215	0.023	0.365
Acid × phytase			0.199	0.421	0.177
Feeding × acid × phytase			0.731	0.915	0.101

SEM: standard error of the mean

4.3. Tibia parameters

The total amount of ash, P, length and weight (fresh and dry) of tibiae were significantly affected by the addition of phytase (Table 7). No differences were found among all treatments in terms of bone density of tibiae. The inclusion of phytase (T₂ and T₄) increased the content of ash and P under both feeding regimes compared to diets without phytase (T₁ and T₃). The width, length and weight of tibiae were improved by adding phytase in the diet (T₂) under *ad libitum* feeding, and these parameters were also significantly improved by *ad libitum* feeding. The interaction among the three main factors had significant effect on width, length and weight (fresh and dry) of tibiae as well.

Table 7. Measurement of tibia parameters at day 36

Treatment			Tibia parameters						
Feeding	Acid	Phytase	WBD ¹ g	WAD ² g	Length mm	Density mg mm ⁻¹	Width mm	Ash ³ %	P ⁴ %
Intermittent	No	No	11.39 ^{ab}	5.10 ^{abc}	90.82 ^{ab}	56.35	9.52 ^{ab}	39.75 ^{bcd}	6.76 ^b
Intermittent	No	Yes	11.47 ^{ab}	5.63 ^{ab}	91.59 ^a	58.01	9.22 ^{ab}	44.42 ^a	7.59 ^a
Intermittent	Yes	No	10.07 ^b	4.81 ^{bc}	89.15 ^{ab}	62.20	8.82 ^b	39.09 ^{cd}	6.54 ^b
Intermittent	Yes	Yes	11.76 ^{ab}	5.83 ^a	92.87 ^a	57.96	9.46 ^{ab}	42.62 ^{abc}	7.24 ^{ab}
<i>Ad libitum</i>	No	No	11.17 ^{ab}	4.73 ^c	85.14 ^b	64.59	9.59 ^{ab}	38.28 ^d	6.50 ^b
<i>Ad libitum</i>	No	Yes	12.40 ^a	5.85 ^a	90.36 ^{ab}	59.07	9.75 ^a	43.44 ^{ab}	7.54 ^a
<i>Ad libitum</i>	Yes	No	11.97 ^{ab}	5.21 ^{abc}	87.88 ^{ab}	61.24	9.67 ^{ab}	39.01 ^{cd}	6.70 ^b
<i>Ad libitum</i>	Yes	Yes	12.00 ^{ab}	5.62 ^{abc}	88.33 ^{ab}	59.70	9.58 ^{ab}	43.34 ^{ab}	7.56 ^a
SEM			0.18	0.08	0.55	1.02	0.55	0.39	0.39
Feeding									
Intermittent			11.17 ^b	5.34	91.11 ^a	58.86	9.25 ^b	41.47	7.03
<i>Ad libitum</i>			11.88 ^a	5.35	87.93 ^b	61.15	9.65 ^a	41.01	7.07
Acid									
No			11.61	5.33	89.48	59.50	9.52	41.47	7.09
Yes			11.45	5.37	89.56	60.27	9.38	41.01	7.01
Phytase									
No			11.15 ^b	5.00 ^b	88.25 ^b	61.10	9.40	39.03 ^b	6.62 ^b
Yes			11.91 ^a	5.73 ^a	90.79 ^a	58.68	9.50	43.45 ^a	7.48 ^a
P values									
Feeding			0.037	0.945	< 0.001	0.2239	0.008	0.460	0.721
Acid			0.637	0.792	0.939	0.7084	0.331	0.460	0.492
Phytase			0.027	< 0.001	0.015	0.2435	0.471	< 0.001	< 0.001
Feeding × acid			0.289	0.559	0.789	0.3021	0.539	0.213	0.115
Feeding × phytase			0.701	0.694	0.772	0.5866	0.631	0.598	0.434
Acid × phytase			0.756	0.967	0.655	0.8177	0.234	0.426	0.530
Feeding × acid × phytase			0.040	0.042	0.062	0.2325	0.043	0.901	0.937

¹ weight before drying (WBD);² weight after drying (WAD);³ percentage of ash in tibiae;⁴ percentage of phosphorus in tibiae

4.4. pH shift in diet 3 after acidification with formic acid

The result of one-way ANOVA test illustrated that there was a linear relation between pH and exposed time after adding formic acid. The pH of diet (T₃) increased significantly with the increase of exposed time within 18 hours (Fig. 4). At the beginning, the pH of diet (T₃) was 4.39 and gradually increased to 4.85 and 5.02 after 8 and 18 h. After 18 h, there was no significant change in pH with the increase of exposed time.

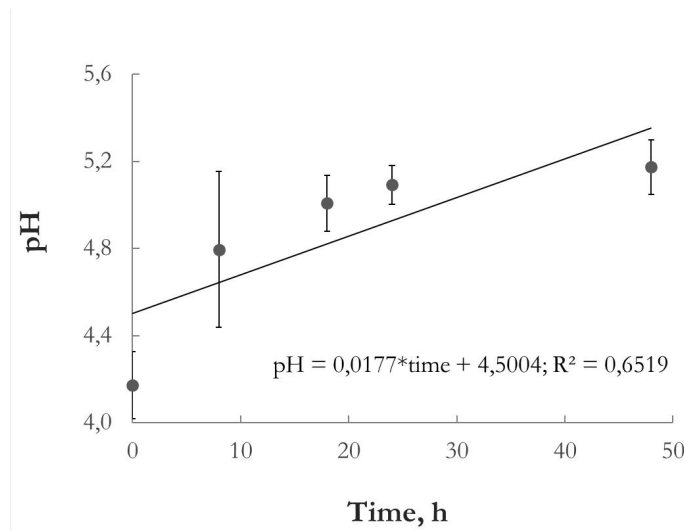


Fig. 4. The changes of pH in diet 3 (T₃) over time

5. Discussion

5.1. Growth performance

In total, the higher FI of *ad libitum* fed chickens resulted in a higher WG than those fed intermittently. Birds usually consume 2–3 times more feed than their maintenance requirement and lead to high level of fat deposition and BW under *ad libitum* feeding (Boekholt et al., 1994, Svihus et al., 2010, Svihus et al., 2013, Sacranie et al., 2012). In the present experiment, broilers reached the commercial standard in terms of their BW and there were no differences in FCR under two feeding regimes, indicating intermittent feeding did not affect the growth and mortality of broilers. Svihus et al. (2013) found that the chicks were able to adapt intermittent feeding rapidly without compromising their growth. Under intermittent feeding, broilers mainly ate feed during the first 20

min of each feeding bout and consumed less during the last 20 min of 1 h feeding (Svihus et al., 2010, Sacranie et al., 2017). In our study, therefore, the relatively shorter feeding period (1–2 h) followed by longer resting interval (3–4 h) most likely facilitated the development of storage and digestive function of the anterior digestive tract of the birds.

Under intermittent feeding, the extended use of crop stimulates the secretion of moisture and microbial fermentation, which are essential for the enhancement of exogenous enzyme activity and nutrient digestibility in small intestine (Classen et al., 2016, Svihus et al., 2010, Sacranie et al., 2017). In the current study, FI, WG and FCR were improved by the supplementation of phytase, which was further facilitated by addition of formic acid. The inclusion of phytase reduced the antinutritional effect of phytate in some of the feed ingredients such as wheat bran, by hydrolysing phytate in anterior digestive tract. As a consequence, the availability of amino acids and minerals increased, leading to increased growth performance (Morgan et al., 2016). One explanation by Mohammadagheri et al. (2016) indicated that the combination of organic acid and phytase improved the intestinal histomorphology, increasing villus height and width. Therefore, the interaction effect among phytase, acid and intermittent feeding entwined with the efficacy of phytase, and the potential mechanism behind the interaction effect still remain unknown.

5.2. Digestibility of phosphorus

Phytase and acid improved the digestibility of P in jejunum, ileum and excreta, in which diet with acid and phytase (T_4) seemed numerically higher than diet with phytase alone (T_2), both in jejunum and ileum, indicating the interaction effect between formic acid and phytase on phytase efficacy. The positive effect of organic acid on phytase efficacy was also concluded in previous studies e.g. using citric acid and phytase (S Radcliffe et al., 1998, Brenes et al., 2003). Phytate can be enzymatically degraded by phytase during the retention in anterior digestive tract, especially in crop (Svihus et al., 2010, Zeller et al., 2016). An appropriate acidic ambient condition in anterior segment of digestive tract is crucial for the phytase activity (Liebert et al., 1993, Lan et al., 2010) since most types of phytases have an optimum pH below 5.5 to function effectively (Wyss et al., 1998, Garrett et al., 2004, Jain et al., 2016, Menezes-Blackburn et al., 2015). Phytase breaks phytase down in a series of steps under acidic condition and releases P that can be absorbed in the posterior segment of digestive tract, i.e. duodenum and jejunum. The pH of ingested feed can be decreased from its initial

neutral pH to 4 or even lower through prolonging the retention time in crop. The adjustment of retention time is associated with the feeding regimes applied, e.g. intermittent feeding.

The digestibility of P in jejunum, ileum and excreta was higher in intermittently fed birds than those fed *ad libitum*, although the FI was lower under intermittent feeding. And there was a tendency that digestibility of P increased along with jejunum and ileum, except for the T₂ and T₄ under intermittent feeding. Compared to *ad libitum* feeding, the interaction between acid and phytase on jejunal P digestibility was more obvious with intermittent feeding in the present study. Interestingly, feeding regime did not induce differences in ileal P digestibility, indicating that the ileal digestibility might be representative regardless of the feeding regimes. In the current study, intermittent feeding prolonged the retention time of digesta in digestive tract resulting in a highest digestibility of P in jejunum. Svihus (2014) demonstrated the beneficial effect of intermittent feeding on prolonging retention time of nutrients in digestive tract of broilers, lending support to the result from the present study. On the other hand, the interaction effect between feeding and acid on P digestibility was significant although the P digestibility was not statistically different between NC (T₁) and the treatment with acid (T₃).

The addition of organic acid, such as formic acid, might compromise the FI, and in this case, the *ad libitum* feeding might have the advantage to compensate the lower FI probably induced by the poor palatability. However, further research is needed to uncover the interaction between feeding regime and organic acid on FI and P digestibility.

Theoretically, the faecal P digestibility should be higher than both jejunal and ileal digestibility, but it was much lower than the digestibility measured in jejunum and ileum in the current study. In the present case, the lower value of P digestibility in faecal material was potentially associated with either endogenous or external sources of P. In this study, therefore, compared to the digesta sampled from ileum, the faecal material sampled after excretion is not representative for determining nutrient digestibility. Similarly, previous study concluded that the digestibility of amino acid in poultry feed (Ravindran et al., 1999).

5.3. Tibia parameters

Phytase had positive effect on the increase of ash and P content in tibiae. In the current study, the interaction among feeding regime, acid and phytase had significant effect on the weight and width of

tibiae, but not on the ash content and P deposition. Bone formation is a complex process and it is associated with many factors, such as strain, age, gender, as well as the proper ratio between Ca and P. Phosphorus plays an important role during the process of bone mineralization that is a process by which the bone, e.g. tibia, is formed. It has been reported that the P digestibility is strongly and positively correlated with the tibia parameters and P retention (Mignon-Grasteau et al., 2016). The apparent ileal P digestibility in the current study was elevated by the supplementation of phytase and acid. Prolonged retention of digesta through intermittent feeding seemed to compensate the lower FI, reaching statistically same ileal P digestibility and deposition in tibiae with *ad libitum* feeding.

5.4. Adjustment of pH in diet 3

The results with T₃ diet showed that the pH of experimental feed increased over time, which is closely related to the volatile characteristics of formic acid at room temperature. The chemical characteristics vary among various organic acids. It was suggested that the stability of formic acid depends on temperature and its concentration (Kieczka, 2016). Formic acid becomes unstable with increasing temperature and concentration (Kieczka, 2016). In current study the concentration of formic acid was 85% and there was a potential of partial decomposition of formic acid before feeding. The pH of experimental feed was probably higher than the expected level when the feeding experiment started. In the current study, the diet with acid (T₃) had no clear positive effect on growth performance, apparent ileal P digestibility and bone mineralization under *ad libitum* feeding, and this result was in accordance with the previous study by (Hernandez et al., 2006). It has been concluded that the blending of formic acid with other types of organic acids such as propionic acid, lactic acid and medium-chain fatty acids have a better effect than the application of formic acid alone due to its instability (Kieczka, 2016), although the mechanism associated with the mixed acid is still unknown.

The feeding experiment started from day 11 for all treatments. But, the amount of acid added was only 0.1% for T₃ and T₄, which was insufficient to reach the expected level of acidification. After adding extra 1% of formic acid (1.1% of acid in total), the feeding experiments using diet with acid (T₃) and diet with acid and phytase (T₄) were started from day 15 under two different feeding regimes. However, feeding experiments with NC diet (T₁) and diet with phytase alone (T₂), started from day 11 under two different feeding regimes. Thus, the possible effect of feeding before day 15

(in T₃ and T₄) should be taken into account because it may lead to the difference between T₃, T₄ and T₁, T₂ treatments in terms of their final results.

6. Conclusion

Phytase, formic acid and feeding regime had positive effect on growth performance (WG, FI and FCR), apparent digestibility of P (in jejunum and ileum) and deposition of P in tibiae. The interaction between acid and phytase on WG was found during day 15 to 22 while the interaction between feeding and phytase on FCR was observed during the period of 22–29 d. The interaction between acid and feeding influenced the jejunal P digestibility while the interaction between feeding and phytase affected the apparent ileal digestibility of P. The P digestibility measured from the excreta was much lower than that measured from both jejunum and ileum, indicating that the faecal material is not an option for determining digestibility of P in broiler chicken. The interaction among three main factors had effect on weight and width of tibiae, but not on the ash content and P deposition. However, it is possible that the interaction effect becomes obvious if the feeding experiment continued further, because the turnover in bone tissue is much lower than other tissues. The pH of the experimental diet (T₃) increased over time when it was exposed to same temperature (27–29 °C) with the chicken house, possibly due to the unstable properties of formic acid, and therefore the future experiment should consider effect of volatile organic acids in feed acidification. It can be concluded that phytase efficacy can be elevated by adding formic acid, and it is much more effective under intermittent feeding.

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Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitenskapelige universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway